Akhlaq A. Farooqui





Neural Membrane Lipidology



Hot Topics in Neural Membrane Lipidology

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In the memory of my beloved father "late Sharafyab Ahmed Saheb", who taught me how a man should act with politeness and set the bar high.

Akhlaq A. Farooqui

Preface

The backbone of neural membranes is the lipid bilayer, which is composed of glycerophospholipids, sphingolipids, and cholesterol. Embedded in the lipid bilayer are proteins of various shapes, sizes, and traits. The intricate interactions among glycerophospholipids, sphingolipids, cholesterol, and proteins provide neural membranes with delicate, dynamic, and stable shape responsible for numerous neural membrane activities. Glycerophospholipids and sphingolipids contribute to the lipid bilayer asymmetry, whereas cholesterol and sphingolipids form microdomains or lipid rafts. The generation of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators is necessary for normal cellular function. Glycerophospholipids contain arachidonic acid (AA) and docosahexaenoic acid (DHA) at the sn-2 position of glycerol moiety. Eicosanoids and docosanoids are enzymically derived lipid mediators of AA and DHA metabolism, respectively. The non-enzymic lipid mediators of AA and DHA metabolism include isoprostanes, neuroprostanes. isoketals, neuroketals, isofurans, 4-hydroxynonenal, and 4-hydroxyhexanal. Sphingolipid-derived lipid mediators include ceramide, ceramide 1-phosphate, and sphingosine 1-phosphate; and cholesterol metabolites include 7-ketocholesterol, 24-hydroxycholesterol, and 25-hydroxycholesterol. These lipid mediators play important roles in neural cell survival and neurodegeneration. Levels of lipid mediators in neural and non-neural tissues are partly regulated by diet. The high intake of food enriched in AA (vegetable oils) elevates levels of eicosanoids and upregulates the expression of pro-inflammatory cytokines. AA and its metabolites (eicosanoids) have prothrombotic, proaggregatory, and pro-inflammatory properties. In contrast, a diet enriched in DHA (fish and fish oil) generates docosanoids, which not only downregulate pro-inflammatory cytokines, but also have anti-inflammatory, antithrombotic, anti-arrhythmic, hypolipidemic, and vasodilatory effects. At present, the threshold concentrations of lipid mediators that promote and facilitate neural cell injury and death are not known. In neurological disorders, cell death depends not only on elevated levels of lipid mediators, but also on cross talk (interplay) among glycerophospholipid-, glycosphingolipid-, and cholesterol-derived lipid mediators. Thus, oxidative stress and neuroinflammation generated by various lipid mediators, along with changes in cellular redox, mitochondrial dysfunction, and alterations in ion homeostasis may be associated with neurodegeneration in neurological disorders.

In recent years, we have been enabled with technological advances in lipidomics, proteomics, and genomics. Investigators are using these techniques not only for identifying and determining levels of molecular species of glycerophospholipids, sphingolipids, and cholesterol, but also for determining threshold levels of lipid mediators that produce neural injury and developing a diagnostic test for neurological disorders. The goal of this monograph is to present readers with cutting edge and comprehensive information on lipid mediators in a manner that is useful not only to students and teachers but also to researchers and physicians. This monograph has 11 chapters. Chapters 1 and 2 describe the generation of various lipid mediators from neural membrane glycerophospholipids, sphingolipids, and cholesterol and their involvement in signal transduction processes. Chapters 3 and 4 describe cutting edge information on roles of various isoforms of phospholipases A₂ in the generation of glycerophospholipid-derived lipid mediators and interactions among phospholipases A₂, C, and D and their lipid mediators in the nucleus. Chapter 5 describes metabolism, roles of bioactive ether lipids, and interactions of ether lipid-derived lipid mediators with ester-linked lipid-derived lipid mediators in brain. Chapters 6 and 7 are devoted to excitotoxicity-mediated modulation of lipid mediators in neurological disorders and recent developments in kainic acid-induced neurotoxicity. Chapters 8 and 9 describe the importance of n-3 fatty acids (docosahexaenoic and eicosapentaenoic acids) in diet and mechanism of their action in cardiovascular and cerebrovascular systems and comparison of n-3 fatty acids with cholesterol-lowering drugs (statins). Chapter 10 describes the contribution of neural membrane lipids in apoptosis and necrosis. Finally, Chapter 11 provides readers and researchers with perspective that will be important for future research work on bioactive lipid mediators. This monograph can be used as a supplemental text for a range of neuroscience courses. Clinicians will find this book useful for understanding molecular aspects of lipid mediators in neurological disorders. To the best of my knowledge no one has written a monograph on the role of lipid mediators in brain tissue and this monograph will be the first to provide a comprehensive description of glycerophospholipid, sphingolipid, and cholesterol-derived mediators, their interactions with each other in normal brain and in brain tissue from patients with neurological disorders. It is anticipated that senior neuroscientists may find some inspiration from this monograph to overcome problems encountered in lipid mediator research in their laboratories and their students may gain insight into the difficulties experienced in their research on lipid mediators in brain.

The choices of topics presented in this monograph are personal. They are not only based on my interest on glycerophospholipid, sphingolipid, and cholesterol metabolism in neurological disorders, but also in areas where major progress has been made. I have tried to ensure uniformity and mode of presentation as well as a logical progression of subject from one topic to another Preface

and have provided an extensive bibliography. For the sake of simplicity and uniformity a large number of figures and line diagrams of signal transduction pathways are also included. I hope that my attempt to integrate and consolidate the knowledge of lipid mediators and signal transduction processes in normal and diseased brain will provide the basis of more dramatic advances and developments on the determination, characterization, and roles of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in neurological disorders.

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> Akhlaq A. Farooqui Columbus, Ohio

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List of Abbreviations

| PtdCho | Phosphatidylcholine |
|----------------------------------|---------------------------------------|
| PtdEtn | Phosphatidylethanolamine |
| PlsCho | Choline plasmalogen |
| PlsEtn | Ethanolamine plasmalogen |
| PtdIns | Phosphatidylinositol |
| PtdIns4P | Phosphatidylinositol 4-phosphate |
| $PtdIns(4,5)P_2$ | Phosphatidylinositol 4,5-bisphosphate |
| Ins-1,4,5- <i>P</i> ₃ | Inositol-1,4,5-trisphosphate |
| PtdH | Phosphatidic acid |
| PtdSer | Phosphatidylserine |
| Cer | Ceramide |
| Sph | Sphingosine |
| AA | Arachidonic acid |
| DHA | Docosahexaenoic acid |
| PLA ₂ | Phospholipase A ₂ |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| COX | Cyclooxygenase |
| LOX | Lipoxygenase |
| EPOX | Epoxygenase |
| РКС | Protein kinase C |

About the Author

Akhlaq A. Farooqui is a leader in the field of brain phospholipases A_2 , bioactive ether lipid metabolism, and glutamate-mediated neurotoxicity. He has discovered the stimulation of plasmalogen-selective phospholipase A_2 activity in brains from patients with Alzheimer disease. Stimulation of this enzyme results in plasmalogen deficiency that may be related to the loss of synapses in brains of patients with Alzheimer disease. Akhlaq A. Farooqui has published cutting edge research on the generation and identification of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in kainic acid neurotoxicity. Akhlaq A. Farooqui has authored three monographs: *Glycerophospholipids in Brain: Phospholipase* A_2 *in Neurological Disorders* (2007); *Neurochemical Aspects of Excitotoxicity* (2008); and *Metabolism and Functions of Bioactive Ether Lipids in the Brain* (2008). All monographs are published by Springer, New York.

Chapter 1 Neural Membranes: A Pandora's Box of Lipid Mediators

1.1 Lipid Composition of Neural Membranes

Neural membranes are composed of glycerophospholipids, sphingolipids, cholesterol, and proteins. The distribution of lipids in two leaflets of lipid bilayer is asymmetric (Ikeda et al., 2006; Yamaji-Hasegawa and Tsujimoto, 2006). Glycerophospholipids and sphingolipids contribute to the lipid asymmetry, whereas cholesterol and sphingolipids form lipid microdomains or lipid rafts. The maintenance of transbilayer lipid asymmetry is a dynamic process, which is necessary for normal neural membrane function. The disruption of asymmetry results in neural cell activation or neurodegeneration. Lipid asymmetry is generated primarily by selective synthesis of lipids on one side of the membrane and maintained by three classes of lipid translocases: P-type ATPases, ABC transporters, and scramblases (Daleke, 2003; Pomorski and Menon, 2006). Energy-independent flippases are involved in equilibration of common glycerophospholipids between the two monolayers. ATP-dependent flippases are associated with the net transfer of specific glycerophospholipids to one leaflet of the membrane and are responsible for the maintenance of its transbilayer lipid asymmetry of membranes (Pomorski and Menon, 2006). Lipid rafts float within the membrane, and certain groups of proteins unite within these rafts. A large number of signaling molecules are concentrated within rafts, which function as signaling centers capable of facilitating efficient and specific signal transduction pathways (Zajchowski and Robbins, 2002; Lucero and Robbins, 2004).

Neural membranes also contain transmembrane and peripheral proteins, which vary in shape, molecular mass, charge distribution, and propensity for aggregation. These proteins are connected to glycerophospholipids and to the intracellular cytoskeleton to restrict their free diffusion. The binding of glycerophospholipids to proteins is also necessary for vertical positioning and tight integration of proteins in the lipid bilayer (Palsdottir and Hunte, 2004). The binding between glycerophospholipid and proteins is stabilized by multiple noncovalent interactions between protein residues and glycerophospholipid head groups and hydrophobic tails (Palsdottir and Hunte, 2004; Farooqui and Horrocks, 2007). Sphingolipids consist of a sphingoid base, a straight-chain alcohol of 18–20 carbon atoms, which is normally attached to a long saturated fatty acid (usually palmitate) through an amide bond. Sphingolipids include glycosphingolipids with a sugar as the head group, ceramide or *N*-acylsphingosine with no head group, and phosphosphingolipids (primarily sphingomyelin, a phosphodiester of ceramide and choline). Sphingomyelin is a major constituent of neural cell plasma membranes where it is concentrated in the outer leaflet (Vaena de Avalos et al., 2004).

Cholesterol is a major constituent of neural membranes. It protects the glycerophospholipid bilayer from oxidative damage and plays a key role in the formation of memory and the uptake of hormones in the brain, including serotonin. When cholesterol levels drop too low, the serotonin receptors cannot function properly. The 3-hydroxy group of cholesterol anchors it at the membrane interface, so that cholesterol inserts into a membrane leaflet approximately parallel to the fatty acid chains of the surrounding glycerophospholipids. Cholesterol plays a crucial role in membrane organization, dynamics, function, and sorting (Simons and Ikonen, 2000). Cholesterol not only serves as a precursor for the synthesis of oxysterol, steroid hormones, but also regulates activities of membrane-bound enzymes, receptors, and ion channels (Simons and Ikonen, 2000; Tun et al., 2002). Dynamic clustering of cholesterol along with sphingolipids results in the formation of specialized structures called microdomains or rafts. In neural membranes, formation of rafts occurs through the interactions between sphingolipids and cholesterol. Cholesterol condenses by positioning itself between hydrocarbon chains below the large head groups of the sphingolipids. These interactions lead to the formation of a less fluid, liquid-ordered phase, separate from a phosphatidylcholine-rich liquid-disordered phase (Simons and Ikonen, 2000). These structures play crucial roles in neural cell functions such as signal transduction, adhesion, sorting, trafficking, and organizing bilayer constituents including receptors, enzymes, and ion channels (Simons and Ikonen, 2000; Farooqui et al., 2000; Farooqui and Horrocks, 2007).

Glycerophospholipid, sphingolipid, and cholesterol metabolism is closely interrelated and interconnected. For example, glycerophospholipid-derived lipid mediators modulate sphingolipid metabolism, and sphingolipid-derived lipid mediators regulate glycerophospholipid metabolism (Farooqui et al., 2007a; Farooqui and Horrocks, 2009). Moreover, many cell stimuli modulate more than one enzyme at the same time, thus adding to the complexity of regulation of glycerophospholipid, sphingolipid, and cholesterol metabolism. Under normal conditions, the status of enzymes of glycerophospholipid, sphingolipid, and cholesterol metabolism in neural cell regulation and proposed roles for these enzymes in integration of cellular responses are based not only on levels of lipid mediators and organization of signaling network, but also on the complexity and interconnectedness of their metabolism. Under pathological situations, marked alterations in levels of lipid mediators disturb the signaling networks and result in loss of communication among glycerophospholipid, sphingolipid, and cholesterol metabolism. This process threatens the integrity of neural cell lipid homeostasis resulting in neural cell death (Farooqui et al., 2004; Farooqui et al., 2007a, b; Farooqui and Horrocks, 2007).

1.2 Glycerophospholipids and Their Metabolism in Brain

Brain tissue contains five major classes of glycerophospholipids. The first four classes include 1,2-diacyl glycerophospholipids, 1-alk-1'-enyl-2-acyl glycerophospholipids or plasmalogens, 1-alkyl-2-acyl glycerophospholipids, and phosphatidic acid. The fifth class is represented by sphingomyelin. It contains ceramide linked to phosphocholine through its primary hydroxyl group. Membrane glycerophospholipids play multiple roles in neural cells. Glycerophospholipids not only provide neural membranes with stability, fluidity, and permeability, but are also required for the proper function of integral membrane proteins, receptors, transporters, and ion channels (Farooqui and Horrocks, 1985; Farooqui et al., 2000).

Brain glycerophospholipids exhibit a high degree of heterogeneity with regard to molecular species, which results from the structural combination of fatty chains at the sn-1 and sn-2 positions. Over 400 glycerophospholipid species with different structures can be identified in a single cell (Farooqui et al., 2000; Ivanova et al., 2004). In neural membranes, each class of glycerophospholipids exists as a heterogeneous mixture of molecular species (Ivanova et al., 2004; Hicks et al., 2006).

In order to understand the roles of glycerophospholipids at the molecular level in brain tissue, it is important to have comprehensive and accurate accounts of molecular species present in various types of neural cells at subcellular levels. This task is very difficult because the methodologies for the characterization of molecular species depend not only on conventional procedures, but also on technical skills for accuracy and reliability. In recent years, lipidomics has emerged as an important procedure for the comprehensive identification and full characterization of molecular species of glycerophospholipids and for the determination and characterization of levels of their lipid mediators in normal brain as well as brain from patients with neurological disorders (Piomelli, 2005; Lee et al., 2005b; Gross et al., 2005; Forrester et al., 2004; Lu et al., 2006). It is hoped that full characterization of glycerophospholipid molecular species at cellular and subcellular levels will be soon forthcoming.

The synthesis of different pools within a glycerophospholipid subclass appears to be compartmentalized according to the fatty acid composition and the source of the head group (Farooqui et al., 2000). Each portion of a glycerophospholipid molecule turns over at a different rate. The turnover time of the phosphate group is different from those of the nitrogenous base and the acyl groups at the sn-1 and sn-2 positions (Porcellati, 1983; Farooqui et al., 2000). Glycerophospholipids are hydrolyzed by a group of enzymes called phospholipases. Phospholipase A_1 (PLA₁) catalyzes the hydrolysis of an ester bond at the sn-1 position forming free fatty acid and 2-acyl lysophospholipid. Phospholipase A_2 (PLA₂) acts on the ester bond at the sn-2 position liberating free fatty acid and 1-acyl lysophospholipid, which in turn can be acylated by acyl-CoA in the presence of an acyltransferase (deacylation/reacylation cycle). Alternatively, a 1-acyl lysophospholipid can be hydrolyzed by a lysophospholipase forming fatty acid and phosphobase. Phospholipase C (PLC) hydrolyzes the phosphodiester bond at the sn-3 position of choline glycerophospholipids forming 1,2-diacylglycerols and phosphocholine. Finally, phospholipase D (PLD) cleaves glycerophospholipids into phosphatidic acid and a free base (Farooqui et al., 2000). PLA₁, PLA₂, PLC, and PLD have been purified and characterized from the brain tissue (Hirashima et al., 1992; Pete et al., 1994; Fukami, 2002; Banno, 2002; McDermott et al., 2004). In brain tissue neurotransmitter release, cPLA₂, PLC, and PLD activities, and arachidonic acid release are coupled to dopamine, glutamate, serotonin, P2-purinergic, muscarinic, cytokine, and growth factor receptors through different coupling mechanisms (Attucci et al., 2001; Shen et al., 2001; Vitale et al., 2004; Farooqui et al., 2006; Axelrod, 1990; Ross, 2003; Qu et al., 2003; Lazarewicz et al., 1990; Qu et al., 2005). Dopamine, serotonin, and muscarinic receptors are linked through G-proteins, whereas glutamate receptors do not involve G-protein coupling (Lazarewicz et al., 1990).

In addition to their roles as neural membrane components and energy storing molecules, glycerophospholipids, sphingolipids, and cholesterol function as precursors for lipid mediators. Brain PLA₂, C, D, and sphingomyelinases are components of a signal transduction network that controls the levels of lipid mediators and intensity of signal transduction processes under normal and pathological conditions. Cross talk among various receptors through the generation of second messengers is essential for maintaining normal neuronal and glial cell growth (Farooqui et al., 2000; Farooqui and Horrocks, 2007).

1.3 Arachidonic Acid and Its Enzymically Derived Oxidation Products

Arachidonic acid (AA) is a major polyunsaturated fatty acid of neural membrane glycerophospholipids. In resting neural cells, isoforms of PLA_2 liberate AA. Some AA is converted to eicosanoids, whereas the majority of AA is reincorporated into brain glycerophospholipids (Rapoport, 1999). Under pathological conditions, AA causes intracellular acidosis and uncouples oxidative phosphorylation, which results in mitochondrial dysfunction (Schapira, 1996). AA produces mitochondrial swelling in neurons and induces changes in membrane permeability by regulating ion channels (Farooqui et. al., 1997).

1.3.1 Arachidonic Acid and Lyso-glycerophospholipids

The concentration of free AA in brain is very low ($<10 \mu$ mol/kg). At this concentration, it not only acts as a lipid mediator itself but also is a precursor of many lipid mediators (Table 1.1). AA modulates activities of many enzymes involved in neural cell survival and death (Fig. 1.1) (Farooqui and Horrocks, 2006). In addition, AA modulates ion channels, neurotransmitter release, induction of long-term potentiation, and neural cell differentiation (Fig. 1.2). AA acts as a facilitatory retrograde neuromodulator in glutamatergic synapses (Katsuki and Okuda, 1995). AA also modulates acetylcholine release in rat hippocampus (Almeida et al., 1999). Low levels of AA are involved in maintaining the structural integrity of neural membranes, determining neural membrane fluidity, and thereby regulating neuronal transmission. In the nucleus, AA may also interact with elements of gene structure, such as promoters, enhancers, suppressors to modulate gene expression in a specific manner that is not shared by eicosanoids or other fatty acids (Farooqui et al., 1997) (Fig. 1.2).

1.3.2 Lysophosphatidylcholine (lyso-PtdCho)

Lyso-PtdCho, the other products of PLA₂-catalyzed reaction, modulates activities of many enzymes (Table 1.2), ion channels, growth factors, and receptors

| 1 able 1.1 | Gijeeropnosphonpi | a dellited lipid | mediators |
|--|--------------------------------|------------------|--|
| Substrate | Lipid mediator | Mechanism | Reference |
| AA-containing glycerophospholipids | Prostaglandins | Enzymic | Phillis et al. (2006) |
| | Leukotrienes | Enzymic | Phillis et al. (2006) |
| | Thromboxanes | Enzymic | Phillis et al. (2006) |
| | Lipoxins | Enzymic | Chiang et al. (2006) |
| | 4-HNE | Enzymic | Esterbauer et al. (1991) |
| | Isoprostanes | Non- enzymic | Fam and Morrow (2003) |
| | Isofurans | Non- enzymic | Fam and Morrow(2003) |
| | Isoketals | Non- enzymic | Fam and Morrow, (2003) |
| DHA-containing glycerophospholipids | Resolvins/ docosatrienes | Enzymic | Hong et al. (2003) |
| | Protectins/ neuroprotectins | Enzymic | Mukherjee et al. (2004); Serhan (2005d) |
| | 4-HHE | Enzymic | Kristal et al. (1996) |
| | Neuroprostanes | Non- enzymic | Yin et al. (2005) |
| | Neuroketals | Non- enzymic | Bernoud-Hubac et al. (2001) |

Table 1.1 Glycerophospholipid-derived lipid mediators



Fig. 1.1 Enzymes affected by arachidonic acid in neural and non-neural tissues



Fig.1.2 Roles of arachidonic acid in brain

(Table 1.3). It induces de-ramification (transformation from ramified morphology into amoeboid morphology) in microglial cells (Schilling et al., 2004). The de-ramification can be retarded by simultaneous inhibition of non-selective cation channels and K⁺-Cl⁻cotransporters, suggesting the functional importance of these channels in microglial activation and de-ramification (Schilling et al.,

| Enzyme | Effect | Reference |
|--|-------------|---------------------------|
| Protein kinase C | Stimulation | Bassa et al. (1999) |
| Tyrosine kinase | Stimulation | Légrádi et al. (2004) |
| c-jun N-terminal kinase | Stimulation | Fang et al. (1997) |
| MAP kinase | Stimulation | Bassa et al. (1999) |
| Cyclooxygenase | Stimulation | Rikitake et al. (2001) |
| Nitric oxide synthase | Inhibition | Durante et al. (1997) |
| Calpain | Stimulation | Chaudhuri et al. (2003) |
| Adenylate cyclase | Stimulation | Yuan et al. (1996) |
| Na ⁺ , K ⁺ -ATPase | Inhibition | Fink and Gross (1984) |
| Cyclooxygenase | Stimulation | Zembowicz et al. (1995) |
| Phospholipase D | Stimulation | Gómez-Muñoz et al. (1999) |
| Protein kinase C | Stimulation | Bassa et al. (1999) |
| Paraoxonase | Inhibition | Park et al. (2006) |

 Table 1.2
 Effect of lysophosphatidylcholine on enzymic activities in neural and non-neural tissues

Modified from Farooqui and Horrocks (2006).

 Table 1.3 Effects of lysophosphatidylcholine on receptors in neural and non-neural cells

| channel | Activity | Reference |
|--|-----------|---|
| Heparin-binding- like growth factor | Enhanced | Kume and Gimbrone, Jr. (1994); Zembowicz et al. (1995) |
| PDGF receptor | Enhanced | Kume and Gimbrone, Jr. (1994); Zembowicz et al. (1995) |
| Dopamine receptor | Inhibited | Lee et al. (2004a, 2005a) |
| G2A receptor | Enhanced | Ikeno et al. (2005) |
| TRPC5 calcium channel | Enhanced | Flemming et al. (2005) |
| TRAM-34 | Enhanced | Schilling and Eder (2007) |
| (HERG) K ⁺ | Enhanced | Wang et al. (2001) |

2004). Lyso-PtdCho also enhances the P2X7 receptor (P2X7R)-associated formation of membrane pores and activates Ca^{2+} influx and p44/42 mitogenactivated protein kinase. These results suggest that lyso-PtdCho regulates microglial functions in the brain by enhancing the sensitivity of P2X7R (Takenouchi et al., 2007).

Injections of lyso-PtdCho into brain produce acute inflammatory demyelination at the injection site, breakdown of the blood–brain barrier, and interstitial edema around the injection site (Lovas et al., 2000; Ousman and David, 2000; Degaonkar et al., 2005). Treatment of cerebellar slices with lyso-PtdCho also causes demyelination in vitro (Birgbauer et al., 2004). A single dose of lyso-PtdCho in the striatum produces the accumulation of dopamine and reduction in levels of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in this region (Lee et al., 2004a, 2005a). Lyso-PtdCho interferes with dopaminergic neurotransmission such as release, binding to postsynaptic receptors, uptake, or metabolism, resulting in retarding dopamine turnover and inducing bradykinesia in animals (Lee et al., 2005a). This process may also relate to an impairment of locomotor activities in rats. It is likely that modulation of dopamine receptor function by lyso-PtdCho may be due to an interaction between dopaminergic receptor and lyso-PtdCho receptor (Lee et al., 2004a).

In brain lyso-PtdCho also modulates exocytosis (Poole et al., 1970) by reversibly blocking it at a stage between triggering and membrane merger (Vogel et al., 1993). Collectively, these studies suggest that in mammalian tissues lyso-PtdCho not only regulates membrane-bound enzymes, receptors, and ion channels but also modulates signal transduction and platelet aggregation (Oishi et al., 1988; Sakai et al., 1994; Yuan et al., 1996).

1.3.3 Eicosanoids

Cyclooxygenases and lipoxygenases convert AA into prostaglandins, leukotrienes, and thromboxanes. These metabolites are collectively known as eicosanoids (Phillis et al., 2006). They are involved in many processes including fever, sensitivity to pain, sleep, inflammation, and oxidative stress and are the target of aspirin-like drugs. These lipid mediators are not stored in neural cells, but are generated in response to receptor-mediated stimulation. Because of their amphiphilic nature, eicosanoids can cross cell membranes and leave the cell in which they are synthesized to act on neighboring cells. Eicosanoids act through specific superficial or intracellular receptors, modulating signal transduction pathways and gene transcription. Some prostaglandins (Fig. 1.3) modulate neural activity by releasing neurotransmitters, whereas others regulate circulatory function. Thromboxane A_2 is a potent vasoconstrictor and produces vasospasm, whereas PGI₂ has opposing effects (Phillis et al., 2006) (Table 1.4).

The generation and accumulation of eicosanoids under pathological conditions is associated with the modulation of cerebrovascular blood flow. Their active production by circulating cells such as platelets and leukocytes may contribute to the onset of alterations in the microcirculation and ultimately to CNS dysfunction (Phillis et al., 2006). High levels of prostaglandins have degenerative affects on differentiated murine neuroblastoma cells in cultures. In vivo, prostaglandins are involved in the regulation of cytokines and maintenance of the inflammatory cascade (Phillis et al., 2006; Farooqui et al., 2007b).

Prostacyclin, a major prostaglandin derived from enzymic oxidation of AA at the nuclear and endoplasmic reticular membrane levels, acts not only through its surface receptors but also via its nuclear receptors in physiological and pathological processes. Prostacyclins interact with peroxisomal proliferator-activated receptors and modulate many biological functions. Similarly, leukotrienes are lipid mediators of arachidonic acid metabolism involved in autocrine and paracrine signaling (Phillis et al., 2006).



Fig. 1.3 Chemical structures of arachidonate-derived lipid mediators. (a) Arachidonic acid; (b) prostaglandin E_2 ; (c) prostaglandin $F_{2\alpha}$; (d) lipoxins A4; (e) lipoxin B4; (f) 15-epi-LX A4; and (g) 15-epi-LXB4

| Metabolite | Role | Reference |
|--|---|--|
| $\begin{array}{c} PGE_2, PGF_{2\alpha}, \\ PGI_2, TxA_2 \end{array}$ | Homeostasis, vasodilation, vasoconstriction, neurotransmitter release, and synaptic plasticity | Vane et al. (1998); Minghetti and Levi (1998); Smith et al. (2000); Minghetti (2004) |
| PGE ₂ , PGI ₂ | Vasodilation, vasoconstriction, inflammation, and apoptosis | Minghetti (2004); Simmons et al. (2004) |
| LTC ₄ , LTD ₄ , LTE ₄ | Vasoconstriction, inflammation, monocyte and T cell trafficking, and apoptosis | Powell and Rokach (2005); Funk (1996); Maccarrone et al. (2001) |
| PGD ₂ | Fever and pain | Chandrasekharan et al. (2002) |
| PGA ₂ , PGJ ₂ | Cell proliferation | Minghetti and Levi (1998); Smith et al. (2000) |
| Isoprostane, isoketal, isofuran | Oxidative stress | Morrow et al. (1991) |
| 4-Hydroxynonenal | Oxidative stress | Esterbauer et al. (1991) |
| Neuroprostane, neuroketal, neurofuran | Oxidative stress | Bernoud-Hubac et al. (2001) |
| 4-Hydroxyhexanal | Oxidative stress | Lee et al. (2000); Lin et al. (2005) |

 Table 1.4 Roles of arachidonic acid and docosahexaenoic acid-derived products in cerebrovascular systems

1.3.4 Lipoxins

Lipoxins (LXA₄, LXB₄, 15 epi-LXA₄, and 15 epi-LXB₄) are generated by the action of lipoxygenases on hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (HETE). Thus, lipoxins, a group of trihydroxyte-traene eicosanoids, are involved in the resolution of acute inflammation. They facilitate resolution by modulating key steps in leukocyte trafficking and preventing neutrophil-mediated acute tissue injury (Fig. 1.3) (Serhan et al., 2004; Kantarci et al., 2003; Yacoubian and Serhan, 2007). In neural and non-neural tissues, these mediators act through ALX and LXA receptors (Serhan and Levy, 2003; Serhan, 2005a; Chiang et al., 2005). Aspirin mediates the generation of lipoxins and promotes resolution of inflammatory reaction (Serhan, 2005a; Chiang et al., 2005).

Leukotrienes and lipoxins also play important roles in neural stem cell (NSC) functions. Thus, LTB₄ and LXA₄ regulate proliferation and differentiation of murine embryo brain NSC. Proliferation of NSCs is stimulated by LTB₄ and retarded by receptor antagonist. In contrast, LXA₄ and its aspirin-triggered stable analog, 15-epi-LXA₄, attenuate the growth of NSC at very low concentrations. Both lipoxygenase inhibitors and LTB₄ receptor antagonists produce apoptotic cell death in NSC. Gene chip analysis indicates that growth-related gene expressions such as epidermal growth factor receptor, cyclin E, p27, and caspase-8 are tightly regulated by LTB₄ and LXA₄ inducing the opposite gene expressions. In addition to proliferation, LTB₄ produces differentiation of NSC into neurons as monitored by neurite outgrowth and MAP2 expression. These studies indicate that LTB₄ and LXA₄ directly regulate proliferation and differentiation of NSC (Wada et al., 2006).

Lipoxins may be involved in the modulation of nociception. LXA_4 receptors are expressed on spinal astrocytes. The delivery of LXA_4 and its stable analogs to spinal cord attenuates inflammation-mediated pain process (Svensson et al., 2007). Furthermore, activation of extracellular signal-regulated kinase and c-Jun N-terminal kinase in astrocytes plays an important role in spinal nociceptive process, which is attenuated in the presence of lipoxins. This observation suggests the possibility that lipoxins regulate spinal nociceptive processing though their actions upon astrocytic activation. Targeting mechanisms that downregulate the spinal consequences of persistent peripheral inflammation provide a novel endogenous mechanism by which chronic pain may be controlled (Svensson et al., 2007).

1.4 Non-enzymic Oxidation of Arachidonic Acid

The accumulation of free fatty acids can trigger an uncontrolled "arachidonic acid cascade." This sets the stage for increased production of reactive oxygen species (ROS). ROS include oxygen free radicals (superoxide radicals, hydroxyl

and alkoxyl radicals, and lipid peroxy radicals) and peroxides (hydrogen peroxide and lipid hydroperoxide). At low levels, ROS can function as signaling intermediates in the regulation of fundamental cell activities such as growth and adaptation responses. At higher concentrations, ROS contribute to neural membrane damage when the balance between reducing and oxidizing (redox) forces shifts toward oxidative stress (Phillis et al., 2006). Free radical scavengers including superoxide dismutase, catalase, and glutathione control, in part, the elimination of ROS. In cerebrovascular arteries, ROS promote vascular tone and facilitate cerebral blood flow (Miller et al., 2006). Major determinants of ROS effect on cerebrovascular tone are the rate of superoxide production and its rate of utilization by superoxide dismutases. Although the major enzymic source of ROS in cerebral arteries has not been clarified, NADPH oxidases, along with cyclooxygenases and lipoxygenases, are the primary sources (Phillis et al., 2006; Sun et al., 2007). In cerebral arteries, activation of NADPH oxidase elicits both an increase in superoxide production and vasodilatation. NADPH oxidase-derived ROS partly contribute to flow-dependent dilatation, and may offset angiotensin II-induced constriction of cerebral arteries, consistent with the hypothesis that NADPH oxidase-derived ROS may play a physiological role in controlling the of cerebrovascular tone (Miller et al., 2006).

In brain tissue, biological targets of ROS are membrane lipids, proteins, and DNA (Farooqui et al., 2002). ROS may also modulate the expression of cytokines in the nucleus. These cytokines not only stimulate the activities of isoforms of PLA₂ and COX but also modulate their expression in reactive astrocytes located in the penumbra (Farooqui et al., 2002; Lin et al., 2004). Thus, an uncontrolled sustained increase in calcium ion influx through increased glycerophospholipid degradation can lead to increased membrane permeability and stimulation of many enzymes associated with lipolysis, proteolysis, and disaggregation of microtubules with disruption of cytoskeleton and membrane structure (Farooqui and Horrocks, 2007).

1.4.1 4-Hydroxynonenal (4-HNE)

Non-enzymic peroxidation of AA generates 4-hydroxynonenal (4-HNE), a nine carbon α , β -unsaturated aldehyde (Fig. 1.4). This aldehyde is one of the major end products of lipid peroxidation and an important mediator of neural cell damage because of its ability to covalently modify biomolecules with disruption of important cellular functions (Esterbauer et al., 1991; Lin et al., 2005; Farooqui and Horrocks, 2006). It reacts not only with lysine, cysteine, and histidine residues in proteins, but also with free amino acids, deoxyguanosine, and aminoglycerophospholipids (Esterbauer et al., 1991; Guichardant et al., 2002). The C3 position of 4-HNE is a highly reactive site that undergoes a Michael addition reaction with cellular thiols and hence readily forms adducts with glutathione or protein-containing thiol groups. 4-HNE may cause a



Fig.1.4 Chemical structures of non-enzymically derived lipid mediators of arachidonic acid and docosahexaenoic acid.

(a) 4-HNE; (b) isoprostane; (c) isoketal; (d) D4 neuroketal; (e) neuroprostane; (f) isofuran; (g) 4-HHE; and (h) 4-hydroxydodeca-(2E,6Z)-dienal, 4-HDDE

number of deleterious effects in cells including inhibition of DNA and RNA synthesis, disturbance in calcium homeostasis, and inhibition of mitochondrial respiration. The modification of adenine nucleotide translocator by 4-HNE causes the inhibition of enzymic activities and suppression of ADP and ATP transport through the inner mitochondrial membrane (Picklo et al., 1999). These events play a substantial role in the disruption of the energy-producing capacity of mitochondria.

In brain tissue, 4-HNE alters the function of key membrane proteins including glucose transporter, glutamate transporter, and sodium, potassium ATPases (Mark et al., 1997; Keller and Mattson, 1998; Lauderback et al., 2001). Inhibition of sodium, potassium ATPase by 4-HNE can result in the depolarization of neuronal membranes leading to the opening of NMDA receptor channels and influx of additional calcium ions into the cell (Kadoya et al., 2003). This calcium entry can be very harmful for neurons. 4-HNE not only inhibits rat brain mitochondrial respiration, but also blocks neurite outgrowth, disrupts neuronal microtubules, and modifies cellular tubulin, which may contribute to the cytos-keletal changes in neurons undergoing a neurodegenerative process (Neely et al., 1999; Farooqui et al., 2004). 4-HNE also decreases cellular ATP levels by impairing glucose transport and by depressing mitochondrial function (Keller

et al., 1997). In non-neural cells, it stimulates caspase-3 activity at low concentrations but inhibits it at higher concentrations. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSH-RD) activities are increased progressively with HNE concentrations (Larini et al., 2004). Collective evidence suggests that 4-HNE triggers multiple signaling cascades that variably affect cell growth, differentiation, and apoptosis.

1.4.2 Isoprostanes

Isoprostanes are prostaglandin-like mediators formed non-enzymically by free radical-catalyzed peroxidation of esterified AA in vivo (Fig. 1.4). The minimum requirement for the production of an isoprostane is a polyunsaturated fatty acid with three contiguous, methylene-interrupted double bonds (Basu, 2004; Nishio et al., 2006). The molecular mechanism by which isoprostanes are formed is analogous to the synthesis of prostaglandins by cyclooxygenases (Morrow et al., 1999). Unlike prostaglandins, the formation of isoprostanes in situ is initiated at the esterified arachidonic acid on the glycerophospholipid molecule (Fam and Morrow, 2003). Non-enzymic synthesis of the family of F₂-isoprostanes (Fig. 1.4) involves the formation of positional peroxyl radical isomers of arachidonic acid, which undergo endocyclization to form PGG₂-like compounds. These compounds are reduced to PGF₂-like compounds. F₂-Isoprostane (F₂-IsoP) is subsequently released in free form by the action of PLA_2 (Morrow et al., 1992; Fam and Morrow, 2003; Montuschi et al., 2007). Another mechanism of isoprostane generation starts with a 4-exocyclization of a peroxyl radical leading to an intermediate dioxetane (Durand et al., 2005). Measurement of F2-IsoP is considered to be one of the most reliable approaches for assessing oxidative stress status in vivo and is the most reliable index of in vivo lipid peroxidation.

Isoprostanes are vasoconstrictors in brain microvasculature. They exert potent biological actions both via receptor-dependent and receptor-independent mechanisms. F₂-IsoP exerts its receptor-mediated effect in vascular beds by promoting interactions between endothelial cells and monocytes (Lahaie et al., 1998; Fam and Morrow, 2003). Isoprostane-mediated monocyte adhesion does not depend on VCAM-1, but involves protein kinases such as protein kinase A and mitogen-activated protein kinase kinase 1. F₂-IsoP also modulates the p38 MAPK pathway during monocyte adhesion (Cracowski, 2004). Thus, F₂-IsoP not only affects vascular and bronchial smooth muscles function but also modulates cellular proliferation (Fam and Morrow, 2003). These processes may relate to inflammation and atherosclerosis. Receptor-independent action of F₂-IsoP is due to adduct formation. These compounds contain reactive α,β -unsaturated carbonyl group on the prostane ring, which readily reacts with thiol-containing compounds to produce many biological effects.

Similar to non-enzymic oxidation of arachidonic acid, in vivo and in vitro oxidation of eicosapentaenoic acid (EPA) generates F₃-IsoPs (Gao et al., 2006).

The amounts of F₃-IsoP formed are extremely large (up to $8.7 + 1.0 \mu g/mg$ EPA) and greater than levels of F₂-IsoPs generated from AA. EPA supplementation markedly reduces levels of arachidonate-derived F₂-IsoPs by up to 64%. These studies provide the evidence that F₃-IsoP is a novel in vivo oxidation product of EPA (Gao et al., 2006).

In isolated bovine retinae, isoprostanes have dual effects. Low concentrations of 8-isoPGF_{2 α} inhibit, whereas higher concentrations of 8-isoprostane stimulate K⁺-mediated [³H]D-aspartate overflow (Opere et al., 2005). The excitatory effect of 8-isoPGF_{2 α} is mimicked by thromboxane receptor agonist, U-46619, and retarded by thromboxane receptor antagonist, SQ 29,548. Pretreatment of retinae with the cyclooxygenase (COX) inhibitor, flurbiprofen, unmasks the inhibitory effect of high concentrations of 8-isoPGF_{2 α} on [³H]Daspartate release that can be attenuated by AH 6809 suggesting that 8-iso-PGF_{2 α} exhibits a dual regulatory effect on K⁺-induced [³H]D-aspartate release in isolated bovine retinae (Opere et al., 2005). The inhibitory effect of 8isoPGF_{2 α} may be due to the activation of EP1/EP2 receptors while the excitatory effects may be caused by the activation of thromboxane receptors.

1.4.3 Isoketals

The formation of isoketals occurs through the rearrangement of H₂-IsoP endoperoxides. Isoketals differ from isoprostanes in containing a characteristic aldehydic group in a 1,4-dicarbonyl array, making them extremely reactive toward primary amino groups in proteins (Boutaud et al., 2005). Unlike F₂-IsoP, isoketals result in modification of biologically important proteins rather than activation of specific receptors (Davies et al., 2004). Isoketals are highly reactive γ -ketoaldehydes that form pyrrole adducts with the ϵ -amino group of lysine residues on protein (Davies et al., 2004). These pyrrole adducts are unstable in the presence of oxygen and are further transformed to lactam and hydroxylactam adducts, which accumulate as stable end products. Collective evidence suggests that isoketals have remarkable ability to crosslink proteins through oxidation of the pyrrole. Oxidative stress activates COX to produce prostaglandin H₂, which can form two specific isomers of IsoK (levuglandin (LG) D_2 and E_2). Isoketals produce several effects in neural cells. They inhibit the activity of proteasomes in glial cells with an IC_{50} of 330 nM and induce cell death with an IC_{50} of 670 nM. Intra-hemispheric injections of 15-E2-IsoK disrupt the blood-brain barrier. Isoketals have been detected in tissues as well as in biological fluids.

1.4.4 Isofurans

Lipid peroxidation under high oxygen tension generates substituted tetrahydrofuran derivatives (Fessel et al., 2002). These compounds are called as isofurans (IsoF) (Fig. 1.4). The molecular mechanism of isofuran synthesis is not fully understood. However, two mechanisms are proposed: a cyclic peroxide cleavage pathway and an epoxide hydrolysis pathway. Oxygen concentration modulates the generation of isofurans. Increased oxygen concentrations favor the formation of isofurans and retard the formation of isoprostanes. Collectively these studies indicate that oxygen concentration differentially modulates the formation of isoprostanes and isofurans. These metabolites are present and readily detectable in normal fluids and tissues, and their levels are dramatically increased in animal model of oxidant injury and chronic neurodegenerative diseases (Fessel et al., 2002). It is proposed that combined measurement of IsoFs and IsoPs provides a more reliable index of oxidant stress severity. The generation of isofurans can be used as an important parameter for evaluating the effectiveness of antioxidant therapies (Roberts, II et al., 2005).

1.5 Enzymic and Non-enzymic Oxidation of DHA

DHA is a major component of neural membrane glycerophospholipids, where it is enriched in plasmalogens and phosphatidylserine. It provides an optimal lipid microenvironment for interactions between membrane proteins and lipid substrates associated with signal transduction processes (Horrocks and Farooqui, 2004). Among neural cell types, it is enriched in the photoreceptor cells. It has hypolipidemic, antithrombotic, and anti-inflammatory effects in neural and nonneural tissues. Since DHA is an essential fatty acid, it is obtained from diet. These best sources of DHA are fish and fish oil.

1.5.1 Enzymically Derived Lipid Mediators of DHA

In brain plasmalogen-selective PLA₂ releases DHA from neural membrane plasmalogens. The action of a lipoxygenase-like enzyme on DHA generates 10,17S-docosatrienes and 17S-resolvins (Hong et al., 2003; Marcheselli et al., 2003; Serhan, 2005c). These second messengers not only antagonize the effects of eicosanoids but also modulate leukocyte trafficking and downregulate the expression of cytokines in glial cells. They are collectively called as docosanoids. They possess potent anti-inflammatory, neuroprotective, and pro-resolving properties. EPA-derived mediators are resolvins of the E series, and those biosynthesized from DHA are resolvins of the D series (RvDs) and protectins.

1.5.2 Resolvins

Resolvin E1 (RvE1; (5S,12R,18R)-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid) is an oxygenase product derived from eicosapentaenoic acid that displays potent anti-inflammation/pro-resolution actions in vivo (Arita et al., 2006). RvE1 is an initial metabolite. The conversion of RvE1 to the oxo product inactivates RvE1. It is proposed that the designed RvE1 analog that resists further metabolism may be used as a useful tool to evaluate the actions of RvE1 in complex disease models (Arita et al., 2006). Isolation, identification, and bioactions of another resolvin, 5S,18-dihydroxy-eicosapentaenoic acid (resolvin E2, RvE2), have also been reported (Tjonahen et al., 2006). RvE2 blocks zymosan-induced polymorphonuclear (PMN) leukocyte infiltration and elicits potent anti-inflammatory properties in murine peritonitis. Similar to RvE1, human recombinant 5-lipoxygenase generates RvE2 from a common precursor of E series resolvins, namely, 18-hydroxyeicosapentaenoate (18-HEPE). Collective evidence suggests that RvE2, together with RvE1, may contribute to the beneficial actions of n-3 fatty acids in human diseases. It is stated that the 5-lipoxygenase in human leukocytes is a pivotal enzyme that generates both pro- and anti-inflammatory chemical mediators.

Aspirin impinges on these systems, triggering formation of the epimeric 17Rseries RvDs (denoted as aspirin-triggered RvDs), which possess bioactivity in vivo equivalent to that evoked by their 17S-series counterparts (RvDs). These bioactive lipid mediators open new avenues and approaches to therapeutic interventions via accelerated resolution of inflammation (Schwab and Serhan, 2006; Ariel and Serhan, 2007). The specific receptors for these bioactive lipid metabolites are found in neural and non-neural tissues. These receptors include resolvin D receptors (resoDR1) and resolvin E receptors (resoER1). Studies on isolation and characterization of these metabolites by lipidomics are in progress.

1.5.3 Protectins and Neuroprotectins

The protectins are a superfamily of lipid mediators that comprise docosatrienes and resolvins of the D series (Fig. 1.5). Aspirin initiates the synthesis of endogenous 18R-resolvins of the E series derived from EPA (i.e., RvE1), 17R-resolvins of the D series from DHA (AT-RvD1 through RvD4), and 17S-resolvins of the D series from DHA (RvD1 through RvD4). In vivo, these mediators display potent antiinflammatory activities that essentially resemble 17S-RvDs (Serhan, 2005c).

Oxidation of DHA by 15-lipoxygenase-like enzyme generates neuroprotectin D1 (10,17S-docosatriene) (Ariel and Serhan, 2007). This metabolite accumulates during reperfusion. The synthesis of this metabolite is enhanced by calcium ionophore A23187, IL-1 β , or the supply of DHA. The infusion of neuroprotectin D1 during reperfusion has neuroprotective effects. Thus, the expression of anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) is upregulated by neuroprotectin D1, whereas the expression of proapoptotic proteins, Bax and Bad, is downregulated by neuroprotectin D1. Moreover, neuroprotectin D1 inhibits oxidative stress-mediated activation of caspase-3 and COX-2 (Bazan, 2005a, b, c). Soluble amyloid precursor protein- α stimulates the synthesis of neuroprotectin D1 have not been



Fig. 1.5 Chemical structures of docosahexaenoic acid and docosanoids. (a) Docosahexaenoic acid; (b) 16,17S-docosatriene; (c) 10,17S-docosatriene; (d) 4S5,17S-resolvin; and (e) 7,16,17S-resolvin. These metabolites retard the actions of eicosanoids

characterized, their occurrence has been proposed (Hong et al., 2003; Marcheselli et al., 2003; Serhan, 2005c; Mukherjee et al., 2004) (Fig. 1.5).

Collectively, these studies suggest that the generation of resolvins and neuroprotectins may be an internal protective mechanism for preventing apoptotic cell death-mediated brain damage (Serhan, 2005d; Bazan, 2005b).

1.6 Non-enzymic Oxidation of Docosahexaenoic Acid

DHA is highly vulnerable to autoxidation because of the presence of six double bonds. Analogous to arachidonic acid-derived oxidation product, 4-HNE and isoprostane, DHA autoxidation generates 4-HHE and neuroprostane (NP) in in vivo and in vitro settings. The autoxidation of DHA is more complex than arachidonate resulting in the formation of primary or secondary end products like conjugated dienes and alkanes. Few autoxidation products (4-HHE and NP) have been characterized (Yin et al., 2005). The generation of NP esterified in glycerophospholipids may induce changes in biophysical properties of neural
membranes. This is particularly important for neurons because one of the functions of DHA is to provide and maintain neural membrane fluidity and permeability that facilitate interactions between DHA and proteins for optimal membrane function.

1.6.1 4-Hydroxyhexenal

Oxidation of DHA produces 4-hydroxy 2-hexenal (4-HHE) (Fig. 1.4). 4-HHE has a conjugated double bond between the α and β carbons; the γ carbon of 4-HHE is electron deficient and reacts readily with nucleophiles such as thiols and amines, while the carbonyl group forms Schiff bases with amino groups such as the N-termini of proteins and the ϵ -amino group of lysine. 4-HHE binds to proteins. These carbonyl derivatives are potential markers of oxidative stress (Yamada et al., 2004). In vivo protein-bound 4-HHE can be detected with monoclonal antibody HHE53 (MAb HHE53).

In spite of the structural similarity between 4-HNE and 4-HHE, the biological actions and efficacies of these aldehydes differ significantly. For example, 4-HHE acts more effectively on mitochondrial permeability transition than 4-HNE (Kristal et al., 1996). 4-HHE more effectively inhibits the mitochondrial ATP translocator than does 4-HNE (Picklo et al., 1999). Recent studies indicate that 4-HHE modulates endothelial nitric oxide synthase (iNOS) through NF- κ B activation (Lee et al., 2004b). In contrast, 4-HNE inhibits NF- κ B activation. These studies suggest that peroxidation of AA and DHA generates end products that have different effects on transcription factor activities of neural and non-neural tissues (Camandola et al., 2000; Lee et al., 2004b).

The occurrence of 4-hydroxydodecadienal (4-HDDE) has also been described. It is shown that 4-HNE, 4-HHE, and 4-HDDE covalently interact and bind to the primary amine moiety of ethanolamine glycerophospholipids, especially with the ethanolamine plasmalogen. Among these alkenals, 4-HDDE shows the highest reactivity with ethanolamine glycerphospholipids. The carboxylic acid metabolites of 4-HNE, 4-HHE, and 4-HDDE, 4-hydroxy-2E-hexenoic acid (4-HHA), 4-hydroxy-2E-nonenoic acid (4-HNA), and 4-hydroxy-2E,6Z-dodecadienoic acid (4-HDDA), respectively, are excreted in human urine and present in higher amounts in chronic diseases such as diabetes (Guichardant et al., 2006).

1.6.2 Neuroprostanes

Non-enzymic oxidation of DHA results in generation of neuroprostanes (NPs) (Roberts, II et al., 1998; Nourooz-Zadeh et al., 1999; Roberts, II and Fessel, 2004; Yin et al., 2005) (Fig. 1.4). Similarly, non-enzymic oxidation of eicosapentaenoic acid (EPA) generates F_3 isoprostane (Nourooz-Zadeh et al., 1997).

NPs have 22 carbons and 4 double bonds and are analogous to isoprostanes (Fig. 1.4). During NP synthesis, oxygen-mediated DHA radicalization generates peroxyl radicals, which undergo endocyclization followed by the addition of molecular oxygen and reduction to form the F ring of NP. As stated above that NP-containing glycerophospholipids may produce changes in neural membrane fluidity and permeability resulting in impairment in optimal neuronal function (Fam and Morrow, 2003; Yin et al., 2005; Greco and Minghetti, 2004).

No information is known about PLA_2 activity that releases NP from NPbound glycerophospholipids. F_4 -NP is the first characterized neuroprostane. It occurs in cerebrospinal fluid (CSF) from normal individuals. The levels of F_4 -NP are significantly increased in CSF from patients with Alzheimer disease (Reich et al., 2001). E_4 -NP and D_4 -NP are also detected in normal rat and human brains. Levels of E_4/D_4 -NP in normal brain were one-third compared to levels of F_4 -NP (Roberts, II and Fessel, 2004).

1.6.3 Neuroketals (NK)

Non-enzymic oxidation of DHA also generates neuroketals (NKs) (Bernoud-Hubac et al., 2001) (Fig. 1.4). NKs not only form lactam and Schiff base adducts, but also generate lysine adducts, suggesting that these metabolites may be involved in protein–protein cross-linking in brain tissue under oxidative stress. Neuroketal lysyl-lactam protein adducts are detected in nonoxidized rat brain synaptosomes at a level of 0.09 ng/mg of protein, which increase 19-fold following oxidation in vitro (Bernoud-Hubac et al., 2001). Neuroketal lysyl-lactam protein adducts are also detected in vivo in normal human brain at a level of 9.9 \pm 3.7 ng/g of brain tissue, but very little is known about their toxic and injurious effects on brain neuropathologies.

The collective evidence suggests that arachidonic acid and docosahexaenoic acid undergo non-enzymic oxidation with the generation of isoprostanes, isofurans, and neuroprostanes. These compounds may produce their neurochemical effects by intensifying oxidative stress in acute neural trauma and neurodegenerative diseases (Roberts, II and Fessel, 2004; Roberts, II et al., 2005; Farooqui and Horrocks, 2006). High levels of isoketals, neuroketals, isoprostanes, and neuroprostanes are reliable indices of oxidative stress in vivo (Roberts, II et al., 1998, 2005; Fam and Morrow, 2003).

1.7 Sphingolipid Metabolism in Brain

Sphingolipids include ceramide, ceramide 1-phosphate, sphingosine, and sphingosine 1-phosphate (Smith and Merrill, Jr., 2002) (Table 1.5). The metabolism of these lipid mediators is closely interconnected, in that a product of one enzyme serves as a substrate for another. Moreover, many cell stimuli regulate

| Substrate | Lipid mediator/metabolite | Reference |
|---------------|---------------------------|---|
| Sphingomyelin | Ceramide | Vaena de Avalos et al. (2004); Luberto et al. (2002) |
| | Ceramide 1-phosphate | Vaena de Avalos et al. (2004); Luberto et al.(2002) |
| | Sphingosine | Vaena de Avalos et al. (2004); Luberto et al. (2002) |
| | Sphingosine 1-phosphate | Vaena de Avalos et al. (2004); Luberto et al.(2002) |
| Cholesterol | 24-Hydroxycholesterol | Ong et al. (2003); Park et al. (2000) |
| | 25-Hydroxycholesterol | Ong et al. (2003); Park et al. (2000) |
| | 27-Hydroxycholesterol | Ong et al. (2003); Park et al. (2000) |
| | 7-Ketocholesterol | Ong et al. (2003); Park et al. (2000) |

 Table 1.5
 Sphingolipid and cholesterol-derived lipid mediators in brain

more than one of these enzymes. This adds to the complexity of regulation of sphingolipid mediators (Hannun et al 2001). These lipid mediators are associated with neural cell proliferation, differentiation, and growth (Smith and Merrill, Jr., 2002). They are also involved in signaling processes associated with cell cycle control, stress and inflammatory responses, and apoptosis (Pettus et al., 2004bb; El Alwani et al., 2006; Cuvillier, 2007).

1.7.1 Ceramide and Ceramide 1-Phosphate

Ceramide, the backbone of sphingolipids, is a well-characterized second messenger. It is synthesized by the de novo synthesis or in response to stress or agonists through the action of sphingomyelinases on sphingomyelin. It participates in numerous biological processes. In addition to serving as a precursor to complex sphingolipids, ceramides participate in a variety of cellular functions ranging from proliferation and differentiation to growth arrest, inflammation, stress responses, and apoptosis (Hannun and Obeid, 2002; Yu et al., 2000; El Alwani et al., 2006). As stated earlier, ceramides reside and function within lipid rafts. Ceramides are also implicated in cellular senescence, oxidative stress responses, and nitric oxide signaling (Mathias et al., 1998). Addition of ceramide produces a variety of effects in neural cells. Neurons are generally less sensitive to ceramide treatment than glial cells, and their response depends on the ceramide concentration (Luberto et al., 2002). Rat hippocampal and spinal cord motor neurons show a biphasic response. C6-ceramide produces cell survival at a low concentration, but at higher concentration promotes apoptosis. These observations indicate the importance of ceramide concentration in determining the survival or death of neural cells (Luberto et al., 2002; Hisaki et al., 2004). Collective evidence suggests that ceramide is an antagonist of cell growth and survival while diacylglycerol (DAG) is a pro-growth agonist of cell growth and survival (Ruvolo, 2001). These lipid mediators are regulated simultaneously but in opposite directions in the sphingomyelin cycle. Ceramide modulates signal transduction pathways that are not only associated with apoptosis but also involved in enhancement of inhibitory pathways to cell growth through the modulation of stress-activated protein kinase, SAPK, pathways. In contrast, DAG not only activates the classical isoform of PKC that is associated with cell growth and cell survival but also stimulates cell proliferation through mitogenactivated protein kinase, MAPK, pathways (Ruvolo, 2001).

Ceramide 1-phosphate (Fig. 1.6) is synthesized by ATP-dependent ceramide kinase. With natural ceramide as a substrate, ceramide kinase has a pH optimum of 6.0–7.5 and shows Michaelis–Menten kinetics, with Km values of 187



Fig. 1.6 Chemical structures of sphingolipid-derived lipid mediators. (a) Ceramide; (b) ceramide 1-phosphate; (c) sphingosine; and (d) sphingosine 1-phosphate

and 32 μ M for ceramide and ATP, respectively. Northern blot analysis reveals that ceramide kinase mRNA is highly expressed in the brain, heart, skeletal muscle, kidney, and liver (Sugiura et al., 2002). It is stimulated by Ca²⁺ and copurifies with neurotransmitter-containing vesicles, suggesting that ceramide 1-phosphate has a role in membrane fusion of brain synaptic vesicles, neurotransmitter release, and neutrophil phagolysosome formation (Sugiura et al., 2002).

Ceramide 1-phosphate has mitogenic properties. It inhibits apoptosis and induces cell survival. It is proposed that ceramide 1-phosphate and ceramide are antagonistic molecules that are interconverted in cells by kinase and phosphatase activities (Shinghal et al., 1993; Gómez-Muñoz, 2006). An appropriate balance between the levels of these two metabolites seems to be crucial for cell and tissue sphingolipid homeostasis. Switching this balance toward accumulation of one or the other may result in metabolic dysfunction that may induce disease processes. Therefore, the activities of enzymes that modulate ceramide 1-phosphate and ceramide 1-phosphate and ceramide 1-phosphate also enhances Ca²⁺ entry in a dose-dependent manner, and phospholipase C inhibitor (U73122) attenuates this response suggesting that ceramide 1-phosphate interacts with phospholipase C. Ceramide 1-phosphate not only invokes significant increase in the formation of inositol phosphates, but also stimulates PLA₂ activity (Pettus et al., 2004a).

1.7.2 Sphingosine and Sphingosine 1-Phosphate

Sphingosine is an 18 carbon amino alcohol with an unsaturated hydrocarbon chain (Fig. 1.6). It forms backbone of sphingolipids. It affects activities of many enzymes. It inhibits protein kinase C, Ca²⁺/calmodulin-dependent kinase, Na⁺/K⁺-ATPase, CTP:phosphocholine cytidylyltransferase, and PLC and stimulates the tyrosine kinase activity that is linked to epidermal growth factor receptor. Sphingosine 1-phosphate (Fig. 1.6) is formed by phosphorylation of sphingosine. This reaction is catalyzed by sphingosine kinase. Two sphingosine kinase isozymes (SphK1 and SphK2) are known to occur in mammalian tissues. Sphingosine 1-phosphate is the natural ligand for specific G-protein-coupled receptors (GPCRs) (Spiegel and Kolesnick, 2002). To date, five members, EDG-1/S1P₁, EDG-5/S1P₂, EDG-3/S1P₃, EDG-6/S1P₄, and EDG-8/S1P₅, have been identified (Spiegel and Kolesnick, 2002). These receptors are coupled to different intracellular second-messenger systems, including adenylate cyclase, phospholipase C, phosphatidylinositol 3-kinase/protein kinase Akt, mitogen-activated protein kinases, as well as Rho- and Ras-dependent pathways (Waeber et al., 2004). Activation of S1P1 receptor is associated with "inside-out" signaling in which cytokines – growth factors – stimulate cytosolic sphingosine kinase and mediate its translocation to the plasma membrane where its substrate sphingosine is located (Spiegel and Kolesnick, 2002). The generation of sphingosine 1-phosphate stimulates cell growth and survival not only through interactions with $S1P_1$ - $S1P_5$ receptors in autocrine/paracrine manner but also via other unknown intracellular effector, which is independent of cell surface S1P₁ receptors. Activation of G-protein-coupled sphingosine receptors (GPCRs) by sphingosine 1-phosphate or dihydrosphingosine 1-phosphate regulates many important processes, including cell migration, angiogenesis, vascular maturation, and neurite retraction (Brindley, 2004). Many studies have indicated that sphingosine 1-phosphate functions as a second messenger. It is involved in the regulation of calcium homeostasis, cell growth, and suppression of apoptosis. In many cases, the intracellular level of sphingosine 1-phosphate is associated with cell death and cell growth arrest (Spiegel and Kolesnick, 2002). Ceramide and sphingosine usually inhibit proliferation and promote apoptosis, while the further metabolite sphingosine 1-phosphate stimulates growth and suppresses apoptosis. The delicate equilibrium between the intracellular levels of each of these sphingolipids is modulated by enzymes that either generate or degrade these metabolites. Sphingosine kinase-1 is a crucial regulator of this two-pan balance, because it produces sphingosine 1-phosphate (a pro-survival mediator) and reduces the content of ceramide and sphingosine, the proapoptotic sphingolipids (Cuvillier, 2007). Because these metabolites are inter-convertible, it has been proposed that it is not the absolute amounts of these metabolites but rather their relative levels that determine cell fate (Maceyka et al., 2002).

1.8 Cholesterol Metabolism in Brain

The brain is the richest source of cholesterol in the body. Most brain cholesterol is present in myelin sheets and in cellular membranes. Cholesterol contents in brain are independent of dietary uptake or hepatic synthesis and are almost completely synthesized in situ (Jurevics and Morell, 1995). Neuronal cholesterol and distribution are necessary for optimal neural plasticity and synaptic transmission. In neural and non-neural cells, the depletion of cholesterol induces autophagy, a process by which cells digest their own components (Cheng et al., 2006; Clark et al., 2008). Autophagy is a homeostatic process for recycling of proteins and organelles that are increased during times of nutrient deprivation. Cholesterol depletion by methyl- β -cyclodextrin or mevastatin treatment is accompanied by a marked increase of lipidated microtubuleassociated protein light chain 3-II (LC3-II) as evidenced by immunoblotting and immunofluorescence microscopy. The increase of LC3-II by methyl- β -cyclodextrin can be prevented by phosphatidylinositol 3-kinase inhibitors and accompanied by dephosphorylation of mammalian target of rapamycin (Cheng et al., 2006). Electron microscopy indicates that autophagic vacuoles induced by cholesterol depletion are indistinguishable from those seen after amino acid starvation. These results demonstrate that a decrease in cholesterol activates autophagy by a phosphatidylinositol 3-kinase-dependent mechanism.



Fig. 1.7 Chemical structures of cholesterol and hydroxycholesterols. (a) Cholesterol; (b) 25hydroxycholesterol; (c) 24-hydroxycholesterol; (d) 27-hydroxycholesterol; and (e) 7-ketocholesterol

Brain contains cytochrome P450-dependent oxygenases that convert cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (Fig. 1.7). Cholesterol is also oxidized to cholesterol oxides and converted into cholesterol ester via acyl-CoA:cholesterol acyltransferase (Björkhem et al., 1998). Conversion of cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol is an important mechanism for the excretion of cholesterol from the brain. It promotes the maintenance of brain cholesterol homeostasis (Kolsch et al., 2001; Velazquez et al., 2006). Hydroxycholesterols are cytotoxic to neural and endothelial cells, which induce apoptotic cell death. In human neuroblastoma cells, SH-SYSY 24-hydroxycholesterol increases caspase-3 and decreases the number of viable cells (Kolsch et al., 2001). Caspase-3 is a cysteine-dependent endoprotease with specificity for aspartate residues in proteins. It is closely associated with apoptotic cell death. It causes proteolytic cleavage of a variety of variety of enzymes (protein kinase C, cytosolic phospholipase A₂, calcium-independent phospholipase A₂, phospholipase C), cytoskeletal proteins (α -spectrin, β -spectrin, actin, vimentin, Bcl-2 family of apoptosis related proteins), and DNA modulating enzymes (poly (ADP-ribose)polymerase) (Kolsch et al., 2001). Other oxysterol such as 25-hydroxycholesterol, 7β - hydroxycholesterol, and 7-ketocholesterol also produce toxic effects on neural cell cultures (Chang et al., 1998; Ong et al., 2003). The molecular mechanism associated with hydroxyl- and ketocholesterol-mediated toxic effect is not fully understood. However, 7-ketocholesterol is known to trigger the stimulation of NADPH oxidase, generation of superoxide anions, loss of mitochondrial transmembrane potential ($\Delta\Psi$ m), release of cytochrome c, and activation of caspase-3. These processes are closely associated with apoptotic cell death (Lizard et al., 2000). 7-Oxocholesterol not only modulates Ca²⁺ signals but also inhibits the phosphorylation of endothelial nitric oxide synthase and cPLA₂ (Millanvoye-Van Brussel et al., 2004). Collective evidence suggests that oxysterols have neurotoxic effects on neural cell cultures, and among them, 24-hydroxycholesterol can be used as a marker for neurodegeneration (Rojo et al., 2006).

1.9 Association of Lipid Mediators with Neurological Disorders

Alterations in neural membrane composition are known to occur in neurodegeneration associated with acute neural trauma (ischemia, spinal cord trauma, and head injury) and neurodegenerative diseases (Alzheimer disease). Changes in neural membrane composition are accompanied by significant increase in lipid mediators derived from glycerophospholipid and sphingolipid and in cholesterol-derived lipid mediators (Yu et al., 2000; Farooqui et al., 2001; Park et al., 2000). These lipid mediators play important roles in neural cell proliferation, cell cycle arrest, apoptosis, and angiogenesis modulating cell survival and neurodegeneration (Farooqui et al., 2001; Farooqui et al., 2007a; Park et al., 2000; Reiss et al., 2004). At present, it is unclear whether the generation of lipid mediators is the initiating point in neurodegeneration (primary target) or whether it is the end result of neurodegenerative process itself. Deleterious alterations in lipid homeostasis and levels of lipid mediators may be key factor in the onset and progression of neurodegenerative diseases (Farooqui and Horrocks, 2007). In recent years, we have been empowered by technological advances in lipidomics, proteomics, and genomics. Investigators are using these techniques not only to identify and determine levels of lipid mediators (F₂-isoprostanes, eicosanoids, lipoxins, docosanoids, nitrotyrosine, carbonyls in proteins, oxidized DNA bases, and 4-HNE) (Serhan, 2005b; Serhan et al., 2006; Adibhatla et al., 2006; Milne et al., 2006; Hunt and Postle, 2006; Morrow, 2006; Lu et al., 2006; Perluigi et al., 2005) but also for developing diagnostic test in CSF from patients with acute neural trauma and neurodegenerative diseases.

1.10 Conclusion

Membranes are essential, highly interactive, and dynamic components of neural cells. Without membranes the neurons and glia cannot assert their identity. They are made up of glycerophospholipids, sphingolipids, cholesterol, and

proteins. Neural membrane bilayer is penetrated by enzymes, receptors, and ion channels. In neural membranes glycerophospholipids, sphingolipids, and cholesterol are in a dynamics flux with continuous biosynthesis countered by continuous degradation. Enzymes of glycerophospholipid, sphingolipid, and cholesterol metabolism are regulated in response to extra- and intracellular stimuli and in turn serve as regulators of levels of bioactive lipids associated with cellular function.

Degradation of glycerophospholipids, sphingolipids, and cholesterol by phospholipases A_2 , sphingomyelinases, and cholesterol hydroxylases generates lipid mediators. These lipid mediators include eicosanoids, docosanoids, lipoxins, platelet activating factor, ceramide, ceramide 1-phosphate, sphingosine, and sphingosine 1-phosphate. In addition, non-enzymic degradation of glycerophospholipids generates a variety of lipid mediators. These mediators include 4-HNE, isoprostanes, isoketals, isofurans, 4-HHE, neuroprostanes, and neuroketal.

Lipid mediators play important roles in internal and external communication and modulate cellular responses such as growth, differentiation, adhesion, and migration. These processes are modulated by PLA₂/cyclooxygenase/ lipoxygenase-mediated generation of eicosanoids, docosanoids, and lipoxins. Action of sphingomyelinases on sphingomyeline generates ceramide, a metabolite closely associated with apoptotic cell death. Further degradation of ceramide generates sphingosine, which in its phosphorylated form induces cell proliferation, and thus produces the balance between cell death and cell survival. Collective evidence suggests that under normal conditions at low levels enzymically- derived lipid mediators are closely associated with neural cell survival, but high levels of enzymic and non-enzymic lipid mediators induce processes that are involved in neural cell injury and death. Thus, neural membranes are not only simple inert barrier, but a Pandora's box of lipid mediators, many of which have powerful neurochemical effects, some beneficial and others harmful (Bazan and Flower, 2002; Levant et al., 2006). Multiple forms of PLA₂, sphingomyelinases, and cholesterol-metabolizing enzymes play the role of Pandora and generate AA, DHA, lyso-glycerophospholipids, and ceramide. These products serve as intracellular second messengers themselves and also act as precursors for eicosanoids, docosanoids, platelet activating factor, ceramide 1phosphate, and sphingosine 1-phosphate. (Farooqui and Horrocks, 2009, 2006).

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Chapter 2 Interplay Among Glycerophospholipid, Sphingolipid, and Cholesterol-Derived Lipid Mediators in Brain: A Matter of Life and Death

2.1 Introduction

Neural membranes contain glycerophospholipids, sphingolipids, cholesterol and proteins. The distribution of these lipids within the neural membrane is irregular between the two leaflets. Glycerophospholipids and sphingolipids are responsible for lipid asymmetry, whereas cholesterol and sphingolipids facilitate the formation of lipid microdomains or lipid rafts. Neural membrane asymmetry is maintained by an aminoglycerophospholipid translocase that phosphatidvlserine (PtdSer) and phosphatidylethanolamine transports (PtdEtn) from outer to inner bilayer leaflet (Farooqui and Horrocks, 2007). Maintenance of proper lipid asymmetry is needed not only for the mechanical stability of neural membranes but also for the vesicular transport. At the same time, local or global changes in lipid asymmetry are essential for cell cycle progression, apoptosis, and platelet coagulation. In neural membrane lipid bilayer, glycerophospholipid and protein composition of outer and inner monolayer halves are quite different. Thus, ethanolamine glycerophospholipids and serine glycerophospholipids are located in the cytofacial site of inner monolayer, whereas choline glycerophospholipids are localized on the exofacial side of outer monolayer. Among the glycerophospholipids, PtdEtn, plasmenylethanolamine (PlsEtn), and PtdSer contain high levels of docosahexaenoyl groups (22:6n-3) at the sn-2 position of the glycerol moiety, whereas phosphatidylcholine (PtdCho), phosphatidylinositol (PtdIns), and phosphatidic acid (PtdH) contain high levels of arachidonovl groups (20:4n-6) (Farooqui et al., 2000a; Tillman and Cascio, 2003). Thus, glycerophospholipids are storage depot for arachidonic acid (AA) and docosahexaenoic acid (DHA). In neural membranes, levels of AA and DHA vary considerably in the various subclasses of glycerophospholipids. AA is distributed rather evenly in gray and white matter and among the different cell types in brain. In contrast, DHA is highly enriched in neuronal membranes including synaptic membranes. Different glycerophospholipids turn over at different rates with respect to their structure and localization in different cells and membranes. In neural membranes, glycerophospholipid homeostasis is based on a balance between glycerophospholipid catabolism via multiple forms of phospholipases A₂ (PLA₂) and resynthesis by the reacylation/deacylation cycle and de novo synthesis pathways (Farooqui et al., 2000a; Farooqui et al., 2000b). Similarly sphingolipids homeostasis involves the balance between sphingomyelinases and sphingomyelin synthetases and galactosyltransferases (Farooqui et al., 2007a). Finally, cholesterol homeostasis in brain is based on continuous synthesis of cholesterol and its conversion in hydroxycholesterols and cholesterol oxides.

In the brain tissue lipid mediators exert a diverse array of effects on cellular activities including those linked to homeostasis, immune responsiveness, and inflammation (Farooqui et al., 2007a). They not only function as intercellular mediators but also act as critical conduits of external stimuli in signal transduction cascades. Glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid-derived mediators and their receptors also may interact with other signaling molecules. Exogenous compounds such as cannabinoids share functionally relevant receptor-binding domains with those for endogenous lipid signaling ligands and have the potential to alter transductional cascades linked to immune functional activities (Farooqui et al., 2007a,b).

2.2 Generation of Glycerophospholipid-Derived Lipid Mediators

Two major mechanisms are involved in the release of AA and DHA from neural membrane glycerophospholipids. A direct mechanism for the release of AA involves paralogs of cPLA₂, and multiple forms of sPLA₂. The release of DHA from plasmalogens is catalyzed by plasmalogen-selective PLA₂ (PlsEtn-PLA₂). The other mechanism of AA release involves the phospholipase C (PLC)/ diacylglycerol lipase pathway (Farooqui et al., 1989) (Fig. 2.1). AA is metabolized into eicosanoids, which include prostaglandins (PG), leukotrienes (LT), and hydroxyeicosatetraenoic acids (HETE). DHA is metabolized to docosanoids, which include resolvins, protectins, and neuroprotectins (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Mukherjee et al., 2004). Lysophospholipid (1-Alkyl-2-lyso-*sn*-glycero-3-phosphocholine, lyso-PakCho) is a precursor of the platelet-activating factor (PAF) (Farooqui et al., 1997; Farooqui and Horrocks, 2006). Rate of generation and levels of these lipid mediators modulate neuroinflammation, neural cell proliferation, differentiation, gene expression, and apoptosis (Farooqui and Horrocks, 2009).

2.3 Enzymically-Derived AA Metabolites and Neuroinflammation

The production of prostaglandins, leukotrienes, and thromboxanes is catalyzed by PLA₂, cyclooxygenases (COX), and lipoxygenases (LOX). These enzymes occur in multiple forms. Stimulation of cPLA₂ and sPLA₂ releases AA from membrane glycerophospholipids (Murakami and Kudo, 2006; Lee et al., 2004). COX-1 and COX-2 enzymes oxidize AA to prostaglandin H₂ (PGH₂) (Fig. 2.2).



Fig. 2.1 Receptor-mediated release of arachidonate and docosahexaenoate and generation of enzymic and non-enzymic lipid mediators.

Plasma membrane (PM); agonists (A1, A2); receptors (R1, R2); phosphatidylcholine containing ether bond at the sn-1 position (PakCho); ethanolamine plasmalogen (PlsEtn); lysophosphatidylcholine containing ether bond at the sn-1 position (lyso-PakCho); lysoethanolamine plasmalogen (Lyso-PlsEtn); arachidonic acid (AA); docosahexaenoic acid (DHA); free fatty acid (FFA); platelet activating factor (PAF); 4-hydroxynonenal (4-HNE); 4-hydroxyhexenal (4-HHE); cPLA₂, cytosolic phospholipase A₂ (PLA₂); plasmalogen-selective phospholipase A₂ (PlsEtn-PLA₂); cyclooxygenase-2 (COX-2); secretory PLA₂ (sPLA₂); inducible nitric oxide synthase (iNOS); reactive oxygen species (ROS); nuclear factor kappaB (NF κ B); inhibitory subunit of NF- κ B (I- κ B); nuclear factor kappaB response element (NF- κ B-RE); tumor necrosis factor- α (TNF- α); interleukin-1 β (IL-1 β); interleukin-6 (IL-6); eicosanoid receptors (EP, DP, FP, TP, and IP); stimulation (+); and inhibition (-)

PGH₂ is a precursor to several prostaglandins, thromboxanes (TXA₂), and prostacyclins (PGI₂). In neurons, cPLA₂ is coupled to many receptors in G protein-dependent and independent manner. These receptors include NMDA, AMPA, P2X, acetylcholine, TNF- α , IL-1 β , and metabotropic type of glutamate receptors (Lazarewicz et al., 1990; Kim et al., 1995; Farooqui et al., 2006). In contrast, sPLA₂ is a secretory enzyme and has its own receptors. It binds to N-type receptors identified on neurons and M-type receptors found on skeletal muscles cell surface (Kolko et al., 2002; DeCoster et al., 2002). Thus sPLA₂ either acts extracellularly through its receptors or can be internalized to reach its intracellular targets (Sun et al., 2004). PLA₂ isoforms signaling pathway are functionally linked with both COX-1 and COX-2 during immediate and



Fig. 2.2 Generation of pro-inflammatory lipid mediators from neural membrane phospholipids in brain tissue.

Phosphatidylcholine containing ether bond at the sn-1 position (PakCho); cytosolic phospholipase A₂ (cPLA₂); arachidonic acid (AA); lysophosphatidylcholine (Lyso-PakCho); cyclooxygenase (COX); lipoxygenase (LOX); acetyltransferase (AT); hydroperoxyeicosatetraenoic acid (5-HPETE); 5-hydroxyeicosatetraenoic acid (5-HETE); Leukotriene (LT); prostaglandin G₂ (PGG₂); prostaglandin H₂ (PGH₂); prostaglandin E₂ (PGE₂); prostaglandin I₂ (PGI₂); thromboxane A₂ (TXA₂); coenzyme A (CoA); platelet activating factor (PAF). Notice that PGI₂, TXA₂, PGE₂, and PAF are pro-inflammatory (+) and lipoxins are anti-inflammatory (-)

delayed eicosanoid synthesis under normal physiological conditions. Three forms of COX enzymes designated as COX-1, COX-2, and COX-3 occur in mammalian tissues (Chandrasekharan and Simmons, 2004; Phillis et al., 2006). COX genes have been cloned from several mammalian tissues. It is not known whether these genes arose from an early single duplication event or from multiple independent duplications during evolution. The intron-exon arrangement of COX genes is completely conserved in vertebrate and invertebrate species. Exon boundaries contain four functional domains encoding (a) the aminoterminal hydrophobic signal peptide domain, (b) the dimerization domain, (c) the membrane-binding domain, and (d) the catalytic domain. The catalytic domain of each enzyme contains distinct peroxidase and cyclooxygenase active sites. All COXs are homodimers and monotopic membrane proteins involved in signal transduction processes in neural and non-neural tissues (Chandrase-kharan and Simmons, 2004; Phillis et al., 2006). COX-1 is constitutively expressed in brain tissue and is responsible for the physiological production of prostaglandins (Bazan et al., 1994). It is involved in several homeostatic processes, and therefore called a "housekeeping" enzyme. COX-2 is an inducible enzyme. Inflammatory mediators such as cytokines, growth factors, and bacterial endotoxin rapidly induce COX-2, which is normally undetectable in healthy tissues. COX-3 is an acetaminophen-sensitive isoform of the COX family (Phillis et al., 2006). Its RNA is derived through the retention of a highly structured, G + C-rich intron 1 of the COX-1 gene.

Under pathological situations a coordinated upregulation of isoforms and paralogs of PLA₂ occurs along with COX-2, COX-3, and LOX. This results in the generation of high levels of pro-inflammatory eicosanoids (Fig. 2.3) (Farooqui et al., 1999). The stimulation isoforms of PLA₂ along with COX and LOX enzymes involves NF- κ B-mediated induction of TNF- α , IL- β , and



Neural membrane PIsEtn/PtdSer

Fig. 2.3 Diagram showing generation of eicosanoids and modulation of their activities by docosanoids.

Cytosolic phospholipase A_2 (cPLA₂); arachidonic acid (AA); cyclooxygenase (COX); lipoxygenase (LOX); prostaglandins (PGs); leukotrienes (LTs); lipoxins (LXs); docosahexaenoic acid (DHA); plasmalogen-selective phospholipase A_2 (PlsEtn-PLA₂); and (-)indicates modulation sites. Notice that PGs and LTs are pro-inflammatory, whereas lipoxins, resolvins, and protectins are anti-inflammatory (-)

chemokines (Hayakawa et al., 1993; Farooqui and Horrocks, 2005; Kronke and Adam-Klages, 2002). The upregulation of isoforms of PLA₂, COX, and LOX can be blocked by inhibitors of PLA₂, COX, and LOX activities, respectively (Anthonsen et al., 2001). These inhibitors also attenuate TNF- α - and IL- 1β -stimulated NF- κ B activation. Exogenous addition of leukotriene B₄ (LTB₄) restores NF- κ B activation that is reduced by 5-lipoxygenase inhibitors or an LTB_4 receptor antagonist, thus identifying LTB_4 as a mediator in signaling to NF- κ B. TNF- α - and IL-1 β -induced AA release from neural membranes is accompanied by the phosphorylation of cPLA₂. Inhibitors of sPLA₂ and of 5-lipoxygenase/LTB₄ reduce AA release and completely abolish cPLA₂ phosphorylation. This observation not only suggests that sPLA₂, through 5-lipoxvgenase metabolites, is an essential upstream regulator of cPLA₂ and AA release, but also indicates the existence of a functional link between sPLA₂ and cytosolic PLA₂ in cytokine-activated non-neural cells providing a molecular explanation for the participation of both sPLA₂ and cPLA₂ in AA signaling and NF- κ B activation in response to pro-inflammatory cytokines (Anthonsen et al., 2001; Woo et al., 2000). Eicosanoids are autocoids, which act in neural and non-neural cells directly as well as through eicosanoid receptors. Four types of eicosanoid receptors (EP, DP, FP, TP, IP) have been cloned (Patrignani et al., 2005; Boie et al., 1997; Breyer et al., 2001; Narumiya et al., 1999; Waschbisch et al., 2006). These receptors have a protein with seven hydrophobic transmembrane segments and evoke cellular responses by distinct intracellular mechanisms (Omote et al., 2002; Nakayama et al., 2004; Minami et al., 1994). Leukotrienes exert their effects through three types of leukotriene receptors such as cysLT1 (LTD4), cysLT2 (LTC4), and hydroleukotriene BLT (LTB4) receptors. These receptors have been characterized and cloned. At the injury site, PGE_2 is involved in modulating the immune response while its proinflammatory signaling is associated with vascular and microglial cell activation (Zhang and Rivest, 2001). Some prostaglandins, PGE₁, PGE₂ and PGD₂ are inflammatory (Mohri et al., 2006), whereas others are anti-inflammatory, for example, PGD₂ and 15-deoxy- $\Delta^{12, 14}$ -prostaglandin J₂ (Itoh and Yamamoto, 2005). However, collective evidence suggests that high levels of eicosanoids contribute to the development of cytotoxicity, vasogenic brain edema, and neuronal damage, and these processes involve the participation of NF- κ B, isoforms of PLA₂, PLC, PKC, and cytokines (Phillis et al., 2006; Waschbisch et al., 2006).

Another class of prostaglandins (J series) is generated by progressive nonenzymic dehydration of PGD₂. Unlike other prostaglandins, the PGJ₂ family has no membrane receptors. Members of J series interact with nuclear peroxisome proliferator receptors (PPARs). Several isoforms of PPARs namely PPAR- α , PPAR- δ , PPAR- γ occur in mammalian tissues. PPAR- γ ligands, 15-deoxy- δ 12,14-prostaglandin J₂ (15-deoxy-PGJ₂), inhibits cell growth and induces apoptosis in two human neuroblastoma cells (SK-N-SH and SK-N-MC) in a PPAR- γ -dependent manner (Kim et al., 2003). 15-Deoxy-PGJ₂ upregulates the expression of proapoptotic proteins caspase-3, caspase-9, and Bax

but downregulates anti-apoptotic protein Bcl-2. 15-Deoxy-PGJ₂ also stimulates extracellular signal-regulated kinase 2 (ERK2). In addition, mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor, PD98059 (2'-amino-3'-methoxyflavone), decreases 15-deoxy-PGJ₂-mediated ERK2 activation and expression of PPAR- γ , capase-3, and cyclin B1 (Kim et al., 2003). Furthermore, MEK1/2 inhibitor PD98059 significantly retards the 15-deoxy-PGJ₂-mediated cell growth inhibition. PPAR- γ antagonist, GW9662 (2-chloro-5-nitro-N-phenylbenzamide), reverses the 15-deoxy-PGJ₂-mediated cell growth inhibition, PPAR- γ expression, and activation of ERK2. Collective evidence suggests that 15deoxy-PGJ₂ inhibits growth of human neuroblastoma cells via the induction of apoptosis in a PPAR- γ -dependent manner through activation of ERK pathway (Kim et al., 2003). Activation of PPAR isoforms also elicits anti-inflammatory activities in neural cells. Although the molecular mechanism involved in antiinflammatory process is not fully understood. However, it is reported that 15-deoxy-PGJ₂ reduces the phosphorylation of STAT1 and STAT3 as well as Janus kinase 1 (JAK1) and JAK2 in activated astrocytes and microglia (Park et al., 2003).

Leukotrienes are another family of paracrine hormones generated from the oxidative metabolism of arachidonic acid. LTC4, LTD4, and LTE4 are important signaling molecules that not only modulate inflammation, but also induce brain-blood barrier (BBB) disruption and brain edema (Wang et al., 2006). In vascular system leukotriene B4 is one of the most powerful chemotactic agent that modulates the recruitment of leukocytes. Microglial cells are pathologic sensors in the brain. Activated microglia are detrimental for brain tissue. It is proposed that leukotrienes, including cysteinyl leukotrienes (CysLTs) and their receptors (CysLT1 and CysLT2), play a very important role in the maintenance of neuroinflammation in ischemia and other neurological disorders (Sheng et al., 2006). N-methyl-D-aspartate (NMDA) injections upregulate CysLT1 receptor expression in neurons, and NMDA-mediated responses are inhibited by CysLT1 receptor antagonists, indicating that the increased CysLT1 receptor may be involved in NMDA-mediated neurotoxicity (Ding et al., 2006). In addition, cysteinyl leukotrienes are also associated with microcirculation (Häggstrom and Wetterholm, 2002).

The action of lipoxygenases on HPETE and HETE results in the generation of lipoxins (LXA₄ and LXB₄, 15-epi-LXA₄ or 15-epi-LXB₄). These lipid mediators are involved in the resolution of acute inflammation (Fig. 2.3) (Serhan, 1994; Serhan and Levy, 2003; Kantarci and Van Dyke, 2003). Lipoxins act through specific G protein-coupled receptors, ALX and LXA receptors (Norel and Brink, 2004; Chiang et al., 2005; Schwab and Serhan, 2006). ALX is the first cloned and identified lipoxygenase-derived eicosanoid receptor with cell typespecific signaling pathways. The activation of these receptors triggers the expression of a suppressor of cytokine signaling (SOCS-2). SOCS-2-deficient mice show uncontrolled synthesis of pro-inflammatory cytokines, aberrant leukocyte infiltration, and increased mortality (Machado et al., 2006). In the absence of LXA₄ biosynthetic pathway, the resulting uncontrolled inflammation can become lethal, despite pathogen clearance (Machado et al., 2006). Collective evidence suggests that lipoxins regulate cellular activities associated with inflammation and resolution (Serhan, 2005; Chiang et al., 2005). LXA₄ also serves as a "stop signal" that regulates key steps in leukocyte trafficking and prevents neutrophil-mediated tissue injury (Gronert, 2005; Kantarci and Van Dyke, 2003; Yacoubian and Serhan, 2007; Serhan, 2005; Chiang et al., 2005).

The action of a lipoxygenase-like enzyme on DHA produces 10,17S-docosatrienes and 17S-resolvins (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004). Aspirin triggers the formation of the epimeric 17R-series RvDs. They are denoted as aspirin-triggered-RvDs. In vivo, they possess biochemical activity similar to that evoked by their 17S-series counterparts (RvDs) (Schwab and Serhan, 2006). These second messengers are collectively called as docosanoids. They control the magnitude and duration of inflammation in neural and non-neural tissues (Ariel and Serhan, 2007). In addition, they may also promote the removal of chemokines from the tissue by apoptotic neutrophils and T cells during resolution. This process involves chemokine receptors (CCR2 and CCR5) and CCR5+ apoptotic leukocytes acting as "terminators" of chemokine signaling during the resolution of inflammation. Enzymes involved in docosanoid metabolism have not been characterized. However, it is becoming increasingly evident that these lipid mediators not only antagonize the effects of eicosanoids but also modulate leukocyte trafficking as well as downregulate the expression of cytokines in glial cells. Thus, detailed investigations are needed on endogenous and exogenous factors that modulate the generation of docosanoids in brain tissue.

As stated in Chapter 1, the non-enzymic oxidation of AA and DHA results in the generation of 4-hydroxynonenal, 4-hydroxyhexenal, isoprostanes, isoketals, isofurans, neuroprostanes, and neuroketals, 4-Hydroxynonenal is the breakdown product of arachidonic acid hydroperoxide, such as 15-hydroperoxy-eicosatraenoic acid and 13-hydroperoxy-octadecadienoic acid (Guichardant et al., 2004). Isoprostanes not only act via irreversible covalent binding to numerous proteins, but also by interacting with prostaglandin and thromboxane-like receptors. Their effects can be blocked by thromboxane receptor antagonists (Takahashi et al., 1992; Morrow et al., 1996; Opere et al., 2005; Morrow, 2006). Thromboxane receptors, like prostaglandin receptors, are linked to different sets of G-proteins resulting in distinct biological effects on brain and other body tissues (Lahaie et al., 1998). At submicromolar concentration, major cyclopentenone IsoP isomer, 15-A2t-IsoP potently induces apoptosis in neuronal cultures (Musiek et al., 2006). 15-A2t-IsoP-mediated neuronal apoptosis is associated with initial depletion of glutathione and upregulation of ROS generation, followed by 12-lipoxygenase activation and phosphorylation of extracellular signalregulated kinase 1/2 and the redox sensitive adaptor protein p66shc, which results in caspase-3 cleavage. 15-A2t-IsoP treatment also dramatically potentiates oxidative glutamate toxicity at low concentrations, demonstrating the functional importance of these molecules in neurodegeneration.

In addition, DHA binds to proteins and forms high molecular weight adducts. For example metal-catalyzed oxidation of bovine serum albumin and DHA results in formation of two adducts: one has a molecular mass of 71.5 kDA and the other with molecular mass of 93 kDa. Although, physiological significance of these results is not understood, but it is proposed that DHA– protein adduct formation may occur during normal aging process and neurodegenerative diseases (Liu et al., 2007).

2.4 Platelet-Activating Factor in Brain

PAF (1-O-alkyl-2-acetyl-*sn*-glycerophosphocholine), a potent pro-inflammatory agent is released from neural and non-neural cells under pathological situations (Snyder, 1995; Ishii et al., 2002; Tokuoka et al., 2003). PAF is synthesized from a specific subclass of PtdCho that contains an ether bond at the sn-1 position of the glycerol backbone (Snyder, 1995; Bazan, 2003). Three different pathways of PAF synthesis occur in mammalian tissues (Honda et al., 2002; Snyder, 1995). They include a remodeling pathway, de novo synthesis, and an oxidative fragmentation pathway. The degradation of PAF is catalyzed by platelet-activating factor hydrolase (PAF-AH), which converts it to inactive lyso-PAF. The balance between PAF biosynthesis and degradation determines its levels in various tissues (Honda et al., 2002; Snyder, 1995; Farooqui et al 2008).

PAF exerts its biological effects by activating the PAF receptors on neural and non-neural cell surfaces (Snyder, 1995; Maclennan et al., 1996; Ishii et al., 2002; Honda et al., 2002; Farooqui et al., 2008). The binding of PAF to PAF receptors results in the activation of diverse cellular functions (Fig. 2.4) and intracellular signal transduction pathways and processes associated with the stimulation of phospholipases A_2 , C, and D as well as increase in Ca^{2+} levels. The stimulation of these enzymes results in the generation of arachidonic acid, diacylglycerol, and inositol 1,4,5-trisphosphate. Arachidonic acid is metabolized to eicosanoids, diacylglycerol activates protein kinase C, and inositol 1,4,5-trisphosphate mobilizes calcium from intracellular stores (Izumi and Shimizu, 1995; Ishii and Shimizu, 2000). PAF receptor antagonists block all these processes. PAF also promotes transcriptional activation of a number of genes including immediate-early genes including c-fos, c-jun, and krox-24, cytokines, enzymes such as cyclooxygenase-2 and growth factors. In neuroblastoma cells, PAF treatment results in a 7- and 12-fold increase in c-fos mRNA in 15 and 30 min, respectively (Bazan et al., 1994; Tokuoka et al., 2003). The activation of these genes by PAF can be blocked by PAF antagonist, BN 52021. PAF stimulates the induction of prostaglandin synthase or cyclooxygenase (COX-2). Preincubation of cells with the PAF antagonist, BN 50730, blocks the induction of COX-2 (Bazan et al., 1997).

During the inflammatory process, PAF activates leukocytes tethered to the blood vessel wall via specific adhesion molecules expressed by endothelial cells.



Fig. 2.4 Roles of platelet-activating factor in brain

The physiological activity of PAF is not limited to its pro-inflammatory function. PAF is also involved in a variety of other settings including allergic reactions, brain function, and circulatory system disturbances such as atherosclerosis (Honda et al., 2002). Collective evidence suggests that PAF, under pathological conditions, behaves as a neuronal injury messenger by at least two mechanisms: (a) enhancing glutamate release and (b) sustained augmentation of COX-2 transcription (Bazan et al., 1997).

PAF has an acetyl group at the sn-2 position of its glycerol moiety. This acetyl group is essential for its pro-inflammatory activity. PAF acetylhydrolase blocks the pro-inflammatory effects of PAF by hydrolyzing the acetyl group. The anti-inflammatory effect of PAF acetylhydrolase is accompanied by inhibition of PAF-induced cellular responses including alterations in intracellular Ca^{2+} (Kuijpers et al., 2001). All these processes are closely associated with pathophysiology of neurological disorders (Farooqui et al., 2008).

2.5 Metabolism of Sphingolipid-Derived Lipid Mediators in Brain

Ceramide (N-acylsphingosine), a minor component of neural membranes, is the second messenger and precursor of all sphingolipids. Transfer of the head group from PtdCho to ceramide results in formation of sphingomyelin and diacylgly-cerol (DAG). This reaction is catalyzed by sphingomyelin synthetase (Fig. 2.5). In neural cells at the plasma membrane level, ceramide is synthesized either through the hydrolysis of sphingomyelin or de novo synthesis. The de novo synthesis of ceramide starts in the endoplasmic reticulum and continues in the Golgi apparatus and plasma membrane. The de novo synthesis of ceramide



Fig. 2.5 Relationship between glycerophospholipid and sphingolipid metabolism in neural and non-neural tissues.

Diacylglycerol (DAG); cytidylyltransferase (CT); phosphatidylcholine (PtdCho); cytidine monophosphate (CMP); phospholipase A_2 (PLA₂); lysophosphatidylcholine (lyso-PtdCho); arachidonic acid (AA); acetyltransferase (AT); ceramide 1-phosphate (Cer 1-P), sphingosine (Sph); sphingosine 1-phosphate (Sph-1-P); platelet-activating factor (PAF); sohingomyelinase (SMase); PtdCho:ceramide phosphocholine transferase (sphingomyelin synthase) (SMS); stimulation (+); and inhibition (-)

starts with the condensation of serine with palmitoyl-CoA. This reaction is catalyzed by the serine palmitoyltransferase. It results in the generation of 3-ketodihydrosphingosine. This metabolite is reduced to dihydrosphingosine by 3-ketoreductase. Acylation of dihydrosphingosine to dihydroceramide is catalyzed by dihydroceramide synthase. Introduction of a 4,5 double bond in the sphingoid base in dihydroceramide is catalyzed by a specific desaturase (Smith and Merrill, 1995; Smith and Merrill, 2002; Vaena de Avalos et al., 2004; Gómez-Muñoz, 2006). The transport of ceramide from endoplasmic reticulum to Golgi apparatus by ceramide transport protein CERT is the crucial step in the biosynthesis of ceramide (Perry and Ridgway, 2005). Ceramide can also be synthesized through other enzymic pathways such as the acylation of sphingosine, hydrolysis of sphingomyelin by sphingomyelinases (SMases) (Clarke et al., 2006; Marchesini and Hannun, 2004), and dephosphorylation of ceramide 1-phosphate (Vaena de Avalos et al., 2004).

In neural cell cultures, low concentrations of ceramides promote differentiation and neuritic outgrowth through its action on p75 low-affinity NGF receptor, p75^{NTR} (Barrett, 2000; Mamidipudi and Wooten, 2002; Song and Posse de Chaves, 2003; Arévalo and Wu, 2006), and the TNF- α receptor, p55 (Adam-Klages et al., 1998). The ceramide-mediated effect on the p75^{NTR} low-affinity NGF receptor not only modulates axonal growth but also promotes myelination (Cosgaya et al., 2002; Roux and Barker, 2002).

Ceramide decreases the phosphorylation of phosphoproteins by stimulation of ceramide-activated protein phosphatase (CAPP) (Galadari et al., 1998). In non-neural cell cultures, ceramide suppresses insulin-induced activation of membrane-associated protein kinase $C\zeta$ (Miura et al., 2003), indicating that ceramide regulates phosphorylation of proteins involved in signal transduction processes. In microglial cells, C8-ceramide enhances the secretion of brainderived neurotrophic factor (BDNF) without induction of TNF- α , IL-1 β , and nitric oxide (Nakajima et al., 2002). Ceramide-induced BDNF secretion is mediated by protein kinase $C\delta$ (PKC δ and/or ε , but not by extracellular signal-regulated kinase (ERK), c-Jun n-terminal kinase (JNK), p38, NF κ B, or cAMP response element-binding transcription factor (CREB). In addition, ceramide also interacts and binds with protein kinases C- α , protein kinases C- δ (Muller et al., 1995; Huwiler et al., 1998) cPLA₂ (Huwiler et al., 2001), and endosomal cathepsin D (Heinrich et al., 1999) and modulates signal transduction processes.

A higher concentration of ceramide is necessary for membrane blebbing and other morphological changes associated with apoptosis (Fig. 2.6). At high concentration, ceramide activates caspase-3 and the protease responsible for the cleavage of polyADP-ribose polymerase (De Stefanis et al., 2002). Activation of caspase-3 results in apoptotic cell death in various types of neuronal and glial cultures. Caspase-3 hydrolyzes a number of proteins related to signal



Fig. 2.6 Roles of ceramide and ceramide 1-phosphate in brain

transduction and glycerophospholipid metabolism such as protein kinase C, cPLA₂, iPLA₂, PLC, and cytoskeletal proteins such as α -spectrin, β -spectrin, actin, vimentin, members of the Bcl-2 family of apoptosis related proteins, presenilins, amyloid precursor protein, and DNA modulating enzymes (Farooqui et al., 2004). The degradation of these proteins results in abnormal signal transduction and key morphological changes that result in apoptotic cell death.

Ceramidase cleaves the amide-linked fatty acid and generates sphingosine and free fatty acid. Sphingosine is further phosphorylated to sphingosine 1-phosphate by sphingosine kinase (Vaena de Avalos et al., 2004). Phosphorylation of ceramide by ceramide kinase generates ceramide 1-phosphate, which is an important lipid mediator of sphingolipid metabolism (Vaena de Avalos et al., 2004). Ceramide 1-phosphate has mitogenic properties and is potent inhibitor of apoptotic cell death. Collective evidence suggests that ceramide and ceramide 1-phosphate are antagonistic molecules that can be converted into each other by phosphorylation and dephosphorylation processes (Gómez-Muñoz, 2006). It is proposed that the ratio between ceramide and ceramide 1-phosphate is crucial for cell survival. Switching optimal balance toward accumulation of one or the other may cause metabolic dysfunction associated with diseases processes. Thus, ceramide 1-phosphate to ceramide ratio may be considered as a switch between life and death (Gómez-Muñoz, 2006).

Ceramide is metabolized by glycosylation, acylation, or by catabolism to sphingosine, which is then phosphorylated to the anti-apoptotic sphingosine 1-phosphate. In nuclear envelop, sphingosine increases the synthesis of phosphatidic acid, whereas in other subcellular fractions phosphatidic acid synthesis is decreased (Baker and Chang, 2001). Increased synthesis of phosphatidic acid in cerebral cortex nuclear envelop is inhibited by PtdIns-specific PLC inhibitors suggesting the participation of diacylglycerol kinase in the generation of phosphatidic acid.

Sphingosine metabolite, sphingosine 1-phosphate modulates proliferation, differentiation, cell migration, regulation of calcium homeostasis, neurite retraction, angiogenic vascular maturation, and cytoskeleton dynamics (Fig. 2.7) (Xin et al., 2004; Donati and Bruni, 2006). Sphingosine 1-phosphate regulates cellular processes by binding to five specific G protein coupled cell surface receptors (GPCRs) called Edg receptors have now been renamed to sphingosine 1-phosphate receptors (S1P₁, S1P₂, S1P₃, S1P₄, S1P₅). As stated in Chapter 1 these receptors are associated with numerous cellular functions.

Thus, S1P₁ and S1P₃ receptors enhance endothelial and vascular smooth muscle cell proliferation and migration associated with pathological angiogenesis (Waeber et al., 2004). In contrast, S1P₂ receptors prevent the migration of endothelial and vascular smooth muscle cells. S1P receptors also modulate the relaxation and constriction of blood vessels. These effects involve the participation of phosphatidylinositol 3-kinase/Akt/endothelial nitric oxide synthase (eNOS) pathway. Finally, sphingosine 1-phosphate also protects endothelial cells from apoptosis through S1P₁ and S1P₃ receptor-mediated stimulation of phosphatidylinositol 3-kinase/Akt/eNOS pathway.



Fig. 2.7 Roles of sphingosine and sphingosine 1-phosphate in brain

Despite of occurrence of S1P receptors, sphingosine 1-phosphate is involved in intracellular events that are independent of S1P receptors. In renal mesangial cells sphingosine 1-phosphate cross-activates the TGF- β signaling cascade, which results in activation of at least three Smad proteins (Smad-1,-2,-3) with subsequent gene transcription that promotes antiinflammatory processes (Xin et al., 2004). Under normal conditions, levels of sphingosine 1-phosphate are very low. However, treatment with growth factors results in increased levels of this lipid mediator in brain tissue. These results strongly suggest that sphingolipid-derived lipid mediators interact with glycerophospholipids and their lipid mediators, and modulate gene transcription associated with neural cell survival and neurodegeneration (Farooqui et al., 2007a).

2.6 Neurochemical Effects and Roles of Ceramides

Ceramides exert their biological activity not only through their metabolites but also through the induction of changes in membrane structure and organization caused by their physicochemical properties. Thus, ceramide displaces cholesterol in artificial membranes. This results in tight lipid packing of membrane components. Minimizing the exposure of cholesterol and ceramide to water may be a strong driving force for the association of ceramide with lipid rafts. It is proposed that the displacement of cholesterol by ceramide produces marked changes in molecular composition, liquid ordered properties, and function of lipid rafts (Megha and London, 2004; Megha et al., 2007).

Ceramides participate in a variety of cellular functions ranging from proliferation and differentiation to growth arrest, inflammation, and apoptosis (Fig. 2.6) (Hannun and Obeid, 2002; Yu et al., 2000; El Alwani et al., 2006). Ceramides are also implicated in cellular senescence, oxidative stress responses, and nitric oxide signaling (Mathias et al., 1998). The generation of endogenous ceramide is regulated by various agonists, which include TNF- α , CD95 (APO-1/Fas), ionizing and ultraviolet radiation, and chemotherapeutic drugs. The targets for ceramide include specific kinases, phosphatases, phospholipases, cyclooxygenases, and various transcription factors including AP1, NF- κ B, and IL-6 (Ohanian and Ohanian, 2001; Sawai et al., 2005). Ceramide also blocks calpain activation and $A\beta$ (beta-amyloid) neurotoxicity in cortical neurons. The molecular mechanism associated with ceramide-mediated effects is not fully understood. However, based on in vitro studies on cortical neurons isolated from wild-type and p35 knockout mice, it is proposed that ceramide regulates A β cell toxicity in a p35/cdk5-dependent manner (Seyb et al., 2007).

2.7 Generation of Cholesterol-Derived Metabolites in Brain

Most brain cholesterol is present in myelin sheets and in cellular membranes. Total cholesterol levels steadily increase between ages 20 and 65 years in both men and women. After 65 years, cholesterol levels begin to decrease. Glial cells (astrocytes) synthesize and secrete 2- to 3-fold more cholesterol than neurons, and secrete it as lipoprotein particles which serve as cholesterol carriers (DeMattos et al., 2001; Vance et al., 2005). Although neurons produce enough cholesterol to survive and grow, but the formation of numerous mature synapses during synaptogenesis demand additional amounts that must be provided by glia. Thus, the availability of cholesterol appears to limit synapse development. This may explain the delayed onset of synaptogenesis after glia differentiation and neurobehavioral manifestations of defects in cholesterol or lipoprotein homeostasis (Mauch et al., 2001; Pfrieger, 2003). Collective evidence suggests that fine-tuning neural cholesterol dynamics are essential for basic synapse function, plasticity, and behavior. In addition, cholinergic function, ionotropic and metabotropic receptor machinery, excessive tau phosphorylation, the change of amyloid β biochemistry, neural oxidative stress reactions, and other features of neurodegeneration are also modulated by brain cholesterol homeostasis (Nelson and Alkon, 2005).

During brain development, neurons reduce or even abandon cholesterol synthesis to save energy and import cholesterol from astrocytes via apolipoprotein particle (Pfrieger, 2003). Brain tissue efficiently recycles cholesterol, but with a surplus of brain cholesterol due to continuing synthesis and/or neuronal death, there is a need to eliminate cholesterol from brain. Cholesterol elimination mechanisms include (a) transportation via apolipotrotein E, (b) conversion of cholesterol to 24-hydroxycholesterol, 25hydroxycholesterol, 27-hydroxycholesterol, (c) conversion of cholesterol to cholesterol oxides, and (d) conversion of cholesterol into cholesterol ester via acyl-CoA:cholesterol acyltransferase (Björkhem et al., 1998). Conversion of cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol is not only an important mechanism to eliminate cholesterol from the brain but also a process that is closely associated with the maintenance of brain cholesterol homeostasis (Kölsch et al., 2001; Velázquez et al., 2006). In brain, cholesterol 24-hydroxylase is expressed almost exclusively in neurons. This enzyme converts cholesterol into 24-hydroxycholesterol (Famer et al., 2007). As stated earlier, this is a major pathway for excretion of excess cholesterol from the brain. There is a significant flux of another oxysterol, 27-hydroxycholesterol from the circulation into the brain. It is becoming increasingly evident that polymorphisms within the CYP46A1 gene, which encodes cholesterol 24-hydroxylase, may be involved in AD. Studies on the effects of 24-hydroxycholesterol and 27-hydroxycholesterol on the α - and β -secretase activity in the human neuroblastoma cell line SH-SY5Y have shown that these oxysterols regulate levels of extra- and intracellular secreted APP α by increasing the α -secretase activity as well as the α/β -secretase activity ratio. It is proposed that the ratio between 24-hydroxycholesterol and 27-hydroxycholesterol is of an important factor for the generation of β -amyloid in the brain (Famer et al., 2007). In brain, these oxysterol also inhibit HMG-CoA reductase activity and in astrocytes, 25-hydroxycholesterol has a dual effect on cell proliferation: at higher concentrations it produces cell proliferation in a dose-dependent manner, while at lower concentrations it promotes neural cell survival (Velázquez et al., 2006).

In brain tissue, 24-hydroxycholesterol and 25-hydroxycholesterol regulate cholesterol homeostasis by two different mechanisms. First, these metabolites are potent inhibitors of cholesterol biosynthesis and LDL receptor activity through inhibition of the sterol regulatory element binding protein (Brown and Goldstein, 1997). Second, they regulate cholesterol metabolism through binding to the orphan nuclear liver X receptor α (LXR α) (Rebeck, 2004; Velázquez et al., 2006). The presence of 24-hydroxycholesterol and 25-hydroxycholesterol in plasma is an indication of neurodegeneration in brain (Lizard et al., 2000; Kölsch et al., 1999; Reiss et al., 2004).

2.8 Interactions Among Phospholipid, Sphingolipid, and Cholesterol-Derived Lipid Mediators

Although considerable progress has been made on the metabolism, uptake, and distribution of glycerophospholipids, sphingolipids and cholesterol in brain tissue, but mechanisms involved in compartmentalization of glycerophospholipids, sphingolipid and cholesterol still remain an enigma. The organization and compartmentalization of glycerophospholipids, sphingolipids, and cholesterol provide neural membranes with structural and functional integrity that facilitates the appropriate interactions with integral membrane proteins. Under normal conditions, interactions among glycerophospholipid, sphingolipid, and cholesterol-derived metabolites modulate regular cellular function through signal transduction processes associated with communication and control adaptive responses involved in neural cell survival (Ivanova et al., 2004). However, under pathological conditions, high levels of glycerophospholipid, sphingolipid, and facilitate neurodegeneration.

2.8.1 Interactions Between Glycerophospholipid and Sphingolipid Metabolism

Platelet-activating factor (PAF), which is generated through acetylation of a lysophospholipid, stimulates the breakdown of sphingomyelin and the release of ceramide (Lang et al., 2005). In erythrocytes, this process is associated with apoptotic cell death. The molecular mechanism involved in apoptotic cell death in this system is not fully understood. However, PAF-mediated activation of sphingomyelinase and generation of ceramide may lead to the activation of scramblase with subsequent phosphatidylserine exposure, a process associated with apoptotic cell death (Lang et al., 2005).

Sphingosine 1-phosphate, a sphingolipid lipid mediator, is metabolized into phosphoethanolamine and hexadecanal. Both these metabolites are prerequisite for the synthesis of glycerophospholipids (Park et al., 2006). Sphingosine 1-phosphate and vascular endothelial growth factor (VEGF) promote endothelial cell (EC) migration and angiogenesis (Bernatchez et al., 2003). Treatment of bovine aortic endothelial cells (BAEC) with sphingosine 1-phosphate results in a dose- and time-dependent increase (3.3-fold) in PAF synthesis. Inhibitors of p38 mitogen-activated protein kinase (MAPK), cPLA₂, and sPLA₂ activities can attenuate this biological response. This suggests that p38 MAPK activation by sphingosine 1-phosphate promotes the conversion of membrane phospholipids into PAF through the combined activation of cPLA₂ and sPLA₂ and acetyl transferases. Interestingly, pretreatment of BAEC with extracellular PAF receptor antagonists, BN52021 and CV3988, reduces sphingosine-mediated migration by up to 42%. This suggests that interactions between sphingosine
1-phosphate and PAF synthesis may contribute to sphingosine 1-phosphatemediated chemotactic activity (Bernatchez et al., 2003).

PtdCho supplies the phosphocholine moiety for sphingomyelin synthesis (Fig. 2.5) (Hannun and Obeid, 2002; Smith and Merrill, 1995). This phospholipid inhibits several lipolytic enzymes including isoforms of PLA₂ (Singh and Subbaiah, 2007; Singh et al., 2007). Fatty acids located at the sn-2 position of PtdCho are used for the synthesis of sphingoid base and ceramide (Meyer et al., 2005). Furthermore, in rat brain slices sphingomyelinase and ceramide decrease the levels of plasmalogens through the activation of PlsEtn-PLA₂ (Latorre et al., 2003). The decrease in plasmalogens by sphingomyelinase or ceramide is prevented by quinacrine, ganglioside, and bromoenol lactone, which are inhibitors of plasmalogen-selective PLA₂ activity (Latorre et al., 2003; Farooqui and Horrocks, 2001). The addition of the caspase-3 inhibitor, acetyl-L-aspartyl-Lglutamyl-L-valyl- L-aspartyl-chloromethylketone (Ac-DEVD-CMK), partially blocks the ceramide-induced stimulation of plasmalogen-selective PLA₂ without altering sphingomyelinase-elicited ceramide accumulation (Latorre et al., 2003; Farooqui et al., 2004). Similarly another sphingolipid metabolite, psychosine (galactosylsphingosine), not only decreases plasmalogen content and the expression of alkyl-dihydroxyacetone phosphate synthase but also reduces levels of glutathione and ATP in C-6 glial cells (Khan et al., 2005) supporting our view on interactions and cross talk between glycerophospholipid and sphingolipid metabolism in maintaining the function of neural membranes (Kihara and Igarashi, 2004). In human myeloid leukemia cells, diacylglycerol opposes ceramide-mediated apoptosis (Jarvis et al., 1994) suggesting that diacylglycerol, a protein kinase C activator, may have a cytoprotective function in survival of leukemic cells.

2.8.2 Interactions Between Glycerophospholipid and Sphingolipid-Derived Lipid Mediators

The hydrolysis of glycerophospholipids and sphingomyelin by PLA₂ and SMases (acid and neural), the synthesis of glycerophospholipids-derived lipid mediators (eicosanoids and platelet activating factor), generation of sphingolipid-derived lipid mediators (ceramide 1-phosphate, sphingosine 1-phosphate), and production of cholesterol-derived mediators (24-hydroxycholesterol and 25-hydroxycholesterol) are closely associated with neural cell survival as well as apoptotic cell death (Farooqui et al., 2004; Hannun and Obeid, 1995). Sphingomyelin has been shown to inhibit activities of isoforms of PLA₂ (Singh and Subbaiah, 2007; Singh et al., 2007). This inhibition may be due to structural similarity with PtdCho and binding of sphingomyelin with catalytic site in the enzyme molecule. A close interaction between the cPLA₂-generated second messenger, AA, and the SMase-generated second messenger, ceramide, occurs at several sites in signal transduction processes induced by the cytokines, tumor



Fig. 2.8 Interactions between glycerophospholipid and sphingolipid-derived lipid mediators. Tumor necrosis factor- α (TNF- α); agonist (A); phosphatidylcholine (PtdCho);lysophosphatidylcholine (lyso-PtdCho); cytosolic phospholipase A₂ (cPLA₂); diacylglycerol (DAG); phosphatidycholine specific phospholipase C (PLC); protein kinase C (PKC); arachidonic acid (AA); cyclooxygenase-2 (COX-2); and reactive oxygen species (ROS)

necrosis factor, and interleukin-1 β (Fig. 2.8) (Vanags et al., 1997; Robinson et al., 1997; Colquhoun, 1998; Malaplate-Armand et al., 2006).

In neural and non-neural cell cultures, AA stimulates SMase (Robinson et al., 1997) and ceramide stimulates PLA_2 activity (Hayakawa et al., 1993, 1996; Sato et al., 1999; Farooqui et al., 2000b; MacEwan, 1996; Jayadev et al., 1997; Malaplate-Armand et al., 2006). This interplay between metabolite of glycerophospholipid and sphingolipid metabolism can be blocked with $cPLA_2$ and SMase inhibitors as well as by their antisense oligonucleotide (Vanags et al., 1997; Gomez-Muñoz, 1998) suggesting that this is an important event in glycerophospholipid and sphingolipid metabolism.

Ceramide 1-phosphate mediates AA release induced by calcium ionophore and interleukin-1 β through the translocation and activation of cPLA₂ (Pettus et al., 2004a). Ceramide 1-phosphate produces a dramatic increase (>15-fold) in cPLA₂- α activity (Fig. 2.7). This activation is highly specific. With the exception of PtdIns 4,5-bisphosphate, the addition of no other lipid has a significant effect on cPLA₂- α activity (Subramanian et al., 2005). The effect of ceramide 1-phosphate on cPLA₂ is through its interaction with the CaLB/C2 domain of cPLA₂ and facilitates its translocation. These interactions require Ca²⁺. In the absence of Ca²⁺, ceramide 1-phosphate is not able to activate cPLA₂ (Pettus et al., 2004a; Pettus et al., 2003; Subramanian et al., 2005; Nakamura et al., 2006).

In H441 cells, tumor necrosis factor TNF- α induced inhibition of CTP:phosphocholine cytidylyltransferase activity involves C2 ceramide (Awasthi et al., 2001). The inhibiting action of C2 ceramide on CTP:phosphocholine cytidylyltransferase requires protein kinase C- α , p38 mitogen-activated protein kinase, and cPLA₂. In H441 cell cultures the actions of C2 ceramide on CTP:phosphocholine cytidylyltransferase activity can be duplicated by the addition of lyso-PtdCho. Furthermore, the effects of C2 ceramide also depends on 5-lipoxygenase. It is proposed that CTP:phosphocholine cytidylyltransferase activity is inhibited by the lyso-PtdCho generated as a consequence of protein kinase C- α and p38 mitogen-activated protein kinase-mediated activation of cPLA₂. The other product of the activation of cPLA₂ (arachidonic acid) is a substrate for the synthesis of leukotrienes, which raise intracellular Ca²⁺ levels and complete the activation of cPLA₂ (Awasthi et al., 2001).

Ceramide also inhibits PLD activation and generation of phosphatidic acid in neural and non-neural cell cultures suggesting that PLD is a target of ceramide. Thus in L6 cells, fumonisin (a mold toxin) increases PLD activity, whereas C6ceramide decreases it. C6-ceramide selectively decreases the expression of PLD1 mRNA transcripts and addition of ceramide synthesis inhibitors increases the PLD1 mRNA transcripts (Mebarek et al., 2007). Sphingosine, a cationic amphiphilic metabolite associates itself with negatively charged polar head group of glycerophospholipids, which facilitate and stimulate PLD2 enzymic activity. Collective evidence suggests that ceramide regulates myogenesis through downregulation of PLD1 expression and activity (Mebarek et al., 2007). Conversely, sphingosine and sphingosine 1-phosphate stimulate PLD, inhibit phosphatidic acid phosphatase, and enhance the accumulation of phosphatidic acid. These processes decrease the formation of DAG resulting in inhibition of PKC activity and induction of apoptosis (Gomez-Muñoz, 1998); Delon et al., 2004).

Sphingosine affects several enzymes of phospholipid metabolism including PLA₂, PLD, PtdH phosphohydrolase, and diacylglycerol kinase (Table 2.1) (Hashizume et al., 1996; Hashizume et al., 1997; Delon et al., 2004). Sphingosine 1-phosphate mediates its action through a family of G protein-coupled sphingosine 1-phosphate receptors. This metabolite also promotes the induction of COX-2 (Pettus et al., 2004b; Nodai et al., 2007). In cultured rat vascular smooth muscle cells (VSMC), sphingosine 1-phosphate stimulates PGI_2 production in a concentration-dependent manner. The generation of PGI₂ is completely suppressed by NS-398, a selective COX-2 inhibitor (Nodai et al.,

| Enzyme | Effect | References |
|---|-------------|-----------------------------|
| Phospholipase A ₂ | Inhibition | Nakamura et al., 2004 |
| Phosphalipase $C\delta$ | Stimulation | Pawelczyk and Matecki, 1997 |
| PtdIns 4-kinase | Stimulation | Hashizume et al., 1996 |
| Protein kinase C | Inhibition | Khan et al., 1991 |
| Protein Kinase A | Stimulation | Ma et al., 2005 |
| Protein kinase K1 | Stimulation | Megidish et al., 1998 |
| DAG-kinase | Stimulation | Yamada and Sakane, 1993 |
| Phosphatidylglycerol phosphate synthase | Stimulation | Xu et al., 1999 |

Table 2.1 Effect of sphingosine on enzymic activities in neural and non-neural tissues

2007). Detailed investigations have also indicated that sphingosine 1-phosphate stimulates COX-2 protein and mRNA expressions in a concentration- and time-dependent manner, but has no effect on COX-1 expression. Suramin, an antagonist of S1P₃ receptor, almost completely prevents sphingosine 1-phosphate-induced COX-2 expression. Pretreatment of VSMC with pertussis toxin (PTX) partially but significantly blocks the sphingosine 1-phosphate-mediated PGI₂ production and COX-2 expression. Collective evidence suggests that sphingosine 1-phosphate stimulates COX-2 induction in rat VSMCs through mechanisms involving Ca²⁺-dependent PKC and Src-family tyrosine kinase activation via S1P₃ receptor coupled to PTX-sensitive and -insensitive Gproteins (Nodai et al., 2007). Since ceramide 1-phosphate and sphingosine 1-phosphate modulate cPLA₂ activity through translocation and activation. It is proposed that these sphingolipid metabolites may be involved in regulation of arachidonic acid release (Fig. 2.6) (Pettus et al., 2004b). Activation of sphingosine 1-phosphate receptors is closely associated with cellular functions such as proliferation, migration, cytoskeletal organization, inflammation, and differentiation (Pyne, 2004; Pettus et al., 2004b). Collective evidence suggests that sphingolipid-derived lipid mediators and their phosphorylated derivatives are involved in signaling processes associated with modulation of neural membrane functions.

Sphingosine and sphingosine 1-phosphate promote the rapid release of Ca^{2+} from internal stores in a manner not sensitive to $InsP_3$ (Einicker-Lamas et al., 2003). An interplay between sphingosine 1-phosphate and PtdIns metabolism through their respective kinases, sphingosine kinase and PtdIns 4-kinase, occurs at the membrane level. The mutual influence of PtdIns 4-kinase and sphingosine 1-kinase on each other is consistent with the cross talk between sphingolipids and glycerophospholipids. The interaction between sphingosine 1-kinase pathways and their synchronized control may represent an important physiological mechanism by which different enzymes associated with ionic homeostasis such as Ca^{2+}/Mg^{2+} -ATPase and Na⁺/K⁺-ATPase are switched on or off (Einicker-Lamas et al., 2003).

2.8.3 Interactions Between Glycerophospholipid and Cholesterol-Derived Lipid Mediators

Lyso-PtdCho, a PLA₂-derived lipid mediator of glycerophospholipid metabolism and oxysterols, are major lipid constitutents of oxidized LDLs and atherosclerotic plaques (Millanvoye-Van Brussel et al., 2004). These lipid mediators regulate the endothelial nitric oxide synthase (eNOS) and cPLA₂. These enzymes generate nitric oxide and arachidonic acid, which are essential for the integrity of the vascular wall. In human umbilical cord endothelial cells, both 7-oxycholesterol and lyso-PtdCho reduce histamine-mediated nitric oxide release and have no effect thapsigargin-mediated nitric oxide release. These lipid mediators reduce nitric oxide release through the participation of phosphoinositide 3-kinase (PI3K)-dependent pathway as well as by downregulating eNOS phosphorylation. It is interesting to note that histamine-mediated Ca²⁺ signals blocked by lyso-PtdCho, but not by 7-oxycholesterol. Oxysterols are known to prevent not only the histamine- and thapsigargin-mediated arachidonic acid release but also the phosphorylation of both cPLA₂ and extracellular-signal-regulated kinases $\frac{1}{2}$ (ERK1/2). Collectively, these results indicate that 7-oxycholesterol inhibits eNOS and cPLA2 activation by altering a Ca^{2+} -independent upstream step of PI3K and ERK1/2 cascades, whereas lyso-PtdCho desensitizes eNOS by interfering with receptor-activated signaling pathways. Although biological significance of these processes remains unknown, but it is proposed that in early stages of atherosclerosis, 7-oxycholesterol and lyso-PtdCho mediated cross talk plays an important role in the initiation of plaque formation (Millanvoye-Van Brussel et al., 2004). It remains to be seen whether or not lyso-PtdCho and oxysterol interaction occurs in neural cells in normal brain and brain from patients with neurological disorders.

2.8.4 Interactions Between Sphingolipid and Cholesterol-Derived Lipid Mediators

Sphingolipids associate with cholesterol to form microdomains or lipid rafts. These microdomains originate in the Golgi apparatus where most sphingolipids are synthesized (Degroote et al., 2004). In vitro studies indicate that interactions of head group in sphingomyelin with cholesterol play a major role in modulating membrane properties such as membrane fluidity, permeability, and formation of lipid rafts (Terova et al., 2005; Ramstedt and Slotte, 2006). Cholesterol has a preference for sphingomyelin over PtdCho with corresponding acyl chains. Several proteins interact and associate with lipid rafts. Studies on interactions between protein kinase C and sphingomyelin and cholesterol model lipid bilayer indicate that these interactions provide suitable hydrophobic environment for protein kinase C binding resulting in penetration of this enzyme into hydrophobic core of the membrane, where protein kinase C can interact with PtdSer, a glycerophospholipid required for the optimal enzymic activity. In contrast, PtdCho and cholesterol model lipid bilayer display weaker interactions. Amyloid precursor protein (APP) processing modulates cholesterol and SM metabolism in neural cell cultures. A β 42 directly activates neutral SMase and downregulates SM levels, whereas A β 40 reduces cholesterol de novo synthesis by inhibiting HMG-CoA reductase activity (Koudinov and Koudinova, 2003). This process strictly depends on γ -secretase activity. Furthermore, soluble $A\beta$ -induced activation of SMases and subsequent cell death are inhibited by antioxidant molecules and a cPLA₂-specific inhibitor or antisense oligonucleotide (Farooqui et al., 2006). Another metabolite of sphingolipid metabolism, sphingosine 1-phosphate is neuroprotects against soluble $A\beta$ oligomer-mediated cell death and apoptosis by inhibiting soluble $A\beta$ mediated activation of acidic sphingomyelinase. Collectively, results suggest that $A\beta$ oligomers induce neuronal death by activating neutral and acidic SMases in a redox-sensitive cPLA₂-dependent manner. Based on these findings, it is proposed that interactions among glycerophospholipid catabolism, sphingomyelin-ceramide cascade, and cholesterol metabolism and modulation of APP processing may be closely associated with the pathogenesis of AD (Yanagisawa, 2002; Kirsch et al., 2002; Ayasolla et al., 2004; Malaplate-Armand et al., 2006; Grimm et al., 2005).

Oxysterols, the lipid mediators of cholesterol metabolism, exert tight control over neural cell cholesterol trafficking by altering cholesterol influx/efflux (Koudinov and Koudinova, 2003). Oxysterols not only modulate Ca^{2+} signals but also inhibit the phosphorylation of endothelial nitric oxide synthase and cPLA₂. They interact with lipid metabolites of glycerophospholipid and sphingolipid metabolism (Millanvoye-Van Brussel et al., 2004; Farooqui et al., 2007a). A β and APP oxidize cholesterol to form 7 β -hydroxycholesterol, a proapoptotic oxysterol, which is neurotoxic at nanomolar concentrations. 7β -Hydroxycholesterol retards secretion of soluble APP from cultured rat hippocampal H19-7/IGF-IR neuronal cells and inhibits tumor necrosis factor- α -converting enzyme α -secretase activity, but has no effect on β -site APPcleaving enzyme 1 activity (Nelson and Alkon, 2005). 7β -Hydroxycholesterol also inhibits α -protein kinase C, an enzyme critical in memory consolidation and synaptic plasticity. Oxidation of cholesterol is accompanied by stoichiometric production of hydrogen peroxide and requires divalent copper. These results suggest that a function of APP may be to produce low levels of 7hydroxycholesterol. It is proposed that higher levels produced by $A\beta$ may contribute to the oxidative stress and neural cell loss observed in AD (Nelson and Alkon, 2005). Similarly, 7-ketocholesterol also induces apoptosis through the production of superoxide anions (Lizard et al., 2000).

Soluble $A\beta$ oligomers induce neuronal apoptosis by activating a cPLA₂dependent SMase pathway (Malaplate-Armand et al., 2006). cPLA₂-specific inhibitor, or cPLA₂ antisense oligonucleotide prevents this PLA₂-dependent activation of SMase (Malaplate-Armand et al., 2006). Sphingosine 1-phosphate blocks soluble $A\beta$ oligomer-mediated apoptosis by inhibiting activation of PLA₂. Collectively, these studies suggest that $A\beta$ oligomer-mediated neuronal death requires interaction between lipid metabolites of glycerophospholipid and sphingolipid metabolism (Malaplate-Armand et al., 2006).

Ethanolamine plasmalogen, a major phospholipids of neural membranes acts as antioxidant against cholesterol oxidation, is also decreased in AD brain (Farooqui and Horrocks, 2004; Maeba and Ueta, 2004). Other cholesterol metabolites, such as 24-hydroxycholesterol are also potent inducers of apoptotic cell death (Lizard et al., 2000; Kölsch et al., 1999). In addition, 7β -hydroxycholesterol and 7-ketocholesterol facilitate interleukin 1β secretion during apoptosis. Thus, marked increases have been reported to occur in lipid mediators of phospholipids, sphingolipid, and cholesterol metabolism in brain tissue. In non-neural tissues (smooth muscle cells), oxysterols bind to the liver X receptor (LXR) and mediate the accumulation of sPLA₂ mRNA and show an increase in enzymic activity (Antonio et al., 2003). Transient transfection experiments indicate that the sPLA₂ promoter is synergistically activated by a combination of oxysterol and 9-cis-retinoic acid, a ligand for the LXR heterodimeric partner RXR (retinoid X receptor). Promoter activity is upregulated in a sterol-dependent manner when cells are co-transfected with LXR α /RXR α or LXR β /RXR α . Based on mutagenesis studies and gel mobility-shift studies, it is proposed that LXR/RXR heterodimers regulate sPLA₂ transcription directly, by interacting with a degenerated LXR response element (LXRE) at position [-421/-406] of the sPLA₂ promoter. Collectively, these studies indicate that in smooth muscle cells oxysterols act independent of cytokine pathway to activate the sPLA₂ promoter (Antonio et al., 2003).

Collective evidence from multiple studies suggests that cross talk and interplay between ceramide and glycerophospholipid signaling may occur in neural cells. This controlled and coordinated signaling may not only vary significantly from one neural cell type to another but also with respect to the nature of stimulus, plus its dosage and/or duration of treatment. Thus, at low concentrations of lipid mediators derived from glycerophospholipids and sphingolipids, interplay and cross talk are necessary for neural cell proliferation, cell mobility, neurite retraction, and survival. However, high concentrations of these metabolites cause oxidative stress, membrane blebbing, and other neurochemical and morphological changes that promote neural cell death and tumor invasiveness. The cross talk between sphingolipid and glycerophospholipid-derived mediators may also be necessary for maintaining the functional lipid asymmetry of lipid bilayers in plasma membranes (Kihara and Igarashi, 2004).

2.9 Conclusion

Glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators have emerged as major players in modulating inflammation and oxidative stress-mediated neural cell death in brain tissue. This has made studies on the determination of levels of glycerophospholipid, sphingolipid, and cholesterolderived lipid mediators by lipidomics a critical area of research (Wenk, 2005; Han and Gross, 2005). The function of glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediator network is to convey extracellular signals from the cell surface to the nucleus to induce a biological response at the gene level. It is becoming increasingly evident that the intensity of interactions among glycerophospholipids, sphingolipids, and cholesterol-derived lipid mediators not only modulate cellular function through signal transduction processes but also adaptive responses (Ivanova et al., 2004). These functions include signal transduction, adhesion, sorting, trafficking, and organizing bilayer constituents (Simons and Ikonen, 2000; Farooqui et al., 2000b;

Farooqui and Horrocks, 2007). Alterations in composition and levels of lipid mediators are associated with various neuropsychiatric, and neurodegenerative diseases (Vigh et al., 2005). Alterations in levels of lipid mediators may be a key factor in the onset and progression of neuropsychiatric and neurodegenerative diseases (Farooqui and Horrocks, 2007). This suggests that neural membranes are not simply an inert physical barrier separating the inside from outside or compartments within cells, regulating passage of nutrients, gasses, and specific ions, but complex, well organized, and highly specialized structures involved in receiving, processing, transporting, and transmitting information from plasma membrane to the nucleus and other subcellular organelles through glycerophospholipids, sphingolipids, and cholesterol-derived lipid mediators. These mediators not only serve as second messenger but also as biosensors. Interplay or cross talk is necessary among glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators for neural cell proliferation, cell mobility, neurite retraction, and neural cell survival (Farooqui et al., 2007a,b). High levels of these metabolites and abnormal signaling cause oxidative stress, inflammation, membrane blebbing, and other neurochemical and morphological changes that promote neural cell death. It should be noted here that neural cell injury and death is not the result of one well-defined signaling cascade but the consequence of extensive cross talk between several neurochemical and molecular events at different cellular and subcellular levels (Farooqui et al., 2000a; Koletzko et al., 2001; Kihara and Igarashi, 2004).

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Chapter 3 Janus Face of Phospholipase A₂: Role of Phospholipase A₂ in Neural Cell Survival and Death

3.1 Introduction

Phospholipases A_2 (PLA₂, EC 3.1.1.4) are a superfamily of enzymes that specifically catalyze the hydrolysis of fatty acid from the sn-2 position of membrane glycerophospholipids to produce a free fatty acid and lysophospholipid (Fig. 3.1). Free fatty acids are oxidized to eicosanoids, and glycerolysophospholipids are either reacylated to native phospholipids or converted to platelet-activating factor (Murakami and Kudo, 2002). Recent advances in molecular and cellular biology have led to the identification of more than 20 mammalian intracellular PLA₂ isoforms, which are subdivided into several classes based upon their structures, enzymic characteristics, subcellular localization, and cellular function (Murakami et al., 1997). PLA₂ superfamily is classified into two subfamilies: (a) intracellular group of enzymes and (b) extracellular group of enzymes. The intracellular group of enzymes includes cytosolic PLA_2 (cPLA₂), calcium-independent PLA_2 (iPLA₂), plasmalogen-selective PLA₂ (PlsEtn-PLA₂), whereas extracellular PLA₂ group includes secretory PLA₂ (sPLA₂) (Farooqui et al., 1997). Several paralogs, splice variants, and multiple forms of cPLA₂, iPLA₂, and sPLA₂ have been identified as a result of cloning strategies. $cPLA_2$ paralogs include $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $cPLA_2\delta$, $cPLA_2\varepsilon$, and $cPLA_2\zeta$. $iPLA_2$ splice variants include iPLA₂ α , iPLA₂ β , and iPLA₂ γ . Multiple forms of sPLA₂ include sPLA2-IB, sPLA2-IIA, sPLA2-IIC, sPLA2-IID, sPLA2-IIE, sPLA2-IIF, sPLA₂-III, sPLA₂-V, sPLA₂-X, sPLA₂-XIIA, and sPLA₂-XIIB. Collective evidence suggests that individual paralogs, spilce variants, and multiple forms of PLA₂ superfamily may have unique enzymic properties, tissue, cellular, subcellular localizations, and role in various physiological and pathophysiological situations (Diaz-Arrastia and Scott, 1999; Farooqui et al., 2000c; Hirabayashi et al., 2004; Stahelin et al., 2003; Ohto et al., 2005; Chiba et al., 2004; Murakami et al., 2005a).

These enzymes are involved in the synthesis of lipid metabolites that have been implicated in fundamental cellular responses including growth, differentiation, adhesion, migration, secretion, and apoptosis (Farooqui et al., 1997; Sun et al., 2004b; Phillis and O'Regan, 2004).



Fig. 3.1 Site of action of phospholipase A₂ on glycerophospholipid molecule

3.2 Multiplicity of PLA₂ in Brain Tissue

3.2.1 Cytosolic Phospholipase A₂ (cPLA₂)

In the nervous system, $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $iPLA_2\alpha$, $iPLA_2\beta$, and $iPLA_2\gamma$, and $sPLA_2$ groups -IB, -IIA, -IIE, -IIF, -V, and -XII have been identified using PCR technique (Molloy et al., 1998; Zanassi et al., 1998; Diaz-Arrastia and Scott, 1999; Balboa et al., 2002; Pickard et al., 1999). $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, and cPLA₂ δ have mol mass of 85, 114, 61, and 109 kDa, respectively (Table 3.1). Thus, $cPLA_2 - \alpha$ has been mapped to chromosome 1, $cPLA_2\beta$ to chromosome 15, and cPLA₂ γ to chromosome 19. Amino acid sequencing indicates that cPLA₂ β and cPLA₂ δ have 120 and 135 amino acid inserts, respectively, between the C2 domain and the catalytic domain A, whereas in cPLA₂ α the two domains are adjacent to each other. In addition, $cPLA_2\beta$ has a unique N-terminal region composed of 242 amino acids, which is not required for the enzymic acitivity (Song et al., 1999). Most cPLA₂ paralogs prefer arachidonic acid over other fatty acids and do not use Ca²⁺ for catalysis, although submicromolar Ca²⁺ concentrations are needed for membrane binding (Clark et al., 1987; Farooqui et al., 2000c). In response to a variety of extracellular stimuli, some paralogs of cPLA₂ translocates from cytosol to intracellular membranes (perinuclear, Golgi, plasma membranes) (Clark et al., 1987; Hirabayashi et al., 2004) through their N-terminal calcium-binding lipid domain (C2 domain), whereas other paralogs utilize prenyl-group-binding motif located at the N-terminus for anchoring to the membrane. Translocation of $cPLA_2$ paralogs is required for the hydrolysis of glycerophospholipid substrates.

In the catalytic domain of cPLA₂ paralogs, a serine as the primary nucleophile. The active site is composed of GXSXG motif. Three amino acids (Arg-200, Ser-228, and Asp-549) play an important role during cPLA₂ catalyzed reaction. The catalytic Ser-228 interacts with carbonyl group at the sn-2 position of glycerophospholipids substrate. During catalysis, Asp-549 transfers

| Phospholipase | Paralog/splice | Molecular | CalB | |
|-------------------|--|------------|---------|---|
| A ₂ | variant/isozyme | mass (kDa) | domain | References |
| cPLA ₂ | $cPLA_2-\alpha^*$ | 85 | Present | Shirai and Ito, 2004 |
| | cPLA ₂ - <i>β</i> 1*, cPLA ₂ - <i>β</i> 2, cPLA ₂ - <i>β</i> 3 | 114 | Present | Ghosh et al., 2006; Shirai and Ito, 2004 |
| | $cPLA_2-\gamma^*$ | 61 | Absent | Ghosh et al., 2006; Shirai and Ito, 2004 |
| | $cPLA_2-\delta$ | 93 | - | Ohto et al., 2005 |
| | $cPLA_2$ - ε | 100 | - | Ohto et al., 2005 |
| | cPLA2- ζ | 95 | _ | Ohto et al., 2005 |
| iPLA ₂ | $iPLA_2-\alpha^*$ | 80 | Absent | Murakami et al., 2005b |
| | iPLA ₂ - β * | 64 | Absent | Murakami et al., 2005b |
| | $iPLA_2-\gamma$ | 80 | Absent | Murakami et al., 2005b |
| | $iPLA_2-\delta$ | _ | Absent | - |
| | iPLA ₂ -ε | _ | Absent | - |
| | $iPLA_2-\zeta$ | _ | Absent | - |
| | $iPLA_2-\eta$ | - | Absent | _ |
| sPLA ₂ | sPLA ₂ -IB | 14–18 | Absent | Macchioni et al., 2004 |
| | sPLA ₂ -IIA* | _ | Absent | Macchioni et al., 2004 |
| | sPLA ₂ -IIC* | _ | Absent | - |
| | sPLA ₂ -IID | _ | Absent | _ |
| | sPLA ₂ -IIE [*] | _ | Absent | - |
| | sPLA ₂ -IIF | - | Absent | - |
| | sPLA ₂ -III | - | Absent | - |
| | sPLA ₂ -V [*] | - | Absent | - |
| | $sPLA_2-X^*$ | _ | Absent | - |
| | sPLA ₂ -XIIA | - | Absent | - |
| | sPLA ₂ -XIIB | — | Absent | _ |

Table 3.1 Some properties of multiple forms of PLA₂ in mammalian tissues

Paralog/splice variant/isozyme expressed in brain (*).

proton causing the departure of lysophospholipid molecule and generation of the serine–acyl intermediate. This acyl intermediate then breaks down and releases free arachidonic acid and native enzyme molecule in the presence of water (Gelb et al., 1994; Wilton, 2005). This is in contrast to sPLA₂ isozymes that utilize Ca^{2+} as a catalytic cofactor and a water molecule as the nucleophile to hydrolyze glycerophospholipids substrate without forming a covalent acyl enzyme intermediate (Wilton, 2005).

The C-terminal region of $cPLA_2\alpha$ contains the phosphorylation sites located at Ser 505 and Ser 727. These serines are phosphorylated by mitogen-activated protein kinase (MAPK) or protein kinase C (PKC) (Hirabayashi and Shimizu, 2000; Hirabayashi et al., 2004). Ser-505 and ser-727 are absent in the $cPLA_2\beta$ and $cPLA_2\gamma$. The activity of $cPLA_2$ paralogs is also upregulated through a cooperative binding mechanism with glycerophospholipids containing arachidonic acid (Burke et al., 1995; Pettus et al., 2004; Lucas and Dennis, 2004; Le Berre et al., 2006), to a pleckstrin homology domain (Mosior et al., 1998). The upregulation of cPLA₂- α is attenuated by the expression of PLC δ and PIP₂-specific 5'-phosphatase (Le Berre et al., 2006). cPLA₂ α also exhibits relatively high lysophospholipase, Ca²⁺-stimulated 7-hydroxycoumarine esterase, and transacylase activities (Murakami et al., 1997; Huang et al., 1996; Reynolds et al., 1993)

Considerable information is available on cPLA₂ α (Hirabayashi et al., 2004). $cPLA_2\alpha$ activity is uniformly distributed in various regions of rat brain (Farooqui et al., 2000c). The basal expression of $cPLA_2\alpha$ mRNA under normal conditions is very low in neuronal and glial cells of brain tissue (Farooqui et al., 2000c; Pardue et al., 2003). Recent cytochemical localization studies have shown that forebrain and midbrain are very lightly stained with cPLA₂ α antibody, except for the arcuate nucleus and mammillary nuclei. The hindbrain, in contrast, contains many densely labeled nuclei. Apart from the facial motor nucleus, dense staining is observed in the initial portions of the ascending auditory pathway, including the dorsal and ventral cochlear nuclei, and the superior olivary nucleus, which receives afferents from the dorsal cochlear nucleus (Farooqui et al., 2000c). In addition, dense staining is also observed in some of the nuclei projecting to the cerebellar cortex, including the external cuneate nucleus and the inferior olivary nucleus. Purkinje neurons of the cerebellar cortex itself are labeled, and deep cerebellar nuclei, which receive afferents from the Purkinje neurons are also labeled. Electron microscopic studies have indicated that most of the $cPLA_2\alpha$ is present in dendrites postsynaptic to unlabeled axon terminals and in a small number of myelinated axons (Sandhya et al., 1998; Kishimoto et al., 1999; Strokin et al., 2003a; Shirai and Ito, 2004). This paralog is involved in the production of lipid mediators such as arachidonic acid, eicosanoids, and platelet-activating factor under physiological and pathophysiological conditions.

cPLA₂ β is found mainly in the cerebellum and shares more similarities with cPLA₂ α than with cPLA₂ γ . Immunolabeling and in situ hybridization studies have indicated that cPLA₂ β is present in the granule cells of rat brain (Shirai and Ito, 2004). Amino acid residues that are necessary for the catalytic activity are conserved in cPLA₂ β . cPLA₂ β has 30% homology with cPLA₂ α . cPLA₂ β contains an N-terminal C2 domain that confers calcium sensitivity and an additional N-terminal extension containing a JmjC domain with an unknown function (Pickard et al., 1999). cPLA₂ β also lacks regulatory phosphorylation sites that are present in cPLA₂ α . Splice variants of cPLA₂ β namely cPLA₂ β 1, cPLA₂ β 2, and cPLA₂ β 3 have been reported to occur in human lung epithelial cells. cPLA₂ β 1 is identical to the originally cloned cPLA₂ β and encodes a 114 kDa protein. cPLA₂ β 2 and cPLA₂ β 3 are smaller in size (100 kDa) because of internal deletion in the catalytic domain(Ghosh et al., 2006). Kinetic studies have indicated that cPLA₂ β 3 hydrolyzes 1-palmitoyl-2-arachidonyl-phosphatidylethano-lamine, but has no activity against 1-palmitoyl-2-arachidonyl-phosphatidylcholine

(Ghosh et al., 2006). cPLA₂ β 3 also hydrolyzes 1-palmitoyl-2-linoleoylphosphatidylethanolamine with a lower rate than 1-palmitoyl-2-arachidonyl-phosphatidylethanolamine. cPLA₂ β 3 activity dependent on calcium. However, some calcium-independent activity is also observed in cPLA₂- β 3 preparations (Ghosh et al., 2006). Studies on the sensitivity of cPLA₂ β splice variants to inhibitors have indicated that pyrrolidine, a potent inhibitor of $cPLA_2\alpha$ (IC50 0.01 µM) weakly inhibits $cPLA_2\beta$ 1 (IC50 80 µM), and has no affect on cPLA₂ β 3. All cPLA₂ β splice variants have lysophospholipase activity, but lysophospholipase activity of cPLA₂ β 3 was 80-fold lower than that of cPLA₂ β 1. Well known cPLA₂ α and cPLA₂ β 1 inhibitors (AZ-1 and methylarachidonyl fluorophosphonate) have no affect on lysophospholipase activity of cPLA₂ β 3. In contrast to cPLA₂ α and cPLA₂ β 1 that are found in cytosol, $cPLA_2\beta 3$ is constitutively associated with mitochondrial and endosomal membranes of unstimulated human lung epithelial cells. It is removed from the membrane by excess of EGTA indicating the involvement of calcium in membrane binding. It is stated that the association of $cPLA_2\beta 3$ with mitochondrial membranes occurs at resting calcium levels. Besides calcium, a variety of other factors such as protein-protein interactions between the mitochondrial and endosomal membranes and a specific region of $cPLA_2\beta 3$ may also facilitate the constitutive association of cPLA₂ β 3 (Ghosh et al., 2006).

 $cPLA_2\gamma$ lacks the C2 domain, but contains a prenyl-group-binding motif that behaves as a lipid anchor and allows binding of the enzyme to the membrane (Diaz-Arrastia and Scott, 1999; Farooqui et al., 2000c; Hirabayashi et al., 2004). cPLA₂ γ also lacks regulatory phosphorylation sites that are present in cPLA₂- α . However, it contains multiple putative protein kinase C phosphorylation sites. $cPLA2\gamma$ is constitutively associated with cellular membranes, and also has farnesylated, palmitoylated, and oleiated sites (Tucker et al., 2005). The role of these sites is not known. However, in addition to membrane anchoring, they may be involved in heterdimeric protein interactions associated with enzyme stabilization. $cPLA_{2}\gamma$ exhibits calcium-independent activity and it is expressed in heart and skeletal muscle. In addition, $cPLA_2\gamma$ also has coenzyme A-independent transacylase and lysophospholipid dismutase activities (Yamashita et al., 2005). Recombinantly expressed cPLA₂ γ liberates arachidonic acid from phosphatidylcholine. Unlike cPLA₂ α , cPLA₂ γ also acts on other fatty acid residues at the sn-2 and sn-1 positions of glycerophospholipids. cPLA₂ α hydrolyzes fatty acids at the sn-2 position, cPLA₂ β prefers to cleave fatty acids at the sn-1 position, and cPLA₂ γ efficiently hydrolyzes fatty acid at sn-1 as well as sn-2 positions of the glycerol moiety (Song et al., 1999). The overexpression of cPLA₂ γ increases the proportions of polyunsaturated fatty acids in phosphatidylethanolamine, indicating that this paralog can modulate the phospholipid composition (Asai et al., 2003). cPLA₂ γ is constitutively expressed in the mitochondrial, endoplasmic reticulum, and Golgi apparatus membranes where it is involved in remodeling and maintaining membrane phospholipid composition under oxidative stress. Among all cPLA₂ paralogs, $cPLA_{\beta}\beta$ displays much lower activity with [2-arachidonyl]PtdCho. The genes

for human cPLA₂ α , β , γ have been mapped to chromosomes 1, 15, and 19 respectively. Collective evidence suggests that mitogen-activated protein kinase phosphorylation sites are only present in cPLA₂ α and are not conserved in cPLA₂ β and cPLA₂ γ .

cPLA₂ δ is mainly found in skin and in contrast to other cPLA₂ paralogs, it has a preference for linoleic acid release instead of arachidonic acid release (Chiba et al., 2004). cPLA₂ δ gene has been mapped to the 15q 13–14 chromosomal locus and is separated from the locus of the cPLA₂ β gene by a physical distance of about 220 kb. cPLA₂ δ , cPLA₂ ε , and cPLA₂ ζ contain one C2 on the N-terminus and one lipase domain on the remaining part. Like cPLA₂ α , newly discovered paralogs (cPLA₂ δ , cPLA₂ ε , and cPLA₂ ζ) contain a conserved Ser-228 and Asp-549 residues and catalytically essential Arg-200 residue in the C-terminal domain. cPLA₂ ζ prefers PtdEtn over PtdCho (Ohto et al., 2005). Detailed investigations on enzymic mechanism have indicated that cPLA₂ α binds to PtdCho through Phe-35, Leu-39, Tyr-96, and Val-97 (Stahelin et al., 2003). These amino acid residues other than leu-39 are not conserved in murine cPLA₂ ζ as well as cPLA₂ δ and ε . It is proposed that these differences in amino acid sequences may be responsible for the preference in hydrolyzing PtdEtn over PtdCho.

3.2.2 Calcium Independent Phospholipase A₂(iPLA₂)

The brain cytosolic fraction contains an 80 kDa Ca²⁺-independent phospholipase A_2 (iPLA₂) activity. This enzyme has been purified from rat brain to homogeneity using multiple column chromatographic procedures with a very low yield. The purified enzyme has a specific activity of 4.3 µmol/min/mg. The peptide sequence of this enzyme has considerable homology to sequences of the iPLA₂ from P388D1 macrophages, CHO cells, and human B lymphocytes (Yang et al., 1999). This iPLA₂ hydrolyzes the sn-2 fatty acid from PtdCho with its preferences linoleoyl> palmitoyl>oleoyl>arachidonyl group. iPLA₂ has an unique amino acid sequence containing a lipase consensus sequence and eight ankyrin repeats. iPLA₂ also exhibits sn-1 lysophospholipase activity, transacylase activity, and platelet-activating factor hydrolase activity. This enzyme is strongly inhibited by bromoenol lactone and ATP augments its activity. Immunochemical studies have indicated that dense iPLA2 immunoreactivity is present in cerebral neocortex, amygdale, hippocampus, caudate nucleus, putamen, and nucleus accumbens of monkey brain. In contrast, thalamus, hypothalamus and globus pallidus are lighly stained. In the midbrain vestibular, trigeminal, inferior olivary nuclei, and the cerebellar cortex are densely stained (Shirai and Ito, 2004). Significant immunoreactivity is observed on the nuclear envelop of neurons and dendrites and axon terminals at electron microscopy (Shirai and Ito, 2004). Immunolabeling studies in rat brain have shown that iPLA₂ variants are present in granule cells, stellate cells, and in the nucleus of Purkinje cells (Shirai and Ito, 2004). Strong signals of iPLA₂ immunoreactivity are observed in olfactory bulb, hippocampus CA1-3, dentate gyrus, and brain stem. Determination of iPLA₂ activity indicates the presence of highest activity in striatum, hypothalamus, and hippocampus.

The gene encoding iPLA₂ has been identified (Molloy et al., 1998). The gene coding for iPLA₂ is located on chromosome 22q13.1. It consists of at least 17 exons spanning >69 kb (Larsson et al., 1998). The putative promotor for the iPLA₂ gene lacks a TATA-box and contains a CpG island as well as several potential Sp-1-binding sites. Furthermore, the 5'-flanking region also contains one medium reiteration frequency repeat (MER53) and an Alu repetitive sequence (Larsson et al., 1998). Northern blot analysis of iPLA₂ mRNA in various human tissues indicates tissue-specific expression of four distinct iPLA₂ transcripts. The native human 3.2-kb iPLA₂ transcript is predominantly expressed in heart, brain, skeletal muscle, prostate, testis, thyroid, and spinal cord, and to a lesser extent in peripheral blood leucocytes, stomach, trachea, and bone marrow (Larsson et al., 1998). Alternative splicing can generate variants of iPLA₂ with distinct tissue distribution and localization (Larsson et al., 1998). iPLA₂ may be negatively regulated by truncated splice variant proteins that prevent the formation of active iPLA₂ tetramers. Native iPLA₂ is a homotetramer that is potentially formed through interactions between N-terminal ankyrin repeats (Ackermann and Dennis, 1995). Ankyrin repeats link integral membrane proteins to the cytoskeleton and mediate protein-protein interactions during signaling processes. Seven iPLA₂ family members including iPLA₂ α , β , γ , δ , ε , ζ , and η occur in cytoplasm and membranes of neural and non-neural tissues (Larsson et al., 1998; Shirai and Ito, 2004; Molloy et al., 1998; Zanassi et al., 1998; Balboa et al., 2002). Most iPLA₂ family members have hydrophobic amino acids which facilitates their attachment to membranes (Larsson et al., 1998; Shirai and Ito, 2004). All iPLA₂ splice variants contain conserved nucleotide-binding (GXGXXG) and lipase (GXSXG) sequence motif. Amino acid residues in iPLA₂ γ active site have been identified. iPLA₂ γ is a membrane bound form that hydrolyzes PtdEtn (the alkenyl species) and PtdIns with polyunsaturated fatty acids. In contrast, iPLA₂ β acts on membrane microdomains enriched in PtdEtn and PtdCho with sn-1-C16:0 irrespective of sn-2 fatty acid moiety. Thus the phospholipids selectivity of iPLA₂ β is different from iPLA₂ γ . iPLA₂ β and iPLA₂ γ mainly show PLA₂ activity, whereas iPLA₂- δ exhibit lysophospholipase activity (Jenkins et al., 2004; Kuwata et al., 2007; van Tienhoven et al., 2002).

Studies on the comparison of catalytic domain with mouse homologue, human cPLA₂ α , and plant PLA₂ patatin indicate that an amino acid sequence of a short segment around Asp-627 of iPLA₂ γ is conserved among these PLA₂ activities in addition to the Ser-483-containing lipase motif. Since the substitution of either Ser-483 or Asp-627 with alanine results in inactivation of iPLA₂ γ , it is proposed that Ser-483 or Asp-627 residues are essential for the catalytic activity of iPLA₂. Several studies have indicated that iPLA₂ γ is associated with membranes from endoplasmic reticulum, Golgi, and mitochondria of non-neural tissues and is a distinctive gene product from iPLA₂ β . iPLA₂ γ protein is expressed as multiple forms with different sizes. Among these forms, the 63 kDa protein is distributed in the peroxisomes (Cummings et al., 2004). iPLA₂ γ hydrolyzes PtdEtn (the alkenyl species) and PtdIns with polyunsaturated fatty acids. In contrast, iPLA₂ β acts on membrane microdomains enriched in PtdEtn and PtdCho with sn-1-C16:0 irrespective of sn-2 fatty acid moiety. Thus, the glycerophospholipids selectivity of iPLA₂ β is different from iPLA₂ γ . Collectively, these studies suggest that these splice variant may act on different pools of membrane glycerophospholipids in different cellular membrane compartments. iPLA₂ γ is coupled with COX-1 for prostaglandin E₂ generation (Cummings et al., 2004).

iPLA₂- β is expressed in cytoplasm of several mammalian tissues including brain, heart, lungs, and kidney (Cummings et al., 2004; Shirai et al., 2005; Saavedra et al., 2006). In the catalytic region, iPLA₂ β is homologous to iPLA_{2 γ}, but the N-terminal regions of these enzymes show no similarity. It is well known that non-neural iPLA₂ is inhibited by bromoenol lactone (BEL). The racemic mixture of BEL can be separated into two enantiomers of BEL, namely (R)-BEL and (S)-BEL. (R)-BEL is 10-fold more selective for $iPLA_2\gamma$, whereas (S)-BEL is 10-fold more selective for iPLA₂ β . However, it is important to know that BEL enantiomers do not display absolute specificity for iPLA₂ isoforms because a significantly enough concentration of either (R)- or (S) blocks both iPLA₂ β and iPLA₂ γ . It is proposed that these enantiomers inhibit iPLA₂ γ and iPLA₂ β with different potencies. (R)-BEL selectively inhibits endoplasmic reticulum iPLA₂ γ in a dose-dependent manner, whereas (S)-BEL inhibits iPLA₂ β (Saavedra et al., 2006). The molecular mechanism of BELmediated inhibition of iPLA₂ β has been recently reported in non-neural cells (Song et al., 2006a). It is shown that BEL-mediated inhibition results in covalent modification(s) of iPLA₂ β . Mass spectrometric studies of proteolytic digests of BEL-treated iPLA₂ β indicate that the GTSTG active site and large flanking regions of sequence are not modified by BEL treatment. However, most iPLA₂ β Cys residues are alkylated at various BEL concentrations to form a thioether linkage to a BEL keto acid hydrolysis product (Song et al., 2006a). The extent of Cys⁶⁵¹ alkylation in iPLA₂ β closely parallels the loss of iPLA₂ β activity. No amino acid residues other than Cys are modified by BEL indicating that Cys alkylation is the main cause of BEL inhibition of iPLA₂ β . BEL inhibition is reversed by dithiothreitol (DTT) suggesting that redox status may play an important role in maintaining enzymic activity (Song et al., 2006a). Physiological concentrations of oxidants such as H₂O₂, NO, and hypochlorous acid (HOCl) also inactivate iPLA₂ β , and this inhibition can be partially reversed by DTT (Song et al., 2006b), once again supporting the view that redox reactions may be important in iPLA₂ β -mediated signaling. W⁴⁶⁰ oxidation may be the mechanism for irreversible $iPLA_2\beta$ inactivation. Irreversible inactivation of iPLA2 β may be caused by the proximity of W⁴⁶⁰ residue to the ⁴⁶³GTSTG⁴⁶⁷ catalytic center. Although the physiological importance of these observations within cells are not fully understood, but in non-neural as well as neural cells mild oxidative stress is known to modulate $iPLA_2\beta$ oligomerization state, its subcellular distribution, and arachidonic acid release from membrane glycerophospholipids. Effect of BEL enantiomers on purified $iPLA_2$ isoforms of neural origin remains unknown.

3.2.3 Secretory Phospholipase A₂ (sPLA₂)

sPLA₂ is synthesized intracellularly, then it is secreted and acts extracellularly as well as intracellularly. sPLA₂ has low molecular mass (14-18 kDa) and is mainly associated with synaptosomes and synaptic vesicle fractions (Matsuzawa et al., 1996; Kim et al., 1995). PLA₂ binds to two types of cell surface receptors, namely the N type, identified in neurons, and the M type, identified in skeletal muscles, of sPLA₂ receptors (Hanasaki and Arita, 2002; Kolko et al., 2002; DeCoster et al., 2002). Brain sPLA₂ contains a secretion peptide and requires mM Ca^{2+} for enzymic activity. sPLA₂ is has 5–8 disulfide bonds. sPLA₂ shows no selectivity for particular fatty acyl chains in the phospholipids. Phospholipid hydrolysis proceeds by the interaction of a water molecule through hydrogen bonding to the active site histidine residue. Adjacent to this histidine, there is a conserved aspartate residue at the catalytic dyad, which together with the calcium-binding loop, acts as a ligand cage for calcium. This enzyme is present in all regions of mammalian brain. The highest activities of sPLA₂ are found in medulla oblongata, pons, and hippocampus; moderate activities in the hypothalamus, thalamus, and cerebral cortex; and low activities in the cerebellum and olfactory bulb (Thwin et al., 2003). At the cellular level, the sPLA₂ transcript is found in astrocytes (Mosior et al., 1998; Zanassi et al., 1998). sPLA₂ is present in differentiated PC12 cells and in rat brain synaptic vesicles indicating that neurons also express sPLA₂ activity (Matsuzawa et al., 1996).

sPLA₂ binds to the presynaptic membrane, enters the lumen of the synaptic vesicle during the vesicle's retrieval from the plasma membrane, and hydrolyzes phospholipids of the inner leaflet of synaptic vesicles, changing the phospholipid composition, and thus impairing its endocytosis. The stimulation of sPLA₂ in synaptic vesicles correlates with the induction of vesicle-vesicle aggregation and this process plays a central role in presynaptic neurotransmission (Moskowitz et al., 1983; Matsuzawa et al., 1996; Wei et al., 2003). Mitochondrial fractions from rat brain, PC12, and U251 astrocytoma cell cultures contain significant sPLA₂ activity (Macchioni et al., 2004; Sun et al., 2005). The mechanism associated with sPLA₂ targeting of intracellular organelles (like mitochondria) remains unknown. However, it is proposed that at the molecular level, heparin sulfate, a glycosaminoglycan, may play an important role in internalization and attachment of PLA₂ isoforms to intracellular organelles (Farooqui et al., 1994c; Boilard et al., 2003). Collective evidence suggests that sPLA₂ acts extracellularly through its receptors, or it can be internalized to reach its intracellular targets (Sun et al., 2004a, 2005).

To date 11 sPLA₂ isoforms, including sPLA₂IB, -IIA, -IIC, -IID, -IIE, -IIF, -III, -V, -X, -XIIA, and -XIIB, have been identified in mammalian tissues (Kudo and Murakami, 2002). The genes for these isoforms are clustered on the same chromosome locus. In brain the expression of $sPLA_2$ isoforms is relatively low; however, mRNAs for sPLA₂-IIA, -IB, -IID, -IIE, -IIF, -IVA, -V, and -XII have been detected (Molloy et al., 1998; Kolko et al., 2004; Kolko et al., 2006). RT-PCR studies have also indicated that the expression of sPLA₂-IIE and -IB is highest in the cortex, hippocampus, and cerebellum region of rat brain. In contrast the expression of sPLA₂-X is quite lowest. mRNAs encoding for sPLA₂-IB, -X, -V, -IIE, -IIA, and -IIF is also found in the retina. A timedependent gene induction of sPLA₂-X, -IB, and -V occurs in light-induced retinal damage. Kainic acid (KA) administration markedly affects the expression of various forms of sPLA₂ in brain tissue. Thus sPLA₂-X is upregulated following KA injections (Kolko et al., 2006). Similarly sPLA2-IB is induced after electroshock and sPLA₂-IB, sPLA₂-V, and sPLA₂-IIE are upregulated in brain tissue following systemic administration of KA in rats (Kolko et al., 2006). Collectively, these studies suggest that multiple forms of sPLA₂ may be upregulated in response to pathologic events. Recent studies have indicated that sPLA₂-X is found in Golgi apparatus and growth cones in PC12 cells (Masuda et al., 2005). In the presence of nerve growth factor, sPLA₂-X facilitates the induction of neurite outgrowth. It is proposed that neurite extending ability of sPLA₂-X depends upon the generation of lyso-PtdCho. This neurite outgrowth extending ability is blocked by sPLA₂-X antibody as well as RNAi for sPLA₂-X (Masuda et al., 2005) (Fig. 3.3). It is proposed that sPLA₂-X may be involved in neuronal differentiation and neural membrane repair under certain conditions.

Neuronal cells, such as peripheral neuronal fibres, spinal DRG (dorsal root ganglia) neurons and cerebellar Purkinje cells express sPLA₂-III. Adenoviral expression of sPLA₂-III in PC12 cells (pheochromocytoma cells) or DRG explants promotes neurite outgrowth, whereas expression of a catalytically inactive sPLA₂-III mutant or use of sPLA₂-III-directed siRNA (small interfering RNA) retards NGF (nerve growth factor)-mediated neuritogenesis. sPLA₂-III also blocks neuronal death mediated by NGF deprivation. It is shown that sPLA₂-III over-expression increases the cellular level of lyso-PtsCho, a PLA₂ reaction product with neuritogenic and neurotropic activities, whereas siRNA knockdown decrease the level of lyso-PtdCho. Collective evidence suggests the potential contribution of sPLA₂-III to neuronal differentiation (Masuda et al., 2008).

3.2.4 Plasmalogen Selective Phospholipase A₂ (PlsEtn-PLA₂)

Docosahexaenoic acid release in astrocytes involves PlsEtn-PLA₂, a calcium-independent enzyme with mol mass of 39 kDa. It is inhibited by bromoenol lactone (Farooqui et al., 1995; Farooqui and Horrocks, 2001b; Strokin et al., 2003b; André et al., 2005). Ceramide decreases the levels of plasmalogens, glycerophospholipids containing vinyl ethers, in rat brain slices through the activation of PlsEtn-PLA₂ (Latorre et al., 2003). The decrease in plasmalogens by ceramide is prevented by quinacrine, ganglioside, and bromoenol lactone, which are inhibitors of PlsEtn-PLA₂ activity (Latorre et al., 2003; Farooqui and Horrocks, 2001b). Furthermore, the addition of the caspase-3 inhibitor, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-chloromethylketone (Ac-DEVD-CMK), partially blocks the ceramide-induced stimulation of PlsEtn-PLA₂ without altering sphingomyelinase-elicited ceramide accumulation (Latorre et al., 2003) suggesting the interaction between plasmalogen hydrolysis and sphingolipid metabolism (Farooqui et al., 2004b). Furthermore, 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphocholine (CoA-independent transacylase inhibitor) prevents ceramide-elicited effects suggesting that phospholipase and transacylase activities are tightly coupled and ceramide may be involved in regulating arachidonic acid release from pools that are enriched in plasmalogens.

3.3 Role of Multiple Forms of PLA₂ in Brain

The relationship among paralogs/splice variants/isozymes of PLA_2 may be quite complex. However, it is becoming increasingly evident that in brain tissue PLA_2 paralogs/splice variants/isozymes are part of a complex signal transduction network and cross talk among various receptors through the generation of second messengers and lipid mediators are essential for maintaining normal neural cell function. Therefore, a tight regulation of PLA₂ paralogs/splice variants/isozymes is very important for normal brain function. The upregulation and expression of PLA₂ paralogs/splice variants/isozymes in neural cell is the rate-limiting step for the production of inflammatory lipid mediators such as eicosanoids, docosanoids, lysophosphatidic acid, and platelet-activating factor (Farooqui and Horrocks, 2007). These processes provide neural cells and brain tissue with great versatility in ensuring that arachidonic acid, docosahexaenoic acid, and their oxygenated metabolites are efficiently utilized by neural cells. In brain tissue, the activity of PLA₂ paralogs/splice variants/isozymes may depend not only on structural, physicochemical and dynamic properties of neural membranes but also on the interaction of extracellular signal with PLA₂-linked neural cell receptors such as dopamine, glutamate, serotonin, P2-purinergic, cytokine, and growth factor receptors (Sun et al., 2005). Collectively, these studies suggest that the regulation of PLA₂ activity is quite complex and is mediated by several factors but also by microenvironment, gene expression, and secretion.

3.3.1 Multiple Forms of PLA₂ and Neurotransmitter Release

In brain tissue, neurotransmitter release is essential for cell-cell communication. This process mainly occurs at the synaptic junction where anterograde information transfer from presynaptic neurons to postsynaptic neurons takes place. The molecular mechanism of neurotransmitter release remains speculative. However, it is reported that calcium ion entry through voltage-sensitive channels and activation of cPLA₂ may facilitate neurotransmitter release in neuronal cell cultures and PC12 cells (Moskowitz et al., 1982; Bloch-Shilderman et al., 2002) (Fig. 3.2). This suggestion is based on the observation that activation of both cPLA₂ and iPLA₂ in synaptic vesicles correlates with the induction of vesicle formation. Inhibitors of cPLA₂, iPLA₂, and sPLA₂ suppress exocytosis and block neurotransmitter release in the rat brain and PC12 cell preparations (Matsuzawa et al., 1996; Abu-Raya et al., 1998; Bloch-Shilderman et al., 2002; Wei et al., 2003). Furthermore, arachidonic acid, a metabolite derived through PLA₂ catalyzed reaction, facilitates the insertion and translocation of protein kinase $C\gamma$ (PKC γ) in neural membranes. PLA₂ inhibitors, bromoenol lactone, and arachidonyl trifluoromethyl ketone shorten the retention time of PKC γ in neural membranes (Yagi et al., 2004). Protein kinase C is also involved in neurotransmitter release in cultured cells of neuronal and glial origin (Bramham et al., 1994). Similarly, in NIH3T3 mouse fibroblasts, multiple forms of PLA₂ have been reported to regulate taurine release (Lambert and Pedersen, 2006). Based on the effects of inhibitors, it is proposed that swelling-mediated taurine efflux depends upon inpart on arachidonic acid release by iPLA₂ and sPLA₂, whereas melittin-mediated taurine efflux is mainly caused by sPLA₂ (Pedersen et al., 2006). Collective evidence thus suggests that cPLA₂, iPLA₂, and sPLA₂ modulate neurotransmitter release by direct and indirect mechanisms in



Fig. 3.2 Proposed roles of multiple forms of cPLA₂ in brain

various brain preparations (Ghijsen et al., 2003; Bloch-Shilderman et al., 2002; Bramham et al., 1994).

In pathological situations, such as ischemia, increase in neurotransmitter release (glutamate and GABA) occurs by depolarization-induced reversal of the sodium-dependent high-affinity amino acid plasma membrane transporter (Ghijsen et al., 2003). This increase in neurotransmitter release can be blocked by quinacrine, a non-specific PLA₂ inhibitor, suggesting the involvement of PLA₂ isoforms in neurotransmitter release (O'Regan et al., 1995). Based on biophysical and neuropharmacological studies, it is proposed that PLA₂ isoforms disrupt the integrity of synaptic vesicle in a calcium-dependent manner (DeVries et al., 1983). This loss of membrane integrity allows the neurotransmitter to diffuse from intracellular compartment into the synaptic cleft suggesting that PLA₂ activity is involved in efflux of neurotransmitter release under pathological conditions (O'Regan et al., 1995). Collectively, these studies suggest that cPLA₂, iPLA₂, and sPLA₂ are associated with neurotransmitter release. However, the identity of multiple forms of cPLA₂, iPLA₂, and sPLA₂ involved in neurotransmitter release still remains unknown.

3.3.2 Multiple Forms of PLA₂ in Long-Term Potentiation and Long-Term Depression

Long-term potentiation (LTP) and long-term depression (LTD) are two major cellular mechanisms involved in learning and memory (Bliss and Collingridge, 1993; Chen and Tonegawa, 1997). They involve depolarization of the postsynaptic membranes and N-methyl-D-aspartate (NMDA) receptor-mediated calcium influx. The induction of LTP in dentate gyrus is accompanied by calcium-dependent PLA₂ (cPLA₂) activation and liberation of arachidonic acid from neural membrane glycerophospholipids. Released arachidonic acid crosses the synaptic cleft to act presynaptically as a retrograde messenger through activation of the γ -isoform of protein kinase C (PKC- γ) (Linden and Routtenberg, 1989; Izquierdo and Medina, 1995). Collective evidence suggests that PKC- γ together with cPLA₂ plays an important role in the induction and maintenance of LTP (Murakami and Routtenberg, 2003; Bernard et al., 1994). In addition, arachidonic acid is also involved in the phosphorylation of a 43-kDa growth associated protein (GAP-43), which is highly expressed in growth cones during neuronal development and synaptic remodeling. The phosphorylation of GAP-43 is closely associated with LTP in the dentate gyrus and CA1 area of hippocampus (Hulo et al., 2002). Lysophosphatidylcholine, the other product of the cPLA₂-catalyzed reaction, is also involved in hippocampal neurotransmission and LTP (Nomura et al., 2001). Thus, both products generated by cPLA2 are involved in the induction and maintenance of LTP. Injections of the cPLA₂ inhibitor, palmitoyl trifluoromethylketone, into rat hippocampus impair memory formation, indicating further that cPLA2 activity



Fig. 3.3 Proposed roles of multiple forms of iPLA₂ in brain

and its metabolites are closely involved in LTP and memory formation (Schaeffer et al., 2005; Schaeffer and Gattaz, 2005).

Calcium-independent PLA_2 (i PLA_2) also facilitates LTP (Wolf et al., 1995) (Fig. 3.3). The treatment of hippocampal slices with bromoenol lactone (BEL), a specific and potent inhibitor of i PLA_2 prior to tetanic stimulation blocks the induction of LTP. Importantly, the addition of arachidonic acid and docosahexaenoic acid during the tetanic stimulation promotes LTP suggesting that i PLA_2 mediates the induction and maintenance of LTP (Wolf et al., 1995; Fujita et al., 2001). Intracerebroventricular injections of BEL in mice markedly affect spatial performance (Fujita et al., 2000), suggesting that i PLA_2 is involved in spatial memory formation. Both the NMDA and the AMPA types of glutamate receptors are coupled to isoforms of PLA_2 in neural membranes (Lazarewicz et al., 1990; Farooqui and Horrocks, 1994a) and therefore participate in the modulation of LTP.

Treatment of rat brain slices with BEL stimulates phosphorylation of serine residue 831 on the AMPA receptor GluR1 subunit in a synaptosomal P2 fraction. In contrast, a cPLA₂ inhibitor, AACOCF₃, enhances the phosphorylation on serine residues 880/891 on the AMPA receptor GluR2/3 subunit. These effects are restricted to the AMPA receptor, and no changes are seen in the NMDA receptor NR1 subunit phosphorylation (Ménard et al., 2005a). The inhibition of protein phosphatases by okadaic acid does not occlude the effects

of BEL and AACOCF₃, indicating that the increase in AMPA receptor phosphorylation mediated by a PLA₂ inhibitor does not rely on a decrease in dephosphorylation reactions. However, pretreatment of rat brain slices with a cell-permeable inhibitor of protein kinase C blocks the phosphorylation induced by BEL and AACOCF₃ on the Ser 831 and Ser 880/891 sites of GluR1 and GluR2/3 subunits, respectively. This suggests that the cPLA₂ and iPLA₂ systems differentially modulate the AMPA receptor properties and function in rat brain through protein kinase C-mediated mechanism (Ménard et al., 2005b). The ability of iPLA₂ inhibitors to increase GluR1 phosphorylation can be mimicked by MK-886, a 5-lipoxygenase inhibitor, but not by blockers of 12-lipoxygenase or cyclooxygenase. Thus, $iPLA_2$ activity through the generation of 5-lipoxygenase metabolites modulates AMPA receptor phosphorylation of GluR1 subunits in the CA1 area of the hippocampus and that AMPA receptors are involved in LTP. Collective evidence suggests that both cPLA₂ and iPLA₂ participate in LTP (Fitzpatrick and Baudry, 1994; Wolf et al., 1995; Fujita et al., 2001). The identity of multiple forms of cPLA₂ and iPLA₂ associated with the induction and maintenance of LTP remains unknown.

Preincubation of hippocampal slices with $iPLA_2$ inhibitor BEL retards LTD formation (Okada et al., 1989), indicating the involvement of $iPLA_2$ in LTD. Treatment of hippocampal slices with arachidonate also mimics the LTD formation (Massicotte, 2000). The identity of multiple forms of $cPLA_2$ and $iPLA_2$ involved in the induction and maintenance of LTD remains unknown.

Recent studies have also indicated that PLA_2/COX product, prostaglandin E_2 (PGE₂) is also involved in LTP (Akaneya and Tsumoto, 2006). PGE₂ is generated in acute visual cortex slices in response to theta burst stimulation (TBS) and is associated with postsynaptically in TBS-mediated LTP. RNA interference (RNAi) for PGE₂ receptor subtypes EP2 and EP3 which upregulate and downregulate the level of cAMP respectively, produces significant decreases and increases of LTP, respectively (Akaneya and Tsumoto, 2006). Collectively, these studies suggest that in TBS-mediated LTP, PGE₂ is released from the postsynaptic cells and then activates PGE2 receptors at the postsynaptic membranes, which is modulated by trafficking of the differential PGE₂ receptor subtypes in an activity-dependent bidirectional manner (Akaneya and Tsumoto, 2006).

3.3.3 Multiple Forms of PLA₂ in Membrane Repair

In neural membranes, reactive oxygen species (ROS) attack on polyunsaturated fatty acids produces lipid hydroperoxides and peroxidized glycerophospholipids (Farooqui et al., 2000b). The presence of peroxidized glycerophospholipids in neural membranes produces a packing defect making the sn-2 ester bond more accessible to the action of PLA₂ isoforms. Glycerophospholipid hydroperoxides and peroxidized glycerophospholipids are better substrates for PLA₂ isozymes than the native non-peroxidized glycerophospholipids (McLean et al.,

1993). The hydrolysis of peroxidized glycerophospholipids by multiple forms of PLA_2 results in the removal of peroxidized fatty acyl chains, which are reduced and re-esterified (Farooqui et al., 2000a). Thus, the action of PLA_2 isoforms repairs and restores the appropriate physicochemical state of the membrane and prevents peroxidative cross-linking reactions. Without such repair, peroxidized glycerophospholipids would accumulate and produce neurotoxicity by altering neural membrane permeability and ion homeostasis. All forms of PLA_2 participate in membrane repair process.

3.3.4 Multiple Forms of PLA₂ in Modulation of Neurite Outgrowth and Regeneration

Paralogs/splice variants/isoforms of PLA₂ play an important role in neurite outgrowth, regeneration, and growth-dependent signal transduction processes (Suburo and Cei de Job, 1986; Katsuki and Okuda, 1995; Obermeier et al., 1995; Hornfelt et al., 1999; Smalheiser et al., 1996; Geddis et al., 2004), and PLA₂ inhibitors impair neurite outgrowth formation (Smalheiser et al., 1996), whereas activators of PLA₂ activity, such as melittin, activate neurite outgrowth. These observations support the view that PLA₂-generated metabolites are involved in neurite outgrowth formation (Figs. 3.2 and 3.3). Although, the molecular mechanism of arachidonic acid induced neurite outgrowth remains unknown, but involvement of arachidonic acid-mediated protein kinase activation has been proposed (Katsuki and Okuda, 1995).

The involvement of paralogs/splice variants/isoforms of cPLA₂ in axonal outgrowth has also been investigated in dorsal root ganglia (DRG) neurons. Methyl arachidonoyl fluorophosphonate (MAFP), a cPLA₂ inhibitor, reduces the axonal outgrowth length about 50%, and causes rapid collapse of the growth cone, an effect that is counteracted by addition of arachidonic acid (AA), suggesting the involvement of cPLA₂ activity. Other studies in DRG indicate that these PLA₂ inhibitors exert a biphasic effect on elongation of axons. They enhance outgrowth at low concentrations and inhibit outgrowth at higher concentration (Suburo and Cei de Job, 1986). Similarly, studies on initial stages of neural regeneration in the snail, Helisoma trivolvis, indicate that PLA_2 activity is necessary for the completion of neuronal regeneration (Geddis and Rehder, 2003). It is proposed that in Helisoma neurons, PLA₂ controls filopodial dynamics during growth cone for targeting and pathfinding (Geddis et al., 2004). Extracellular signals that modulate paralogs/spilce variants/isozymes of PLA₂ may not only control growth cone morphology but also affect neuronal pathfinding by regulating the sensory radius of navigating growth cones (Geddis et al., 2004). Furthermore, activation of cPLA2 and generation of eicosanoids have been reported to occur during thrombin-mediated growth cone collapse (Rintala et al., 1999). Growth cone collapsing can be retarded by lipoxygenase inhibitors indicating that 12/15 hydroxyeicosatetraenoic acids

(HETE) are involved in thrombin-induced growth cone collapse. Collective evidence thus suggests the importance of $cPLA_2$ activity for growth cone motility and axonal outgrowth in the adult central and peripheral nervous systems. The molecular mechanism of PLA_2 -mediated induction of neurite outgrowth remains unknown. However, arachidonic acid and its metabolites through peroxisome proliferator-activated receptor-mediated process may modulate the expression of genes related to neurite outgrowth formation and differentiation. Furthermore, lysophospholipids, the other product of PLA_2 catalyzed reaction, stimulate mitogen-activated protein kinase and protein kinase C. Both of these enzymes are closely associated with neuronal cell proliferation and differentiation. These studies strongly link multiple forms of $cPLA_2$ activity to axonal outgrowth and growth cone function.

PlsEtn-PLA₂ releases docosahexaenoic acid (DHA) from plasmalogens (Farooqui and Horrocks, 2001a; Strokin et al., 2003b). Like arachidonic acid, treatment of hippocampal cultures with DHA also increases neurite length and number of branches indicating that DHA also facilitates neuritic growth in vitro (Calderon and Kim, 2004). The molecular mechanism of DHA-induced neuritic outgrowth in not fully understood, however, DHA induces the synthesis of phosphatidylserine content in neuronal membranes, which in turn promotes the activation of Raf-1 and the PtdIns-3 kinase pathways (Kim et al., 2000; Akbar and Kim, 2002). Raf-1 and the PtdIns-3 kinase pathways are closely associated with neurite outgrowth formation in PC12 and H19-7 hippocampal cell lines (Kobayashi et al., 1997; Kita et al., 1998). Finally, both arachidonic acid and DHA can also act as endogenous ligands for nuclear retinoid receptor RXR, which modulates activities of transcription factors and gene expression in developing and adult brain (Lengqvist et al., 2004; Antony et al., 2003).

In PC12 cell cultures sPLA₂-X, an isoform of sPLA₂ participates in neurite outgrowth formation (Fig. 3.4). It is proposed that this process involves the ability of sPLA₂-X to generate lysophosphatidylcholine (lyso-PtdCho), which induces G2A, a G-protein-coupled receptor involved in signaling mediated by lyso-PtdCho (Ikeno et al., 2005; Masuda et al., 2005). Other sPLA₂ isoforms such as sPLA₂-IIA have no effect on neurite outgrowth, but bee venom sPLA₂ and fungal sPLA₂ induce neurite outgrowth in a manner dependent upon their catalytic activity. A combination of sPLA₂ and nerve growth factor induces more neurite outgrowth than sPLA₂ alone. The nerve growth factor-mediated neurite extension of PC12 cells is significantly attenuated by both an anti-sPLA₂ antibody and a siRNA (Masuda et al., 2005), indicating that sPLA₂ is an important player in neuritogenesis (Fig. 3.4).

3.3.5 Multiple Forms of PLA₂ in Tubule Formation and Membrane Trafficking

The Golgi complex contains several isoforms of PLA_2 including $cPLA_2$, $iPLA_2$, and $sPLA_2$ -X (Masuda et al., 2005; Grewal et al., 2005; Shirai et al., 2005). These isoforms are implicated in maintaining normal Golgi structure and



Fig. 3.4 Proposed roles of multiple forms of sPLA₂ in brain

function during Golgi formation (Choukroun et al., 2000), tubulation (de Figueiredo et al., 1999), and membrane remodeling (Schmidt et al., 1999; Yamashita et al., 2005). PLA₂ inhibitors treatment not only prevents trafficking between the Golgi complex and endoplasmic reticulum (Drecktrah and Brown, 1999; de Figueiredo et al., 1998; de Figueiredo et al., 1999; Polizotto et al., 1999), endocytosis (Mayorga et al., 1993) but also blocks exocytosis (Slomiany et al., 1998) and the intracellular trafficking of secretory proteins (Choukroun et al., 2000; Tagaya et al., 1993). Treatment with Brefeldin A (BFA), a fungal metabolite, has been used to develop in vivo and in vitro models for the formation of tubules from the Golgi complex to endoplasmic reticulum and from the trans-Golgi network to endosomes in hepatocytes. The PLA2 inhibitors ONO-RS-082, arachidonoyl trifluoromethylketone, and bromoenol lactone prevent tubule formation, and the PLA₂ activators melittin and PLA₂-activating protein peptide stimulate, brefeldin A (BFA)-mediated Golgi tubulation and retrograde trafficking in vivo and in vitro (de Figueiredo et al., 1998; de Figueiredo et al., 1999; Kuroiwa et al., 2001; Brown et al., 2003; Herbert et al., 2005). These observations implicate PLA₂ isoforms in membrane tubule formation from the Golgi complex in non-neural tissues (Polizotto et al., 1999).

3.3.6 Multiple Forms of PLA₂ in the Cell Cycle

Little is known about activities of PLA₂ paralogs/splice variants/isoforms and glycerophospholipid alterations in maturation of neuronal and glial cells during

brain development (Ledeen and Wu, 2004; Farooqui et al., 2004a). However, alterations in activities of $cPLA_2$ and $iPLA_2$ paralogs/splice variants/isoforms and glycerophospholipid contents occur during the cell cycle in non-neural cells. $cPLA_2$ activity is high in mitosis, decreased afterwards, and increases again in the G1 and G1/S phases. During these phases, $cPLA_2$ activity is increased due to its phosphorylation rather than increase in $cPLA_2$ protein expression. This suggestion is based on the observation that phosphatase treatment of $cPLA_2$ preparation reduces its activity (van Rossum et al., 2002). Inhibition of $cPLA_2$ activity with arachidonoyl trifluoromethyl ketone in early G1 phase markedly reduces DNA synthesis suggesting that $cPLA_2$ plays an important role in cell cycle progression.

It is becoming increasingly evident that glycerophospholipid turnover and accumulation of PtdCho are increased during late G1 and early S phases of cell cycle (Manguikian and Barbour, 2004; Roshak et al., 2000; Ng et al., 2004). Decline in iPLA₂ activity during G1 phase is not caused by a loss in iPLA₂ protein mass, but rather due to decreased catalytic activity of the iPLA₂ protein. This decrease in iPLA₂ directly regulates cell proliferation by arresting cells in the G1 phase (Fig. 3.3). The G1 phase arrest requires activation of the tumor suppressor p53 and expression of the cyclin-dependent kinase inhibitor p21. These observations suggest that iPLA₂ cooperates with p53 to monitor membrane phospholipids turnover in G1 phase. Similarly, in endothelial cells iPLA₂ activity is critical for the progression of endothelial cells through S phase and is needed for the expression of the cyclin A/cdk2 complex (Herbert and Walker, 2006). The blockade of iPLA₂ prevents S phase progression and results in exit from the cell cycle. Collective evidence suggests that iPLA₂ activity is involved in regulation of endothelial cell S phase progression and angiogenesis (Herbert and Walker, 2006).

3.3.7 Multiple Forms of PLA₂ in Neuroinflammation

Neuroinflammation is a complex defensive process designed to remove or inactivate noxious agents and inhibits their detrimental effects for the restoration of normal tissue structure and function. Neuroinflammation is a hallmark of all major CNS diseases. The main mediators of neuroinflammation are microglial and astroglial cells. In vascular system, cells such as neutrophils, monocytes, and macrophages support and facilitate inflammatory responses. Microglial cells initiate a rapid response that involves cell migration, proliferation, release of cytokines/chemokines, and trophic and/or toxic effects. Cytokines/chemokines stimulate multiple forms of PLA₂ PLC, and COX enzymes (Fig. 3.5) (Farooqui and Horrocks, 2009). This results in breakdown of membrane glycerophospholipids with release of arachidonic acid. Oxidation of arachidonic acid through COX and LOX enzymes produces pro-inflammatory prostaglandins, leukotrienes, and thromboxanes (Farooqui et al., 2007; Yedgar


Fig. 3.5 Receptors-mediated degradation of phosphatidylcholine, by cPLA₂ and PtdIns-4,5-P₂ by PLC, and sPLA₂ induction in the nucleus.

Agonists (A1 and A2); receptors (R1 and R2); phosphatidylcholine (PtdCho); phosphatidylinositol 4,5-bisphosphate (PtdIns-5,5- P_2); cytosolic phospholipase A₂ (cPLA₂); phospholipase C (PLC); secretory phospholipase A₂ (sPLA₂); sPLA₂ receptor (sPLA₂-R); calcium-independent phospholipase A₂ (iPLA₂); arachidonic acid (AA); cyclooxygenase-2 (COX-2); inducible nitric oxide synthase (iNOS); lipoxygenase (LOX); protein kinase C (PKC); inositol-1,4,5-trisphosphate (Ins-1,4,5- P_3); diacylglycerol (DAG); tumor necrosis factor- α (TNF- α); interleukin-1 β (IL- β); interleukin-6 (IL-6); endoplasmic reticulum (endo); and plasma membrane (PM)

et al., 2006). One of the lyso-glycerophospholipids, the other products of reactions catalyzed by PLA₂, is used for the synthesis of pro-inflammatory plateletactivating factor (Phillis et al., 2006). These pro-inflammatory mediators intensify neuroinflammation. The release of cytokines further stimulates PLA₂ isoforms through positive loop (Fig. 3.5) and provides a link between inflammatory cells and specific immunity because they can stimulate T and B lymphocytes. Lipoxin, an oxidized product of arachidonic acid through 5-lipoxygenase, is involved in resolution of inflammation and is anti-inflammatory (Serhan and Savill, 2005).

PlsEtn-PLA₂ releases docosahexaenoic acid from plasmalogen (Farooqui and Horrocks, 2001b). Docosahexaenoic acid is metabolized to resolvins and neuroprotectins through the action of 15-LOX and 5-LOX like enzyme (Bazan, 2005a,b; Serhan, 2005; Tjonahen et al., 2006). Resolvins and neuroprotectins act through their receptors and inhibit the generation of prostaglandins,

leukotrienes, and thromboxanes. Thus, docosahexaenoic acid-derived lipid mediators prevent neuroinflammation by inhibiting transcription factor NF κ B, preventing cytokine secretion, blocking the synthesis of prostaglandins, leukotrienes, and thromboxanes, and modulating leukocyte trafficking. In murine peritonitis, resolvin E2 (5S, 18-dihydroxy-eicosapentaenoic acid) blocks zymosan-mediated polymorphonuclear leukocyte infiltration and displays potent anti-inflammatory properties (Tjonahen et al., 2006). Depending on its timing and magnitude in brain tissue, inflammation serves multiple purposes. It is involved in protection of uninjured neurons and removal of degenerating neuronal debris and also in assisting repair and recovery processes (Serhan, 2004). Inhibitors of multiple forms of PLA₂ prevent chronic inflammation and neural cell injury (Meyer et al., 2005; Malaviya et al., 2006; Farooqui et al., 2006).

3.3.8 Multiple Forms of PLA₂ in Nociception and Vacuous Chewing Movements

Injections of carrageenan in the paw or face have been widely used as models to study pain sensitization (Walters, 1994; Ng and Ong, 2001). Intracerebroventricular injections of a sPLA₂ inhibitor, 12-epi-scalaradial; a cPLA₂ inhibitor, AACOCF_{3:} and a iPLA₂ inhibitor, bromoenol lactone (BEL) reduce the response to the development of allodynia after facial carrageenan injections in C57BL/6 J (B6) and BALB/c mice (Yeo et al., 2004). The effect of PLA₂ inhibitors on allodynia is not due to arachidonic acid release and generation of prostaglandin but due to lysophospholipids (Yeo et al., 2004). Based on these observations, it is proposed that lysophosphatidylcholine mediates pain transmission in the central nervous system. The pronounced and long-lasting antinociceptive effect of 12-epi-scalaradial, AACOCF₃, and BEL is consistent with studies on neurotransmitter release in neurons and supports a key role of central nervous sPLA₂, cPLA₂, and iPLA₂ in synaptic and pain transmission. These results suggest that PLA₂ isoforms play an important role not only in pain transmission but also in non-painful, touch, or pressure sensation. Findings on the anti-nociceptive effect of PLA₂ inhibitors are supported by recent studies on intrathecal injections of MAFP and AACOCF₃ in rats. MAFP has a significant anti-nociceptive effect in the rat formalin test (Ates et al., 2003). Similarly, in vivo, the systemic and intrathecal delivery of AX048, a potent cPLA₂ inhibitor prevents carrageenan hyperalgesia as well as spinally mediated hyperalgesia induced by intrathecal substance P. Collectively, these studies (Kokotos et al., 2004; DeMar et al., 2006) suggest that cPLA₂ inhibitors mediate anti-nociceptive activity by blocking $cPLA_2$ activity and reducing lysophosphatidylcholine levels.

In addition to nociception, BEL also affects vacuous chewing movements (VCMs) in rats. Thus, BEL interferes with iPLA₂ activity in various components of motor system. Rats receiving bromoenol lactone and highdose MAFP injections in the striatum, thalamus, and motor cortex show a significant increase in vacuous chewing movements, compared to those injected with PBS vehicle. Vacuous chewing movements mediated by bromoenol lactone can be prevented by intramuscular injections of the anticholinergic drug benztropine, commonly used to treat extrapyramidal side effects in humans. The involvement of iPLA₂ in VCM is supported by using antisense oligonucleotides to iPLA₂. Intrastriatal injections of scrambled sense sequence of iPLA₂ have no effect on VCM. In contrast, increased VCM are observed after intrastriatal injection of antisense oligonucleotides to iPLA₂. The increased in VCM can be correlated with decrease in protein levels of iPLA₂ in the striatum in Western blot experiments, confirming a role of iPLA₂ inhibition in the appearance of VCM. These observations strongly support the involvement of iPLA2-mediated signaling in VCMs (Lee et al., 2007). Vacuous chewing and tongue protrusions are commonly accepted as a rodent model for tardive dyskinesia, a common side effect in human patients on antipsychotic drugs (Iversen et al., 1980; Ellison et al., 1987). The VCMs can be abolished by treatment with benztropine, a drug commonly used for the treatment of tardive dyskinesia and other movement disorders. It is proposed that effect of BEL on VCMs may provide insights on molecular mechanism of the development of tardive dyskinesia in humans (Lee et al., 2007).

3.3.9 Multiple Forms of PLA₂ in Oxidative Stress

The brain consumes large quantities of oxygen relative to its contribution to total body mass. This, together with low antioxidants levels (vitamin E, glutathione, lipoic acid) and antioxidant enzymes (superoxide dismutase, catalase and peroxidase), places neural cells at risk for damage mediated by reactive oxygen species (ROS) (Farooqui et al., 2008). Oxidative stress is defined as cytotoxic consequences produced by ROS (superoxide, singlet oxygen, peroxvnitrite and hydrogen peroxide). Oxidative stress is caused by an imbalance between increased levels ROS and cellular antioxidants. Oxidative stressmediated damage affects lipids, proteins, and DNA (Berlett and Stadtman, 1997; Farooqui et al., 2008). The reaction between ROS and proteins or unsaturated lipids in the plasma membrane leads to a chemical cross-linking of membrane proteins and phospholipids and a reduction in membrane unsaturation. This depletion of unsaturation in membrane lipids is associated with decreased membrane fluidity and decreases in the activity of membrane-bound enzymes, ion channels, and receptors (Ray et al., 1994). Furthermore, oxidative modification of phospholipids alters the physiological state of the neural membranes, which in turn affects the susceptibility of oxygenated and non-oxygenated fatty acid residues toward multiple forms of PLA₂.

Paralogs/spilce variants of cPLA₂ are known to interact with NADPH oxidase and regulate ROS generation (Shmelzer et al., 2003; Sun et al., 2007). The mechanism by which paralogs of cPLA₂ modulate NADPH oxidase activity is not known. However, it is recently demonstrated that upon non-neural cell stimulation, cPLA₂ is transiently recruited to the plasma membranes by NADPH oxidase (Shmelzer et al., 2003; Levy, 2006). Coimmunoprecipitation experiments and double labeling immunofluorescence analysis indicate unique co-localization of multiple forms of cPLA₂ and the NADPH oxidase in plasma membranes of stimulated cells. Furthermore, in vitro studies also indicate a specific affinity binding between NADPH oxidase subunit (GST-p47phox or GST-p67phox) and cPLA₂ in extracts of stimulated cells. The interactions between two enzymes provide the molecular basis for the activation of membrane associated NADPH oxidase by PLA2-derived arachidonate (Shmelzer et al., 2003). The ability of paralogs/splice variants of cPLA₂ to regulate two different functions (superoxide generation and eicosanoid production) at the same time in indicates a close relationship between NADPH oxidase and $cPLA_2$ in mediating oxidation and degradation of neural membrane glycerophospholipids located in various subcellular structures (Levy, 2006).

Ischemic injury increase in cPLA₂, sPLA₂, and PlsEtn-PLA₂ activities is not only accompanied by the generation of phospholipids degraded products and increased in oxidative stress but also by reduction in the levels of vitamin E and glutathione (Farooqui et al., 1994b; Farooqui and Horrocks, 1994b; Wullner et al., 1999). The neurochemical consequences of increased PLA₂ activities and high levels of arachidonic acid and its oxidation products include not only the generation of highly reactive oxygen free radical species with their potent damaging effects on neural membrane phospholipids, proteins, and DNA but also induction of inflammatory reactions. Supplementation of vitamin E is valuable in reducing cerebral damage in patients with stroke (Ascherio et al., 1999; Garcia-Estrada et al., 2003). It is stated that vitamin E reduces generation of ROS, decreases the rate of lipid peroxidation, and stabilizes neuronal membranes. Involvement of iPLA₂ in the translocation of hypoxia-inducible factor-1 α (HIF- 1α) to the nucleus following hypoxic injury has been reported (Osada-Oka et al., 2006). BEL, a specific inhibitor of iPLA₂ suppresses erythropoietin mRNA expression and HIF-1 α translocation to the nucleus indicating that this PLA₂ also participates in hypoxic injury. Collective evidence suggests that upregulation of multiple forms of PLA₂ during acute trauma results in oxidative stress which can be reversed by antioxidants.

3.3.10 Multiple Forms of PLA₂ in Apoptotic and Necrotic Cell Death

Apoptosis and necrosis are the two basic mechanisms of cell death that occur in brain and spinal cord. Apoptosis occurs in neurons during development of the nervous system. Approximately 50% of the neurons apoptose during neurogenesis

before the nervous system matures. However, recent studies implicate premature apoptosis and/or aberrations in the fine control of neuronal apoptosis in the pathogenesis of a variety of neurodegenerative disorders such as Alzheimer disease, Parkinson disease, Huntington disease, amvotrophic lateral sclerosis, spinal cord trauma, stroke, and head injury. Apoptosis is an active process in which caspases, a family of cysteine-dependent endoproteases with specificity for aspartate residues in proteins, are stimulated. Caspases act either as an upstream initiator of a proteolytic cascade, such as caspase-8 and caspase-9, or as a downstream effector such as caspase-3 that hydrolyzes intracellular proteins related to signal transduction. These proteins include protein kinase C, cPLA₂, iPLA₂, PLC, and cytoskeletal proteins such as α -spectrin, β -spectrin, actin, vimentin, presenilins, Bcl-2 family of apoptosis related proteins, and DNA modulating enzymes (Sastry and Rao, 2000). Apoptotic cell death is accompanied by cell shrinkage, dynamic membrane blebbing, chromatin condensation, DNA laddering, loss of plasma membrane asymmetry, maintenance of ATP, mitochondrial oxyradical generation, and a mild calcium ion overload (Sastry and Rao, 2000).

The mild alteration in calcium homeostasis and its short duration may lead to neuronal degeneration through caspase-3-mediated PLA₂ resulting in apoptotic cell death. Inhibitors of caspases and PLA₂ block apoptotic cell death (Wissing et al., 1997; Atsumi et al., 1998; Pirianov et al., 1999; Atsumi et al., 2000). Several isoforms, cPLA₂, iPLA₂, sPLA₂, and PlsEtn-PLA₂, participate in apoptotic cell death in brain tissue. During Fas-mediated apoptosis, iPLA₂ activity is stimulated and bromoenol lactone (BEL), an iPLA₂ inhibitor retards apoptotic cell death supporting the view that iPLA₂ is associated with Fasmediated cell death (Atsumi et al., 1998, 2000; Balsinde et al., 2006). Induction of apoptosis in non-neural cells generates lyso-PtdCho (16:0-lyso-PtdCho and 18:0-lyso-PtdCho), which acts as chemotactic factors for macrophages. The inhibition of caspases or iPLA₂ blocks the enhanced release of lyso-PtdCho from apoptotic cells (Lauber et al., 2003).

The other mechanism of apoptosis in rat brain involves the release of mitochondrial cytochrome c in the presence of truncated forms of BID (tBID) and BAX. This process is independent of the mitochondrial permeability transition, but depends upon generation of reactive oxygen species (ROS) and augmentation of iPLA₂ activity (Brustovetsky et al., 2005). The main target of ROS is mitochondrial cardiolipins. These glycerophospholipids are enriched in polyunsaturated fatty acids and located in the inner mitochondrial membrane near the ROSproducing sites. Under physiological conditions mitochondria have ability to repair peroxidative damage through the deacylation/reacylation cycle mediated by PLA₂ isoforms and acyl-coenzyme A-dependent monolysocardiolipin acyltransferase (Farooqui et al., 2000a; Seleznev et al., 2006). Treatment of INS-1 cell with staurosporine (STS) results in location of iPLA₂ to mitochondrial membrane where it not only blocks the loss of mitochondrial membrane potential and attenuates the release of cytochrome c, Smac/DIABLO, but also reduces mitochondrial reactive oxygen species production. Furthermore, STS downregulates endogenous iPLA₂ transcription in both INS-1 and iPLA₂-expressing INS-1 cells without affecting the expression of group IV Ca^{2+} -dependent PLA₂. Collective evidence suggests that iPLA₂ is associated with the protection of mitochondrial function from oxidative damage during apoptosis (Seleznev et al 2006). The treatment of brain mitochondria with a mixture of tBID and BAX not only inhibits cytochrome c release, but also suppresses iPLA₂ activity. This indicates a correlation between the iPLA₂ activity and the release of cytochrome c from brain mitochondrial membrane may lead to the release of cytochrome c, which may initiate apoptotic cell death (Brustovetsky et al., 2005).

Stimulation of sPLA₂ is observed in cytokine-mediated apoptosis in neuronrich cultures. sPLA₂ inhibitors retard apoptotic cell death in neuronal cultures supporting the view that sPLA₂ may also be involved in apoptotic cell death (Yagami et al., 2002, 2003, 2005; Zhao et al., 2002; Lee et al., 2006).

PlsEtn-PLA₂ is another target for ceramide-induced apoptosis (Latorre et al., 2003). Based on the effect of caspase-3 inhibitors, it is suggested that caspase-3 activates PlsEtn-PLA₂, and quinacrine, a non-specific PLA₂ inhibitor inhibits ceramide-mediated apoptosis. This suggests an interaction between plasmalogen and sphingomyelin catabolism. Thus, many isoforms of PLA₂ are involved in apoptotic cell death.

Necrosis occurs due to excessive physical or chemical injury. It is characterized by passive cell swelling, loss of membrane permeability, high calcium ion influx, drastic decrease in ATP levels, high levels of ROS, alterations in cellular redox due to the depletion of reduced glutathione, and intense inflammatory reactions accompany necrotic cell death ((Sastry and Rao, 2000; Weber, 1999). The activation of PLA₂ isoforms releases arachidonic acid and sets in motion an uncontrolled "arachidonic acid cascade" generating not only more ROS, but also 4-hydroxynonenal (4-HNE), which disrupts transmembrane signaling and the glucose and glutamate transporters in astrocytes (Camandola et al., 2000). The arachidonic acid cascade also potentiates the accumulation of lipid hydroperoxides. These lipid hydroperoxides inhibit reacylation of glycerophospholipids in neural membranes (Zaleska and Wilson, 1989). This inhibition constitutes another mechanism whereby oxidative processes contribute to necrotic cell death in neural cells. All together, these studies suggest that isoforms and paralogs of PLA2 and PLA2-generated metabolites play an important role in apoptotic and necrotic cell death.

3.3.11 Multiple Forms of PLA₂ in Chemotaxis

Recently iPLA₂ and cPLA₂ have been identified as regulators of chemotaxis in monocytes. These enzymes are localized in cytosol in unstimulated monocytes. Upon MCP-1 stimulation, iPLA₂ β is translocated to the membrane-enriched pseudopod (Mishra et al., 2008). In contrast, cPLA₂ α is translocated to the endoplasmic reticulum. Although iPLA₂ β or cPLA₂ α antisense oligodeoxyribonucleotide

(ODN)-treated monocytes display reduced rate of translocation, iPLA₂ β also modulates directionality and actin polymerization. PLA₂ β or cPLA₂ α antisense ODN-treated adoptively transferred mouse monocytes display a profound defect in migration to the peritoneum in vivo. These observations suggest that iPLA₂ β and cPLA₂ α regulate monocyte migration from different intracellular locations, with iPLA₂ β acting as a critical regulator of the cellular compass, and identify them as potential targets for antiinflammatory strategies (Mishra et al., 2008).

3.4 Regulation of Multiple Forms of PLA₂ Activity in Brain

Many stimuli regulate multiple forms of PLA₂ and modulate the generation of PLA₂-derived lipid mediators. They include neurotransmitters, growth factors, cytokines, glycosaminoglycans, ganglioside, and endogenous inhibitors like annexins. Regulation of multiple forms of PLA₂ not only depends on endogenous and exogenous stimuli but also on type of neural cells involved (neurons versus glia or neural cells versus non-neural cells). In addition to signaling role PLA₂, regulatory processes also maintain neural cell glycerophospholipid mass and therefore glycerophospholipid homeostasis by modulating the recycling of fatty acid moieties.

3.4.1 Regulation of cPLA₂

The most thoroughly investigated class of PLA₂ is cPLA₂ (Shirai and Ito, 2004; Clark et al., 1995). In neural membranes, cPLA₂ activity and arachidonic acid release are linked to dopamine, glutamate, serotonin, P2-purinergic, cytokine, growth factor receptors, and RA receptors through different coupling mechanisms. Some receptors involve G-proteins and others do not (Farooqui et al., 2006). As stated above that cPLA₂ activity is regulated by translocation in the presence of Ca^{2+} and by phosphorylation. Calcium mediates binding of the enzyme to phospholipid substrate without being involved in the catalytic mechanism itself. An increase in the intracellular calcium produces translocation of cPLA₂ to nuclear and other cellular membranes through a calcium-dependent lipid-binding motif (Clark et al., 1995). The translocation of cPLA₂ not only allows the interaction between enzyme protein and its phospholipid substrate but also brings cPLA₂ into close proximity with other downstream enzymes responsible for the conversion of arachidonic acid into eicosanoids. cPLA₂ also contains a consensus sequence for mitogen-activated protein kinase (Pro-Leu-Ser-505-Pro). In astrocytes, Ser-505 is phosphorylated by both p38-MAP kinase and c-Jun N-terminal kinase (Hernández et al., 1999, 2000) (Fig. 3.5). Phosphorylation of cPLA₂ increases its intrinsic activity by several folds. sPLA₂ interacts with cPLA₂ during agonist-mediated receptor activation. In keeping with this view, stimulation

of 1321N1 cells with sPLA₂ elicits the decrease in electrophoretic mobility that is characteristic of the phosphorylation of cPLA₂, as well as activation of p42 mitogen-activated protein (MAP) kinase, c-Jun kinase, and p38 MAP kinase (Hernández et al., 1999). Thus, cPLA₂ paralogs may be regulated both by docking to the cell membrane for accessing its glycerophospholipid substrate and by phosphorylation (Clark et al., 1995; Hernández et al., 1999).

In neural and non-neural systems, cPLA₂ inhibitory proteins (annexins) modulate cPLA₂ activity (Gerke and Moss, 1997; Kaetzel and Dedman, 1995; Clemen et al., 2001). The ability of annexins, particularly annexin 1, to inhibit PLA_2 is well known and a substrate depletion mechanism is now widely accepted as the explanation for most inhibitory studies (Kim et al., 2001a,b). Studies on the regulation of cPLA₂ activity by annexins in non-neural cells are complicated not only by the presence of several annexins (at least five) but also by the occurrence of several paralogs of cPLA₂ activity. Annexin 1(ANXA1) specifically targets cPLA₂ through direct enzyme inhibition and suppression of cytokine-mediated activation of the enzyme. ANXA1 also downregulates the activity of enzymes such as inducible nitric oxide synthase (iNOS) in macrophages and inducible cyclooxygenase-2 (COX-2) in activated microglia. The inhibition of iNOS expression may be due to ANXA1-mediated IL-10 release in macrophages (Parente and Solito, 2004). Detailed investigations are required on the type of $cPLA_2$, binding parameters, and the type of annexin involved in cPLA₂-mediated signaling in neural cells. The occurrence of PLA₂ activating proteins has also been reported in non-neural cells such as monocytes and endothelial cells (Clark et al., 1987). cPLA₂-activating proteins occur in Aplysia neurons and rat cerebral cortex (Calignano et al., 1991). The partially purified cPLA₂ stimulatory protein is phosphorylated by protein kinase C. It has been proposed that phosphorylation of this stimulatory protein by protein kinase C regulates cPLA₂ activity in neurons (Calignano et al., 1991).

3.4.2 Regulation of iPLA₂

Very little is known about the regulation of iPLA₂ activity in brain tissue. However, iPLA₂ activity in non-neural cells is regulated by several mechanisms. iPLA₂ splice variants are homotetramers formed through interactions between N-terminal ankyrin repeates (Larsson Forsell et al., 1998). iPLA₂ is negatively regulated by truncated splice variant proteins that block the formation of active iPLA₂ tetramers. Sterol and sterol regulatory element binding proteins have been shown to modulate iPLA₂ expression. Thus, Chinese hamster ovary cells grown under sterol-depleted conditions show 2- to 3-fold increased in catalytic activity, mRNA, and iPLA₂ protein expression (Seashols et al., 2004). Induction of iPLA₂ is suppressed when cell cultures are supplemented with exogenous sterols suggesting that sterol regulatory element in iPLA₂ gene (Seashols et al., 2004).

iPLA₂ γ is a membrane bound iPLA₂ isoform that is associated with nuclear, mitochondrial, and peroxisomal membranes (Kuwata et al., 2007). In rat fibroblastic 3Y1 cells, iPLA₂ γ participates in the induction of sPLA₂-IIA. The molecular mechanism associated with sPLA₂-IIA induction is not fully understood. However, based on the effects of inhibitors of PLA₂ and 12/15-LOX, it is suggested that in rat fibroblastic 3Y1 cells, 12S or 15S-hydroperoxyeicosatetraenoic acid, a metabolite-derived through the action of 12/15-LOX on arachidonic acid contributes to sPLA₂ IIA induction (Kuwata et al., 2007). This suggestion is supported by the observation that the R-enantiomer of bromoenol lactone, an iPLA₂ γ inhibitor, suppresses the cytokine-induced sPLA₂-IIA expression, whereas (S)-bromoenol lactone, an iPLA₂ β inhibitor, has no effect. Moreover, lipopolysaccharide-stimulated sPLA₂-IIA expression is retarded in iPLA₂ γ knockdowns. These findings open new insight into a novel regulatory role of iPLA₂ γ in stimulus-coupled sPLA₂-IIA expression.

3.4.3 Regulation of sPLA₂

High concentrations of calcium stimulate brain sPLA₂ activity not only in brain tissue but also in astrocytic cultures. The other mechanism involved in regulation of sPLA₂ activity is through its gene expression. IL-1, IL-6, tumor necrosis factor- α (TNF- α), and growth factors with tyrosine kinase receptors upregulate cPLA₂ and sPLA₂ expression, whereas glucocorticoids downregulate cPLA₂ and sPLA₂ activities in astrocytic cultures (Hernández et al., 2000). This sensitivity to glucocorticoids is consistent with the presence of a putative glucocorticoid response element in the promoter region of the cPLA₂ gene (Tay et al., 1994). The presence of sPLA₂, iPLA₂, and cPLA₂ transcripts (Zanassi et al., 1998; Yang et al., 1999; Balboa et al., 2002) in astrocytes is in agreement with the notion that these cells are capable of responding to cytokines with the induction of PLA₂ isozyme activities. At present, nothing is known about the regulation of PlsEtn-PLA₂. However, it has been suggested that glycosaminoglycans and gangliosides may be involved in regulation of this enzyme (Yang et al., 1994a,b).

3.5 Conclusion

Several paralogs/splice variants/isoforms of cPLA₂, iPLA₂, and sPLA₂ are expressed in brain tissue. The relationship among multiple forms of PLA₂ and the cellular processes in which their diverse lipid mediators are involved is becoming more complex because of recent discovery of new paralogs/splice variants/isoforms of cPLA₂, iPLA₂, and sPLA₂. Most of our knowledge about the role of PLA₂ isoforms in brain comes from in vitro cell biology, neuropharmacology, and neuropathology studies. In brain tissue, paralogs/splice variant/

isoforms of PLA₂ perform housekeeping as well as signaling functions. Housekeeping functions of PLA₂ multiple forms include phospholipid turnover. neural membrane remodeling, and removal of peroxidized fatty acids from phospholipid hydroperoxides. Under normal conditions, changes in activity of PLA₂ multiple forms induce alterations in the level of essential phospholipids affecting membrane fluidity, permeability, and ion homeostasis transiently. The reacylation of lysophospholipids through a series of energy-dependent reactions results in restoration of the normal phospholipid content of neural membranes (Farooqui et al., 2000a). The PLA₂ signaling functions in neural membranes are modulated by the generation of various lipid mediators. These mediators include eicosanoids (prostaglandins, leukotrienes, and lipoxins), docosanoids (resolvins and neuroprotectins), lysophospholipids, and plateletactivating factor (PAF). They modulate cellular function by acting through extracellular as well as intracellular receptors (Farooqui et al., 2000b,c). Besides modulating the generation of lipid mediators, PLA₂ isoforms are also involved in maintaining neural cell membrane homeostasis through the recycling of fatty acid moieties in glycerophospholipid molecular species of brain tissues. Although the relative contributions of PLA₂ isoforms in the release of various lipid mediators and maintenance of phospholipid molecular mass in neural membrane are still unknown, it is evident that PLA₂ isoforms play important roles in the efficient control of the acylation/deacylation cycle and in regulation of the production of eicosanoids, platelet-activating factor, and docosanoids. These lipid mediators are involved in modulation of neurotransmitter release, long-term potentiation, membrane repair, cell cycle, regeneration, and neurodegeneration.

In contrast, under pathological conditions (ischemia) PLA_2 paralog/splice variant/isozyme-mediated hydrolysis of neural membrane phospholipids produces free fatty acids and lysophospholipids at rates greater than the rate of membrane repair (reacylation), resulting in an accumulation of free fatty acids (Sun and MacQuarrie, 1989). This process produces irreversible changes in membrane integrity, loss of ion homeostasis, and abnormalities in cellular function (Farooqui and Horrocks, 1991, 1994, 2006). Collective evidence suggests that multiple forms of PLA_2 are necessary for neural cell survival. However, upregulation of these enzymes in pathological conditions causes neuroinflammation, oxidative stress, and neural cell injury.

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Chapter 4 Glycerophospholipid Metabolism in the Nucleus: Cross Talk Among Phospholipase A₂, Phospholipase C and Phospholipase D

4.1 Introduction

In brain, neural cell viability and functions are maintained through a network of signaling molecules (lipid mediators) that modulate neural cell metabolism, roles, and homeostasis by conveying messages from plasma membrane to various subcellular structures including the nucleus. These signaling molecules originate from neural membranes that are made up of glycerophospholipids, sphingolipids, cholesterol, and proteins. At the sn-1 and sn-2 position of glycerol moiety, glycerophospholipids contain saturated and long polyunsaturated fatty acids, respectively. In contrast, sphingolipids bear mostly saturated aliphatic hydrocarbon chains. As stated earlier, glycerophospholipids and sphingolipids contribute to the lipid asymmetry, whereas cholesterol and sphingolipids form lipid microdomains or lipid rafts. Lipid raft population is heterogeneous. Some lipid rafts lack structural protein components. while others are enriched in structural proteins. These differences not only modulate the morphology but also the stability and functions of lipid rafts. Alterations in levels of signaling molecules in response to mechanical or metabolic stress cause drastic functional consequences and result in modulation of neuronal survival as well as neurodegeneration (Farooqui et al., 2007a). Several lines of evidence support the view that neural cell survival or death involves the activation of discrete signaling pathways and generation of mediators associated with compromised plasma membrane, mitochondrial, and nuclear lipid and protein metabolism (Farooqui et al., 2004b; SanGiovanni and Chew, 2005; Farooqui et al., 2007a).

The occurrence of glycerophospholipids, sphingolipids, and cholesterol in neural and non-neural cell nuclei has been established by biochemical and electron microscopic procedures (Fraschini and Fuhrman Conti, 1995; Albi et al., 1996; Ledeen and Wu, 2006; Albi and Magni, 2004). The total glycer-ophospholipid content of nuclei is reported as 3% by weight compared with 75% for protein and 22% for DNA. Major nuclear glycerophospholipids include phosphatidylinositol (PtdIns), phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer), and sphingomyelin (Cer*P*Cho). Small amounts of phosphatidylethanolamine (PtdEtn) are also found in the nucleus. This is in

contrast to plasma membranes, which have considerable amount of PtdCho, PtdEtn, PtdSer (phosphatidylserine), and PtdIns (Hunt et al., 2001; Irvine, 2003, 2006). The glycerophospholipid contents (per mg protein) of the nuclear membrane are approximately nine times that of whole nuclei. Changes in nuclear glycerophospholipid metabolism occur during cell activation, differentiation, proliferation, and degeneration. Although significant information is available on metabolism and proportions of molecular species of glycerophospholipids in plasma membrane (Farooqui et al., 2000a, 2002; Farooqui and Horrocks, 2007), but very little is known about metabolism and occurrence of glycerophospholipid molecular species in the nucleus (Hunt et al., 2001; Delton-Vandenbroucke et al., 2004). Nuclear glycerophospholipids have a composition and turnover rate different from plasma membranes, microsomal, and mitochondrial glycerophospholipids. Based on metabolism of phosphoinositides, it is proposed that the nucleus is a site of an active and autonomous glycerophospholipid metabolism (Fraschini et al., 1999; Tamiya-Koizumi, 2002; Martelli et al., 2004b; Ledeen and Wu, 2004; Irvine, 2006; Ledeen and Wu, 2006; Luo et al., 2006). Like plasma membrane preparations, nuclear preparations also contain various types of receptors, which not only modulate nuclear signal transduction network but also cross talk with plasma membrane and other subcellular structure receptors (Table 4.1).

Histones, the cationic proteins found in the nucleus, bind to anionic glycerophospholipids (cardiolipin, Ptd_2Gro , and PtdSer) with high avidity, but they do not interact with zwitterionic phospholipids (PtdCho). Surface plasmon resonance (SPR) studies on interactions of PtdSer, PtdEtn, PtdCho, and cardiolipin with histones (H1, H2A, H2B, H3, and H4) indicate interactions of glycerophospholipids with all histones. Among histones, H2A displays the highest binding affinity for glycerophospholipids. It is proposed that histones/ nucleosomes complex not only promote autoimmunity toward nuclear compounds, but also contribute to the binding of histones with surfaces and blebs of apoptotic cells (Furnrohr et al., 2007).

| Receptor | Reference |
|--|--|
| Retinoic acid receptor | Farooqui et al. (2004) |
| PAF receptor | Gobeil et al. (2003b) |
| Ins1,4,5-trisphosphate receptor | Malviya and Klein (2006) |
| Thrombaxane receptor | Lin et al. (2005), Ramamurthy et al. (2006) |
| Peroxisome proliferator-activated receptor | Hwang (2000) |
| PGE ₂ receptor | Gobeil et al. (2003b) |
| Liver X receptor | Crestani et al. (2004), Pelton et al. (2005) |
| Farnesoid receptor | Favard et al. (2001), Pelton et al. (2005) |

 Table 4.1 Receptors associated with glycerophospholipid metabolism in the nucleus of neural and non-neural cells

4.2 Phospholipid Metabolism in the Nucleus

Nuclear glycerophospholipids are not only concentrated in the nuclear envelop but also found inside the nucleus. After nuclear envelope membranes removal, the isolated nucleus contains about 6% of mammalian cell glycerophospholipid within the nuclear matrix (Hunt, 2006a,b). Among nuclear glycerophospholipids, phosphoinositides have received a lot of attention (Maraldi et al., 1999; Cocco et al., 2001; Martelli et al., 1999, 2003, 2002a, 2004b, a, 2006; Cocco et al., 2006; Goto et al., 2006). However, there has been considerable development on metabolism of other glycerophospholipids such as sphingomyelin, PlsEtn, and PlsEtn in the nucleus (Albi and Magni, 2004; Albi et al., 2004; Antony et al., 2001b, 2003a; Farooqui et al., 2004a). The occurrence of phospholipases A_2 (PLA₂), phospholipase C (PLC), and phospholipase D (PLD) and generation of lipid mediators such as arachidonic acid, eicosanoids, diacylglycerol, plateletactivating factor, and various inositol phosphates in the nucleus has been described in recent years. Multiple forms of PLA₂ hydrolyze neural membrane glycerophospholipids at the sn-2 position generating lysophospholipids and arachidonic or docosahexaenoic acids (Farooqui et al., 2000a,b). Multiple



Fig. 4.1 Hypothetical diagram showing the interactions in among second messengers generated by PLA₂, PLC, and PLD in the nucleus. Phosphatidylcholine (PtdCho); phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5) P_2); lysophosphatidylcholine (Lyso-PtdCho); inositol 1,4,5-trisphosphate (InsP₃); phosphatidic acid (PtdH); platelet-activating factor (PAF); phospholipase A₂ (PLA₂); phospholipase C (PLC); phospholipase D (PLD); protein kinase C (PKC), nuclear membrane (NM), and plasma membrane (PM)

forms of PLC hydrolyze the phosphodiester bond on the glycerol side forming diacylglycerols and a phosphobase, whereas isoforms of PLD break the bond between the base and the phosphate forming phosphatidic acid and a free base, choline, ethanolamine, or inositol (Farooqui et al., 2000a) (Fig. 4.1). Nuclear matrix contains dynamic pools of PtdCho and PtdIns that serve as precursor for lipid mediators and other glycerophospholipids such as plasmalogens appear as cells mature and proliferate. Applications of highly sensitive and specific methods based on tandem electrospray ionization mass spectrometry (lipidomics) and the use of stable isotopes have indicated the presence of glycerophospholipid static and dynamic pools in the nuclear matrix (Hunt, 2006a.b). Since many enzymes that synthesize and hydrolyze glycerophospholipid are not only present in the nuclear membrane but also in nuclear matrix, it is suggested that nucleus has its own glycerophospholipid metabolism. Interactions among PtdCho, PlsEtn, and PlsEtn-derived lipid mediators in the nucleus and interplay between similar lipid mediators in other subcellular organelles found in the cytoplasmic compartment is essential for maintaining normal neuronal and glial cell growth (Farooqui et al., 2004a).

4.3 Importance of Phospholipases and Glycerophospholipid Metabolism in the Nucleus

PLA₂, PLC, and PLD-derived lipid mediators modulate neural cell proliferation, differentiation, and apoptosis. These processes are modulated by a signal transduction network that is controlled by activities of PLA₂, PLC, and PLD in various subcellular compartments under normal and pathological conditions. Cross talks among various receptors through PLA₂, PLC, and PLD-derived lipid mediators are essential for neural cell survival (Farooqui et al., 2004a). Agonist-mediated cross talk among PLA₂, PLC, and PLD may determine the cellular behavior in response to environmental challenges. Within PLA₂, PLC, and PLD signal transduction network, many neural cell functions are modulated at the transcriptional level and consequently at gene level. Activities of PLA₂, PLC, and PLD are modulated by the coordinated expression of different genes associated with the expression of transcription factors, neurotrophins, and cytokines (Haddad, 2004). During proliferation, differentiation, and apoptosis, the quantitative ratios among various glycerophospholipids undergo significant alterations depending upon the functional state of neurons, astrocytes, and oligodendrocytes. Proliferation, differentiation, and apoptosis are not only modulated by the activities of PLA₂, PLC, and PLD network but also through interactions among neurons, astrocytes, oligodendrocytes, and microglia (Lang et al., 1995; D'Santos et al., 1998; Farooqui et al., 2000a, 2002). Depending upon agonists, receptor-mediated activation of PLA₂, PLC, and PLD generates lipid mediators such as diacylglycerol, arachidonic acid, eicosanoids, platelet-activating factor, and various phosphorylated inositides not only in plasma membrane and cytoplasmic compartment but also in the nucleus. Thus, some extracellular stimuli, such as retinoic acid, produce bioactive lipid metabolites in the nucleus while other agonists, such as glutamate generate lipid mediators in cytoplasmic and plasma membrane compartments (see below) (Farooqui et al., 2003a; Antony et al., 2001b, 2003a; Farooqui et al., 2004a). In neural cell cultures, agonist-mediated generation of lipid mediators in nuclear fraction is not a duplication of lipid mediator changes that occur in the cytoplasm and at the plasma membrane level. This suggestion is supported by studies on PLC activity and generation of PtdIns-derived lipid mediators in the nucleus (Martelli et al., 1999, 2004b; Cocco et al., 2001; Maraldi et al., 1999). Furthermore, the enzymic properties of glycerophospholipid metabolizing enzymes in the nuclear fraction are different from those found in plasma membrane, microsomes, and cytoplasm (Maraldi et al., 1999; Albi and Magni, 2004). For example, bombesin, a powerful mitogen, stimulates phosphoinositide metabolism at the plasma membrane level, but has no effect on phosphoinositide metabolism in the nucleus. Furthermore, insulin-like growth factor-1 (IGF-1) stimulates DAG-kinase activity in the nucleus but not in whole homogenate (Martelli et al., 2004b). In addition to their role in the generation of second messengers, nuclear inositol polyphosphates serve as essential co-factors for several nuclear processes such as DNA repair and transcription regulation. Nuclear PtdIns modulate RNA dynamics not only by stimulating RNA polymerase activity but also by modulating chromatin organization (Maraldi et al., 1999). Metabolites of the phosphatidylinositol cycle interact with nuclear lamin B and DNA topoisomerase and nuclear phospholipase C (Martelli et al., 2002b,a; Ledeen and Wu, 2004). Nuclear inositol lipids may also be involved in mRNA transcription and/or processing, DNA replication or repair potentially resulting in cell differentiation, proliferation, or apoptosis. Furthermore, the interaction of glycerophospholipids with histones and non-histone chromosomal proteins suggests that the regulation of RNA polymerase with PtdIns derivatives occurs at the level of template availability and modulate pre-mRNA splicing (D'Santos et al., 1998; Martelli et al., 2002a,b).

The nuclear fractions from neural and non-neural tissues contain many enzymes of glycerophospholipid metabolism (Table 4.2) that synthesize and regulate the levels of second messengers. These enzymes include PLA₂, PLC, PLD, diacylglycerol kinase (DAG-kinase), diacylglycerol lipase (DAG-lipase), phosphatidylinositol 4-kinase, Mg²⁺-dependent sphingomyelinase, CTP: phosphocholine cytidylyltransferase, and sphingosine kinase. The α isoform of CTP: choline phosphate cytidylyltransferase, the main enzyme of Kennedy pathway (Antony et al., 2000; Albi and Magni, 2004) also occurs in the nucleus, and its activity is involved in temperature-sensitive mutation associated with cell survival. Collective evidence thus suggests that glycerophospholipids and their second messengers play a major role in modulation of differentiation, apoptosis, and growth suppression (Ledeen and Wu, 2004; Hammond et al., 2004; Goto et al., 2006; Irvine, 2006).

| Enzyme | Reference |
|---|---|
| Phosphatidylinositol synthetase | Baker and Chang (1990b) |
| Acyl- <i>sn</i> -glycero-3-phosphate acyltransferase | Baker and Chang (1990a) |
| CTP:phosphocholine cytidylyltransferase | Morand and Kent (1989) |
| Diacylglycerol acyltransferase | Baker and Chang (1987) |
| Diacylglycerol kinase | Payrastre et al. (1992) |
| Lysophosphatidic acid phosphohydrolase | Baker and Chang (1990b) |
| Sphingosine kinase | Kleuser et al. (2001) |
| Acetyltransferase | Baker and Chang (1997) |
| Diacylglycerol lipase | Farooqui et al. (2004) |
| Monoacylglycerol lipase | Baker and Chang (1990b) |
| PtdIns-specific PLC (isoforms) | Payrastre et al. (1992) |
| PtdCho-specific PLC (isoforms) | Antony et al. (2000) |
| PLA ₂ (isoforms) | Antony et al. (2000) |
| PLD | Kanfer et al. (1996) |
| PtdH phosphatase | Kanfer et al. (1996) |
| Mg ²⁺ -dependent neutral sphingomyelinase | Tamiya-Koizumi (2002), Albi et al. (2006) |
| PtdIns 3-kinase, 4-kinase, 5-kinase | Martelli et al. (2004b) |
| CDP-choline: 1,2-diacylglycerol cholinephosphotransferase | Fernández-Tome et al. (2004) |
| Ceramidase | Shiraishi et al. (2003) |
| Leukotriene C4 synthase | Svartz et al. (2006) |
| 5-Lipoxygenase | Hanaka (2002), Svartz et al. (2006) |

 Table 4.2 Glycerophospholipid metabolizing enzymes involved in generation and modulation of phospholipid metabolism in the nucleus

4.4 Occurrence of Isoforms of Phospholipase A₂, Phospholipase C, and Phospholipase D in Nucleus

In neural cells, major proportions of the PLA₂, PLC, and PLD activities occur mainly in cytoplasm and the subcellular organelles found in cytoplasmic compartment with small amounts (5–10%) of PLA₂, PLC, and PLD localized in the nucleus (Antony et al., 2001b, 2003a; Farooqui et al., 2004a). The low levels of PLA₂, PLC, and PLD must be viewed in the context that the isolated nuclei contain less than 3% of the total protein present in homogenate.

In general, the function of the signal transduction network is to convey extracellular signals from the cell surface to the nucleus to induce a biological response at the gene level. This is performed by nuclear pore complexes (large proteinaceous assemblies) that provide the sole gateway for the exchange of material between cytoplasm and nucleus at the interphase. Thus, nuclear pore complexes are the sole gateways that mediate all trafficking between nucleus and cytoplasm (Fahrenkrog, 2006; Naim et al., 2007). Nuclear pore complexes support two modes of transport (a) passive diffusion of ions, metabolites, and

(b) intermediate-sized macromolecules. These processes facilitate receptormediated translocation of proteins, RNA, and ribonucleoprotein complexes. Faithful, continuous nuclear pore complex assembly is the key for maintaining normal physiological function and is closely tied to proper cell division. It is generally assumed that both modes of transport occur through a single diffusion channel located within the central pore of the nuclear pore complex. As such, the nuclear pore complex and nuclear transport play central roles in translocating death signals from the cell membrane to the nucleus where they initiate biochemical and morphological changes occurring during apoptosis (Fahrenkrog, 2006; Naim et al., 2007).

Brain PLA₂, PLC, and PLD are major signaling enzymes involved not only in neural cell proliferation, differentiation, and growth under normal conditions but also in neuroinflammation, oxidative stress, and cell death under pathological situations. These enzymes are linked to various receptors through different coupling mechanisms at the plasma membrane and nuclear domain levels. PLA₂, PLC, and PLD control the intensity and duration of the signal transduction process by modulating the levels of second messengers including arachidonic acid, diacylglycerols, platelet-activating factor, eicosanoids, and lipid mediators derived from polyphosphoinositides (Farooqui et al., 1997, 2000a; Farooqui and Horrocks, 2004).

The kinetic properties of PLA₂, PLC, and PLD in the nuclear fraction from LA-N-1 cells are shown in Table 4.3. The response of nuclear PLA₂, PLC, and PLD to their inhibitors is different from that of the PLA₂, PLC, and PLD associated with non-nuclear compartment (Misra and Pizzo, 2000; Martelli et al., 2004b) suggesting that nuclear phospholipases either differ from non-nuclear enzymes in their active site or may require different environment for their optimal activity.

| Kinetic parameter | Value | Reference |
|-------------------------|----------------|-----------------------|
| PtdEtn-PLA ₂ | | |
| pH optimum | 7.4 | Antony et al. (2001b) |
| Km value (µM) | 35.0 ± 5.0 | Antony et al. (2001b) |
| Vmax (pmol/min/mg) | 14.65 ± 1.5 | Antony et al. (2001b) |
| PlsEtn-PLA ₂ | | |
| pH optimum | 8.0 | Antony et al. (2001b) |
| Km value (µM) | 50.0 ± 7.0 | Antony et al. (2001b) |
| Vmax (pmol/min/mg) | 25.7 ± 2.0 | Antony et al. (2001b) |
| PLD | | |
| pH optimum | 6.5 | Antony et al. (2003b) |
| Km value (µM) | 330 | Antony et al. (2003b) |
| Vmax (pmol/min/mg) | 3.8 | Antony et al. (2003b) |

Table 4.3 Kinetic properties of nuclear PLA₂, PLC, and PLD in LA-N-1 cells

Nuclei are isolated from LA-N-1 cells and used for the determination of enzymic activities (Antony et al., 2000, 2001b, 2003a). At present nothing is known about the kinetic parameters of PtdIns-PLA₂ and PtdCho-PLC in LA-N-1 cell nuclei.

4.4.1 PLA₂ Activities in the Nucleus

As stated in chapter 3 that mammalian brain PLA_2 includes secretory phospholipase A_2 (sPLA₂), cytosolic phospholipase A_2 (cPLA₂), plasmalogen-selective phospholipase A_2 (PlsEtn-PLA₂), and calcium-independent phospholipase A_2 (iPLA₂) (Farooqui et al., 1997; Sun, et al., 2004; Farooqui and Horrocks, 2004). Some isoforms of PLA₂ have been partially purified and characterized from brain tissue (Hirashima et al., 1992; Yang et al., 1999). However, none of the above isoforms have been cloned and fully characterized from brain tissue.

LA-N-1 cells resemble neurons in many characteristics. Like neurons they undergo differentiation in the presence of agonists such as retinoic acid (Fig. 4.2). Subcellular distribution studies have indicated that LA-N-1 cell nuclei (Fig. 4.3) and rat cerebral neuron-enriched cultures contain at least two calcium-independent PLA₂ activities. The 110 kDa PLA₂ hydrolyzes 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PtdEtn), whereas the 39 kDa PLA₂ selectively





Fig. 4.2 Morphology of isolated LA-N-1 cells before and after treatment with retinoic acid. LA-N-1 cells before RA treatment (**a**) and after RA treatment (**b**). RA treatments are performed as described by Antony et al. (2001a, 2003b). Magnification: $\times 400$



Fig. 4.3 Morphology of intact isolated LA-N-1 cell nuclei with nuclear envelop. Isolation of nuclei and RA treatments has been performed as described by Antony et al. (2001a, 2003b). Magnification: ×2500 (**a**) and **B**, ×5000 (**b**)

acts on plasmenylethanolamine (PlsEtn). The treatment of LA-N-1 cell cultures with retinoic acid, an agonist that diffuses through cellular membranes, transported to the nucleus by retinal-binding proteins, results in a marked stimulation of PLA₂ activities hydrolyzing PtdEtn and PlsEtn. The specific activity of PtdEtn-PLA₂ increases during the first 5 days, while that of PlsEtn-PLA₂ increases rapidly during the first 2 days after retinoic acid treatment (Antony et al., 2001b, 2003a). The specific activities of PLA₂ hydrolyzing PlsEtn increase about 5- and 3.5-fold between 6 and 10 hours in the nuclei while that of PtdCho hydrolyzing cytosolic PLA₂ are elevated only 1.3- and 1.7-fold during the same period (Antony et al., 2001b, 2003a). This indicates that the effect of retinoic acid in LA-N-1 cells is mediated through the nuclear glycerophospholipids.

The stimulation of PLA₂ activities in nuclei by RA is in agreement with labeling studies. Thus, the treatment of prelabeled LA-N-1 cells with RA has indicated that arachidonic acid release in the nuclear fraction is a biphasic process. A rapid release within the first minute is followed by a sustained arachidonic acid release at 30 min that continues even after 10 h (Antony et al., 2003a). This observation is supported by studies on the effect of PLA₂ inhibitors that are known to exert a biphasic effect over several days on elongation in dorsal root ganglion axon, enhancing outgrowth at low concentrations, and inhibiting outgrowth at higher concentration (Suburo and Cei de Job, 1986). It is interesting to note here that cPLA₂ inhibitors delay the initial outgrowth of neurites on laminin and exogenous arachidonic acid enhances neurite outgrowth (Smalheiser et al., 1996).

Treatment of neuron-enriched cultures with kainate, a glutamate analog (Fig. 4.4), has no effect on nuclear fraction PlsEtn-PLA₂ activity, while PlsEtn-PLA₂ activity in the cytosolic fraction is increased 5-fold upon kainic acid treatment (Table 4.4). In contrast, treatment of neuron-enriched cultures with retinoic acid results in 3-fold increase in PlsEtn-PLA₂ activity in the nuclear fraction. Retinoic acid has no effect on PlsEtn-PLA₂ activity in non-nuclear and cytosolic fractions (Table 4.5). The collective evidence suggests that nuclear PLA₂ activities are stimulated earlier, then cytosolic PLA₂ activities, while plasma membrane PLA₂ activities are not changed as a result of retinoic acid treatment (Antony et al., 2001a, 2003a).



Fig. 4.4 Chemical structures of agonists that differentially effects PLA_2 activities in plasma membrane and nuclear fractions. Glutamate (**a**); kainate (**b**); all-*trans* retinoic acid (**c**), and 9-*cis* retinoic acid (**d**). Isolation of nuclei is described by Antony et al. (2001b, 2003a) and rat cerebellar cultures are prepared as described earlier (Farooqui et al., 2003b)

| Subcellular fraction | Control culture | KA-treated culture | KA + CNQX-treated culture |
|-------------------------------|-----------------|--------------------|---------------------------|
| Homogenate | 3.05 ± 0.62 | 10.21 ± 1.72 | 2.54 ± 0.26 |
| Nuclear fraction | 2.72 ± 0.21 | 2.34 ± 0.42 | 1.23 ± 0.25 |
| Non-nuclear membrane fraction | 2.20 ± 0.31 | 4.62 ± 0.48 | 1.80 ± 0.30 |
| Cytosol | 4.7 ± 0.42 | 15.22 ± 2.78 | 3.42 ± 0.50 |
| G 16 11 1 | 1/ 1/ | | 6.1 |

Table 4.4 Effect of kainate on $PlsEtn-PLA_2$ activity of subcellular fractions prepared fromrat cerebral neuron-enriched cultures

Specific activity is expressed as pmol/min/mg protein and represent mean of three experiments performed in triplicate. Kainic acid (50 μ M) and CNQX (10 μ M).

Table 4.5 Effect of retinoic acid on $PlsEtn-PLA_2$ activity of subcellular fractions preparedfrom rat cerebral neuron-enriched cultures

| Subcellular fraction | Control | RA-treated |
|----------------------|---------------|----------------|
| Homogenate | 2.72 ± 0.54 | 4.72 ± 0.52 |
| Nuclear fraction | 1.82 ± 0.35 | 10.73 ± 1.53 |
| Non-nuclear fraction | 2.00 ± 0.45 | 2.27 ± 0.53 |
| Cytosol | 3.72 ± 0.60 | 2.53 ± 0.28 |

Specific activity is expressed as pmol/min/mg protein and represent mean of three experiments performed in triplicate. Retinoic acid ($10 \,\mu$ M). Treatment was performed as described earlier (Antony et al., 2001b, 2003a).

The pretreatment of LA-N-1 cells with the pan retinoic acid receptor antagonist, BMS493, blocks the stimulation of nuclear PLA₂ activities suggesting that the nuclear PLA₂ stimulation by retinoic acid is a receptor-mediated process (Fig. 4.5). The retinoic acid-mediated stimulation of PtdEtn-PLA₂ and PlsEtn-PLA₂ activities in the nuclear fraction is also inhibited by a low concentration of

Fig. 4.5 Effect of BMS493 and cycloheximide on PlsEtn-PLA₂ and PtdEtn-PLA₂ in the nuclear fraction from LA-N-1 cell cultures. RA (1.0 μ M); BMS493 (10.0 μ M); cycloheximide (cycloH) (0.5 μ g). Modified from earlier studies (Antony et al., 2003a). Specific activity is expressed as pmol/min/mg protein. Values are mean \pm SD for three determinations



cycloheximide, but the extent of inhibition produced by cycloheximide is lower than that of BMS493. This observation suggests that the cycloheximideinduced inhibition of PLA₂ activities is due to a general decrease in protein synthesis. In contrast, the BMS493-mediated inhibition is due to its specific antagonistic activity toward retinoic acid receptor (Antony et al., 2003; Farooqui et al., 2004a). Little is known about the ligands that activate RXR in vivo. Unsaturated fatty acids, particularly linoleic, linolenic, arachidonic, and docosahexaenoic acids interact with RXR (Wolf, 2006). It is suggested that DHA and AA, products of PlsEtn-PLA₂ and cPLA₂ catalyzed reactions respectively may act as natural RXR ligands in brain tissue (Radominska-Pandya and Chen, 2002; Lengqvist et al., 2004; Farooqui et al., 2004a; de Urquiza et al., 2000). DHA is a more potent RXR ligand than AA in mediating robust RXR activation at low micromolar concentrations. This suggests that the non-covalent interactions between the RXR ligand-binding domain (LBD) of RXR and the DHA may influence neural function growth, differentiation, and apoptosis through activation of RXR signaling pathway. AA also interacts with RXR but less efficiently than DHA. DHA and its metabolites docosanoids inhibit AA release and oxidation of AA into eicosanoids (Fig. 4.6). AA and eicosanoids turn on genes associated with neuroinflammation, oxidative stress and apoptosis,



Fig. 4.6 Hypothetical diagram showing interactions between docosahexaenoic acid and arachidonic acid-generated metabolites in the nucleus. Plasma membrane (PM); retinoic acid (RA); retinoic acid binding protein (RBP); retnoic acid receptor (RXR); docosahexaenoic acid (DHA); arachidonic acid (AA); lipoxygenase (LOX); cyclooxygenase (COX). Positive sign (+) indicates stimulation and negative sign (-) indicates inhibition

whereas DHA and docosanoids turn on genes that block inflammation, oxidative stress, and apoptotic cell death (Farooqui et al., 2007b; Farooqui and Horrocks, 2007). Levels of DHA and AA in diet modulate inflammation, oxidative stress, and apoptotic cell death in brain tissue. In addition to above genes, DHA modulates genes for the expression of glucose and glutamate transporters, cyto-kines, transcription factors, cell adhesion molecules, cytoskeleton, and hormone receptors (Pifferi et al., 2005; Kitajka et al., 2002; Puskás et al., 2003). Alterations in activities of glucose and glutamate transporters, cytokines, transcription factors are produced, and modulated by the cooperation between retinoic receptor and peroxisome proliferator-activated receptors (Hwang, 2000).

It is well established that cytosolic $cPLA_2$ translocates to nuclear membranes during cellular stimulation (Perisic et al., 1999). This enzyme contains a calcium-dependent lipid-binding domain. In response to intracellular calcium, this domain facilitates the binding of $cPLA_2$ to endoplasmic reticulum and perinuclear membranes (Evans et al., 2001; Evans et al., 2003). A transient calcium flux causes reversible translocation without the release of arachidonic acid, while a sustained calcium flux results in a prolonged perinuclear translocation with accompanying arachidonic acid release. During hypoxic injury, the depletion of ATP results in translocation of $cPLA_2$ to the nucleus. This translocation is partially blocked by okadaic acid, a phosphatase 2A inhibitor (Sheridan et al., 2001). This observation suggests the involvement of phosphorylation/ dephosphorylation processes in the translocation of $cPLA_2$.

At present, nothing is known about the relationship between the $cPLA_2$ translocated to the perinuclear membrane and the activities of intrinsic nuclear PLA_2 isoforms. In contrast, $sPLA_2$ translocates from the inside to the outside of cells during stimulation and hydrolyzes fatty acids from outer cellular membranes. These observations suggest that multiple forms of PLA_2 act on different phospholipid molecular species, which reside at different subcellular locations indicating that in neural cells different subcellular sites are enriched with specific glycerophospholipid species that are degraded by various forms of $sPLA_2$ and $cPLA_2$ (Farooqui et al., 2000a,b).

Lysophosphatidylcholine, the other product of PLA₂ catalyzed reactions, is either reacylated to phosphatidylcholine (Fig. 4.1), further hydrolyzed, or metabolized to platelet-activating factor (PAF). A neuronal nuclear acetyltransferase is found in young rat brain (Baker and Chang, 1997, 2004). In nuclear membranes, these enzymes are involved in the generation of plateletactivating factor (PAF) (Squinto et al., 1989; Doucet et al., 1990). PAF is known to induce the expression of c-fos and c-jun and prostaglandin synthase. The expression of c-fos, c-jun, and prostaglandins is blocked by BN-50730, a specific PAF antagonist (Bazan et al., 1997; Miguel et al., 2001).

In the nucleus, PLA_2 isoforms are involved in the cell cycle and in apoptotic cell death (Farooqui et al., 2004b). Inhibitors of PLA_2 activity block apoptosis (Wissing et al., 1997; Pirianov et al., 1999). In permeabilized HeLa cells, PLA_2 isoforms play an important role in nuclear shrinkage during hypoxic injury. Bromoenol lactone, a potent inhibitor of iPLA₂ (Zupan et al., 1993), blocks the

hypoxia-mediated nuclear shrinkage not only in HeLa cells but also in PC12 cell cultures. These observations suggest that PLA_2 isoforms are required for nuclear shrinkage in caspase-independent apoptotic cell death (Shinzawa and Tsujimoto, 2003; Farooqui et al., 2004b).

Treatment of tumor P-388 cells with cPLA₂ inhibitors suppresses cell proliferation by inducing apoptosis (Korystov et al., 1998). Determination of cPLA₂ activity in various phases of the cell cycle indicate that enzyme activity is high in mitosis, decreases afterwards, and is increased again in G1 and following the G1/S transition phases. During cell cycle, elevations in cPLA₂ activity are due to increased phosphorylation rather than increased cPLA₂ protein expression. This observation is supported by studies in which phosphatase treatment of $cPLA_2$ reduces its activity (van Rossum et al., 2002). The inhibition of cPLA₂ with arachidonyl trifluoromethylketone in early G1 phase markedly reduces DNA synthesis suggesting that cPLA₂ plays a crucial role in progression of the cell cycle. Cyclooxygenase inhibitors have no effect on cell cycle progression into S phase of cell cycle (van Rossum et al., 2002), indicating that the cPLA₂-dependent progression is not mediated by arachidonic acid metabolites generated by cyclooxygenase. However, the lipoxygenase inhibitors, caffeic acid and nordihydroguaiaretic acid, inhibit DNA synthesis when added in early G1 phase (van Rossum et al., 2002) indicating that lipoxygenase is closely associated with the progression of cell cycle.

Retinoic acid blocks apoptosis in some cells populations (Ahlemeyer and Krieglstein, 2000), whereas it induces apoptosis in others (Tong et al., 1997). The mechanism of retinoic acid-mediated apoptosis is not fully understood. However, two mechanisms have been proposed (Farooqui et al., 2004a). According to the first mechanism retinoic acid induces apoptosis through mitochondrial dysfunction (Zhang et al., 2003). The second mechanism involves cytokine-mediated stimulation of PLA₂ activity (Farooqui et al., 2004b), resulting in the generation of excess of arachidonic acid. This excess of arachidonic acid affects the transcription of many genes not only through the generation of eicosanoids but also due to direct affect of arachidonic acid on gene expression (Jump et al., 1999). At present, it is not possible to specify genes involved at various stages of proliferation, differentiation, and apoptosis (Balmer and Blomhoff, 2002; Obermeier et al., 1995). However, proliferation, differentiation, and apoptosis controlling genes may include those genes that regulate synaptic plasticity, cytoskeleton, membrane association, signal transduction, and energy metabolism (Barceló-Coblijn et al., 2003). Beside this arachidonic acid also modulate activities of isoforms of protein kinase C and PLC in the nucleus (Farooqui et al., 2004a). Both these enzymes are involved in signal transduction processes associated with cell proliferation and differentiation (Han et al., 2002; Tong et al., 1997). Arachidonic acid is metabolized to eicosanoids and reactive oxygen species (ROS) that may induce oxidative stress contributing to either cell proliferation or apoptotic cell death depending on cell type (Ahlemeyer and Krieglstein, 2000). Arachidonic acid, eicosanoids, and ROS modulate gene expression through the activation of the redox-sensitive transcription factors, nuclear factor- κ B (NF- κ B) (Maziere et al., 1999; Haddad, 2004) and activator protein-1 (AP-1) (Becuwe et al., 2003). NF- κ B has binding sites on cPLA₂ promotor and this binding may induce that expression of other cytokines such as IL-1 β and TNF- α and neurotrophins (Tay et al., 1994; Anthonsen et al., 2001). AP-1 transcription factors are among the best characterized DNA-binding proteins in the brain (Herdegen and Leah, 1998). They consist of either a jun–jun homodimer or a jun–Fos heterodimer and bind to a specific site present in the promoter region of a wide variety of genes implicated in cell proliferation and tumorigenesis (Angel and Karin, 1991). These observations strongly suggest that the nucleus is an important site of control for the incorporation and redistribution of arachidonic acid from nuclear phospholipids into other cellular membranes, and nuclear PLA₂ activities and transcriptional factors like NF- κ B and AP-1 play important roles in the modulation of various stages cellular proliferation, differentiation, and apoptosis.

4.4.2 Nuclear PLC Activities

Enzymes of the phosphoinositide cycle are found in the nucleus (Fig. 4.1). The nuclear phosphatidylinositol cycle is distinct and independent of that found at the plasma membrane (D'Santos et al., 1998; Martelli et al., 1999; Cocco et al., 2001; Martelli et al., 2004b). Thus, many agonists that stimulate phosphoinositide cycle at the plasma membrane may not have any effect on nuclear phosphoinositide cycle. Isoforms of PtdIns-specific PLC are present in the nuclear fraction prepared from neural and non-neural tissues. Four isoforms of the PtdIns-specific PLC are known to occur in brain tissue (Asano et al., 1994; Fukami, 2002), but PLC δ 4 and PLC β 1 are the only isoforms specific to the nucleus. These isoforms are differentially regulated in the nucleus. The PLC β_1 is constitutively expressed in the nucleus and is activated very early during nuclear signaling, while PLC δ 4 is expressed later during the transition phase. Two forms of PLC β_1 with molecular mass of 150 and 140 kDa are generated from a single gene by alternative RNA splicing. Both forms have COOH-terminal tail with a cluster of lysine residues responsible for nuclear localization (Faenza et al., 2000). In the nucleus, overexpression of both forms of PLC β_1 is related to the expression of cyclin D_3 , along with its kinase (cdk4). These observations suggest a direct involvement of nuclear PLC β_1 signaling in G₁ phase progression through cyclin D_3/cdk_4 (Faenza et al., 2000). Nuclear PLC β_1 up-regulates the expression of CD24, a gene coding for an antigen involved in differentiation and hematopoiesis indicating that nuclear $PLC\beta_1$ constitutes a key step in cellular differentiation in vitro (Fiume et al., 2005). PLC β_1 is predominantly expressed in the neurons of granular layer of rat cerebellar cortex (Vitale et al., 2004). It is absent in the molecular and Purkinje cell layers of rat cerebellar cortex. The significance of differential expression pattern of PLC β_1 is not fully understood. However, it is likely that differential expression pattern of $PLC\beta_1$
not only modulates the intensity of signal transduction in the nucleus during cell cycle, but also cross talk with lipid mediators derived from the hydrolysis of nuclear glycerophospholipids by PLA₂ and PLD. The generation of polyphosphoinositide-derived lipid mediators is also modulated by extracellular stimuli, such as retinoic acid, affecting cell proliferation, differentiation, or neoplastic transformation. Thus, the amount and activity of PtdIns-PLC β is increased in the nucleus following the incubation of HL60 cells with RA (Bertagnolo et al., 1997).

Collective evidence suggests that nuclear PLC δ 4 and PLC β_1 play a pivotal role in regulating levels of nuclear phosphoinositides but also in controlling the initiation of DNA synthesis in S phase of cell cycle. Spatio-temporal alterations in the levels of PtdIns4,5 P_2 seem to be another major determinant for the localization and regulation of the PLC δ and PLC β_1 isoforms (Faenza et al., 2000; Martelli et al., 2005). High nuclear PtdIns4,5 P_2 levels are associated with the G₁/S phases. After entering M phase, PtdIns4,5 P_2 synthesis at sites of cell division occurs, and PLCs seem to localize to the cleavage furrow during cytokinesis. Coordinated PLC isoforms-mediated signaling in the nucleus and nuclear membrane and its association with the cell cycle suggests that changes in intranuclear environments are closely associated with the modulation of proliferation and differentiation (Martelli et al., 2005).

The degradation of phosphatidylinositol 4,5-bisphosphate by PLC isozymes generates diacylglycerols and inositol 1,4,5-trisphosphate. Diacylglycerols stimulate nuclear protein kinase C isoforms, and inositol 1,4,5-trisphosphate mobilizes calcium via its receptors on inner nuclear membrane (Fig. 4.1). Thus, inositol phosphates have a role in calcium homeostasis within the nucleus. Nuclear calcium homeostasis is known to modulate a number of critical nuclear events such as the regulation of transcription factors, cell cycle regulation, gene transcription, DNA replication, and nuclear envelope breakdown. Furthermore, inositol 1,4,5-trisphosphate generated during the intranuclear phosphatidylinositol cycle can also be converted via successive kinases to inositol tetrakisphosphate ($InsP_4$), inositol pentakisphosphate ($InsP_5$), and inositol hexakisphosphate ($InsP_6$). $InsP_4$ inhibits chloride channel conductance, InsP₅ inhibits signal transduction pathway involving phosphatidylinositol 3-kinase, and $InsP_6$ has been proposed to play an important role in mRNA transport and regulation of the transcription of some genes (Odom et al., 2000; Martelli et al., 2004b, 2005). This suggests that nuclear phosphatidylinositol metabolites may not just act as substrates for second messenger generation, but they may be directly involved in mRNA splicing, regulation of transcription factors, cell cycle regulation, gene transcription, and DNA replication (Martelli et al., 2003, 2004b, 2005). Furthermore, highly phosphorylated inositols have also been implicated in chromatin remodeling (Steger et al., 2003). Inositol polyphosphates are essential co-factors for many nuclear processes including DNA repair, transcription regulation, and RNA dynamics. These observations strongly support the view that lipid mediators of inositol metabolism play an essential role in modulation of nuclear activities.

PtdCho and trace amounts of PtdEtn also occur in neuronal nuclei (Baker and Chang, 1996, 1998). Very little information is available on their metabolism in neural cell nuclei (Irvine, 2003). Although, the occurrence of PtdCho-specific PLC in nerve cell has been well established, but attempts on its purification and cloning from mammalian tissues have been unsuccessful (Li et al., 1998; Ramoni et al., 2004). Determination of PtdCho hydrolyzing PLC in the nuclear fraction obtained from LA-N-1 cells indicates that significant enzymic activity is present in LA-N-1 cells nuclei (Antony et al., 2000, 2001b,a, 2003a,b). The kinetic properties of nuclear PtdCho-PLC are shown in Table 4.2. The treatment of LA-N-1 cells with TPA as well as RA increases nuclear DAG levels indicating the stimulation of PLC and CTP: phosphocholine cytidylyltransferase activities RA-mediated stimulation of PtdCho-PLC is inhibited by D609. This not only suggests that the stimulation of PLC by RA is a receptor-mediated process but also supports the view that the PtdCho cycle occurs in nuclear preparations from LA-N-1 cells. D609 also blocks ceramide phosphocholine transferase activity indicating that D609 is not a specific inhibitor of PtdCho-specific PLC. These observations complicate the analysis of PLC activity in the presence of D609 at the nuclear level. Thus, PtdCho-derived DAG in the nuclei can be reutilized for the synthesis of nuclear PtdCho and is required for the activation of CTP: phosphocholine cytidylyltransferase (Antony et al., 2001a).

Nuclear PtdCho has been implicated in cell proliferation through the activation of intranuclear PtdCho-specific PLC and diacylglycerol formation. The increase in diacylglycerol stimulates phosphatidylcholine synthesis through the major pathway from cytidyltriphosphate (Albi and Viola Magni, 2007). An inhibition of PtdCho synthesis is responsible for the initiation of apoptosis. The presence of reverse sphingomyelin synthase favors the generation of PtdCho. the donor of phosphorylcholine, from sphingomyelin. Very little information is available on other glycerophospholipids (PtdEtn) metabolism in the nucleus, but PtdIns appears to be involved and influence cell differentiation and proliferation. This effect is due to interactions between lipid mediators generated by PtdCho-specific PLC and PC-PLCy 1. As stated above that phosphoinositides also may have an important role in promoting the translocation of protein kinase C through the generation of diacylglycerol. The overexpression of inositol polyphosphate-1-phosphatase blocks the DNA synthesis by 50%. Nevertheless, an enhanced rate of phosphorylation has been demonstrated in cells destined to differentiate (Albi and Viola Magni, 2007). Collective evidence suggests that cross talk between PtdCho and PtdIns metabolism is closely associated with cellular differentiation.

PLC isoforms also play an important role in cell cycle progression. The two subtypes of PLC β 1, when overexpressed in the nucleus, cause the overexpression of cyclin D3 and its kinase (cdk4). Based on various cell biology studies, nuclear PLC β 1 signaling is involved in the G1 progression phase of the cell cycle through cyclin D3 and its kinase (Faenza et al., 2000). In contrast, the cytosolic enzyme PLC γ 1 does not fluctuate during the cell cycle. Stimulation of quiescent 3T3 cells with IGF-1 increases the activity of the phosphatidylinositol-specific

| Subcellular fraction | DAG-lipase (1) | MAG-lipase (2) | DAG-lipase (3) | MAG-lipase (4) |
|--------------------------|----------------|----------------|----------------|----------------|
| Homogenate | 0.53 ± 0.21 | 1.37 ± 0.33 | 1.17 ± 0.32 | 2.32 ± 0.32 |
| Cytosol | 0.27 ± 0.07 | 0.63 ± 0.09 | 0.52 ± 0.11 | 0.57 ± 0.17 |
| Non-nuclear membranes | 2.32 ± 0.71 | 2.87 ± 0.35 | 3.52 ± 0.40 | 3.56 ± 0.25 |
| Nucleus | 1.35 ± 0.33 | 2.56 ± 0.37 | 2.57 ± 0.38 | 4.21 ± 0.75 |
| | | | | |

 Table 4.6 Specific activities of diacylglycerol and monoacylglycerol lipases in subcellular fractions prepared from LA-N-1 cell homogenate

DAG-lipase activity in control LA-N-1 cells (1); MAG-lipase in control LA-N-1 cells (2); DAG-lipase in RA-treated LA-N-1 cells (3); and MAG-lipase in RA-treated LA-N-1 cells (4). Specific activities are expressed as nmol/min/mg protein. Values are mean of three experiments performed in triplicate

PLC- β 1 by 2- to 3-fold within a few minutes. This suggests that phosphatidylinositol-specific PLC- β 1 may be involved in control of cell proliferation.

DAG- and MAG-lipase activities are present in subcellular fractions prepared from LA-N-1 cell homogenates (Table 4.6). Non-nuclear pellets that contained plasma membranes, mitochondria, and microsomes displayed the highest specific activity for DAG- and MAG-lipases. The nuclear fraction had significant DAG-and MAG-lipase activities. These enzymes hydrolyze DAG which is generated in LA-N-1 cells by the action of PLC and PLD on nuclear phospholipids (Antony et al., 2000). DAG cannot be regarded as a single entity, as there are as many as 50 different DAG molecular species that are present in LA-N-1 cells (Lang et al., 1995). Many of these molecular species are involved in the activation of nuclear protein kinase C isozymes (Lang et al., 1995, 1996). In the nucleus, DAG is also a substrate for DAG-kinase (Martelli et al., 2002a; Hozumi et al., 2003). This enzyme phosphorylates DAG to phosphatidic acid (PtdH). Thus, nuclear DAG-lipase and DAG-kinase attenuate the DAG-generated signal by regulating the amount of nuclear DAG available for the activation of protein kinase C isozymes. It has been suggested that DAG derived from PtdIns is shuttled directly to a DAG-kinase present in the nucleus, but DAG derived from PtdCho are not accessible to DAG-kinase. This suggests that nuclei contain at least two distinct pools of DAG that are generated by the action of two distinct phospholipases.

In brain tissue, DAGs are a fundamental lipid second messenger that is generated not only at the plasma membrane level but also in the nucleus. At both subcellular levels, DAG are distributed in several pools that may be involved in modulating PKC activity in signal transduction processes associated with cellular proliferation and differentiation. The levels of nuclear DAG fluctuate during the cell cycle progression indicating that DAG molecular species have an important regulatory role not only in signal transduction but also in cell division (Martelli et al., 2002a). In contrast, the cytoplasmic levels of DAG remain constant. A major increase in DAG and marked decrease of phosphatidylinositol in the nucleus coincides with the S phase of the cell cycle (York and Majerus, 1994).

4.4.3 Nuclear PLD Activities

Action of PLD on PtdCho generates membrane-bound phosphatidic acid (PtdH) and soluble choline (Fig. 4.1). Whereas choline formation may be important for some aspects of PLD function in the nucleus, it is suggested that the generation of PtdH (and its downstream metabolites, DAG) constitutes an important step in PLD-mediated signaling pathways. PtdH is also metabolized to lysophosphatidic acid (lyso-PtdH). This metabolite interacts with its receptor in the nucleus and modulates neural cell proliferation and differentiation (Gobeil et al., 2003a; Luquain et al., 2003). In neural cells, PLD isozymes activate other signaling enzymes, facilitate membrane vesicle fusion events, or serve as a lipid anchor for membrane-associated proteins (Farooqui et al., 2000a; Banno, 2002). The kinetic properties of PLD in LA-N-1 nuclei are shown in Table 4.3. Rat brain neuronal nuclei contain a PLD that is activated by unsaturated fatty acids, particularly oleic acid (Kanfer et al., 1996). PLD, in addition to having PtdCho hydrolyzing activity, also catalyzes a transphosphatidylation reaction generating a phosphatidylalcohol. GTPyS, ATPyS, phosphatidic acid, and phosphatidylethanol inhibit the neuronal nuclear PLD. The PLD activity of neuronal nuclei is higher than that detected in the nuclei of glial cells or extracellular neural cells (Kanfer et al., 1996).

Treatment of LA-N-1 cell nuclei with TPA and GTP γ S in the presence of 1.0% ethanol generates PtdEt. This reaction is catalyzed by a G-protein-linked phospholipase D in LA-N-1 cell nuclei (Antony et al., 2000). Preliminary investigations have indicated that LA-N-1 cell nuclei contain two PLD isoforms, a G-protein-dependent isoform that is stimulated by phorbol ester, and an oleate-dependent isoform that is stimulated by RA (Antony et al., 2003b). G-protein-dependent isoform triggers the activation of multiple signal transduction pathways that act in a synergistic and combinatorial fashion with PLA₂ and PLC to relay the information signals from the nucleus to other subcellular organelles in the cytoplasm during cell proliferation. Thus, receptor-mediated rapid increase in the activity of PLA₂, C, and D leading to the synthesis of lipid mediators, Ca²⁺ fluxes, and subsequent activation of protein phosphorylation cascades, including PKC/PKD, Raf/MEK/ERK, and Akt/mTOR/p70S6K is an important early response to various toxic agonists.

The non-nuclear PLD (PLD₁ and PLD₂) activities are stimulated by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family proteins in a GTP-dependent manner. PLD₂, however, only requires the presence of phosphatidylinositol 4,5-bisphosphate for optimal activity. In contrast, phorbol 12-myristate treatment results in 14-fold increase in the basal activity of PLD₁ activity (Liscovitch, 1996). These observations suggest that distinct isoforms of PLD in the nucleus may be involved in the generation of different molecular species of PtdH and ultimately DAG by the action of phosphatidate phosphohydrolase during cellular proliferation and differentiation (Lang et al., 1996). A transient increase in the ADP-ribosylation factor-dependent nuclear PLD is reported to occur in the S phase of regenerating rat hepatocytes (Banno, 2002), supporting the view that PLD activity may also be associated with the cell cycle.

RA-mediated stimulation of PLA₂, C, and D activities in the nucleus may be useful for metabolic processes in LA-N-1 cells because (a) it aids the generation of eicosanoids and these metabolites may directly bind and activate nuclear transcription factors (Han et al., 2002), (b) it accelerates the acylation–deacylation cycle for maintenance of essential phospholipids in the nuclear membrane (remodeling), (c) it protects the nuclear membrane from lipid peroxidation (Antony et al., 2001b), and (d) it induces neuritogenesis not only by activating protein kinase C isozymes (Miloso et al., 2004) but also by generating and modulating the levels of arachidonic acid and its metabolites (Farooqui et al., 2004).

4.5 Interplay Among Nuclear and Non-Nuclear PLA₂, PLC, and PLD Activities

Very little is known about the interactions among nuclear and non-nuclear PLA₂, PLC, and PLD (Farooqui et al., 1992, 2004a). Studies on this topic are complicated not only by the occurrence of isoforms of PLA₂, PLC, and PLD in cytoplasm and other subcellular organelles. The multiplicity of PLA₂, PLC, and PLD in brain tissue provides diversity in function and specificity of various isoforms in the regulation of enzymic activity in response to a wide range of extracellular signals. This complicates the analysis of PLA₂, PLC, and PLD function at cellular and subcellular levels. The complexity of this problem becomes obvious when one considers the coupling mechanisms of various isoforms of PLA₂, PLC, and PLD with different receptors in a single neural cell in nuclear and non-nuclear membrane fractions and then tries to associate PLA₂, PLC and PLD activities with neuronal function.

Multiple forms of PLA₂, PLC, and PLD are regulated not only by calcium ions but also by covalent modification mediated by intrinsic protein tyrosine kinases, mitogen-activated kinases, and protein kinases C. Some isoforms of these enzymes are inducible to a further extent with cytokines and growth factors such as IL-1, IL-3, tumor necrosis factor- α (TNF- α), and nerve growth factor. Isoforms of PLA₂, PLC, and PLD may not function interchangeably, but act in parallel to transducer signals (Farooqui et al., 2004a). It is likely that various isoforms of PLA₂, PLC, and PLD act on different cellular pools of phospholipids located in different subcellular organelles of various types of neural cells and these isoforms may be regulated by different coupling mechanisms generating common second messengers.

Free unsaturated fatty acids, the product of a PLA₂ reaction, and DAG, the product of PLC and PLD catalyzed reactions, act synergistically to stimulate PKC activity. Coordination and integration of these second messengers in nuclear and non-nuclear compartments is necessary for optimal functioning

of signal transduction processes. The PLC- and PLD-mediated release of DAG results in the stimulation of various isoforms of protein kinase C. This leads to the activation of both PLA₂ as well as PLD (Clark et al., 1995). Similarly, activation of nuclear PLA₂ isoforms generates arachidonic acid and eicosanoids, and these lipid mediators have been shown to activate isoforms of PLC, PLD, and PKC (Klein et al., 1995).

The interactions among metabolites generated by PLA₂, PLC, and PLD at subcellular levels may provide neural cells and brain tissue with great versatility in ensuring that DAG, arachidonic acid, and eicosanoids are efficiently utilized. Thus, the cross talk among PLA₂, PLC, and PLD isozymes is essential for maintaining normal neuronal and glial cell growth. In the nucleus, signaling mediated by PLA₂, PLC, and PLD has an advantage over plasma membrane signaling in that second messengers generated by these enzymes during differentiation may directly interact with nuclear factors producing physiological and morphological changes (Farooqui et al., 2004a). In brain tissue, the activity of PLA₂, PLC, and PLD isoforms may depend not only on structural, physicochemical, and dynamic properties of neural membranes but also on the type and metabolic state of neural cells. The activation of PLA₂, PLC, and PLD isoforms at the subcellular level in neural cell is the rate-limiting step for the production of lipid mediators such as arachidonic acid, eicosanoids, and DAG. Therefore, tight regulation of PLA₂, PLC, and PLD isozymes is very important for normal brain function. As stated above, the regulation of PLA₂, PLC, and PLD activities is quite complex and is mediated by several factors such as translocation and phosphorylation and mechanisms such as gene expression and cross talk among isoforms of these enzymes at cellular and subcellular level (Farooqui et al., 2004a). The collective evidence thus suggests that formation of PLA₂, PLC, and PLD-generated second messengers in the nucleus may be linked to neuronal growth and differentiation via the activation of certain PKC isozymes and subsequent phosphorylation (Farooqui et al., 2004).

4.6 Nuclear PLA₂, PLC, and PLD and Nuclear Inclusions in Neurological Disorders

Many neurodegenerative diseases are accompanied by the abnormal accumulation of intranuclear protein aggregation in the form of intranuclear inclusion bodies (Woulfe, 2007). Studies on lipid composition of these intranuclear inclusions have provided important clues regarding the cellular pathophysiology of these diseases. Although the precise role of intranuclear inclusion bodies in the pathogenesis of neurological is not understood. However, it is well known that activities of PLA₂, PLC, and PLD and abnormal accumulation of glycerophospholipid and sphingolipid are closely associated with the morphological and pathophysiological abnormalities at plasma membrane, mitochondrial, and nuclear levels in many neurological disorders (Farooqui and Horrocks, 2006; Farooqui et al., 2006, 2007a). We have observed abnormal intranuclear inclusions in fresh autopsy brains from patients with stroke, AD, PD, and peroxisomal disorders. It is likely that elevated activities of nuclear PLA_2 , PLC, and PLD network may be related to the accumulation of intranuclear inclusion bodies and may represent a cellular protective mechanism that is induced under pathological conditions (Woulfe, 2007).

4.7 Conclusion

Phospholipids are integral components of the nuclear membranes and intranuclear domains. Alterations in phospholipid metabolism occur during cellular differentiation, proliferation, and apoptosis, but the molecular mechanism involved in the above processes remains unknown. In brain tissue, arachidonic acid is mainly released by the action of PLA_2 and phospholipase C/ diacylglycerol lipase (PLC/DAG-lipase) pathways. Treatment of LA-N-1 cells and primary cultures of neuronal and glial origin with retinoic acid (RA) produces a marked increase in the activities of the PLA₂ hydrolyzing PtdEtn and PlsEtn, the PLC specific for PtdCho, and the PLD in the nuclear fraction. RA also stimulates DAG-lipase and MAG-lipase activities in LA-N-1 cell cultures. RA has no effect on the activities of PLA₂, PLC, and PLD in nonnuclear membranes, indicating that autonomous signaling mediated by PLA₂, PLC, and PLD operate within the nucleus. Lipid mediators generated by the action of PLA₂, PLC, and PLD on nuclear phospholipids markedly affect neuritic outgrowth and neurotransmitter release in cells of neuronal and glial origin. Under normal conditions, transcription factor, neurotrophin, and cytokine-mediated modulation of PLA₂, PLC, and PLD activities in the nucleus may play an important role in the redistribution of arachidonic acid and its metabolites and DAG in nuclear and non-nuclear neuronal membranes during differentiation and growth suppression and apoptosis. However, excessive production of arachidonic acid metabolites, PAF, and diacylglycerols under pathological situations results in oxidative stress, inflammation, and neurodegeneration (Farooqui et al., 1997, 2004).

Many questions remain to be answered regarding the involvement of signaling in the nucleus induced by PLA₂, PLC, and PLD. For example, how many isoforms of PLA₂, PLC, and PLD are present in the nucleus and what are their proportions and characteristics? Two forms of calcium-independent phospholipase A₂ (PtdEtn-PLA₂ and PlsEtn-PLA₂) are present in LA-N-1 cell nuclei (Antony et al., 2001b, 2003a). The kinetic properties of these enzymes are similar to the corresponding enzymes in the cytosol. However, others have reported differences between nuclear and cytosolic enzymes in response to PLA₂ inhibitors (Maraldi et al., 1999; Misra and Pizzo, 2000). At present, nothing is known about the regulation of nuclear PLA₂, PLC, and PLD. Detailed investigations are required on the extent of cross talk between nuclear and cytosolic PLA₂, PLC, and PLD activities. Nuclear PLA₂, PLC, and PLD activities should be characterized and antibodies should be prepared against the isoforms. The availability of antibodies against nuclear PLA₂, PLC, and PLD isoforms would not only aid in their localization in the nucleus but also be useful in determining their role in lipid transport and trafficking.

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Chapter 5 Ether Glycerophospholipids: The Workhorse Lipids of Neural Membranes

5.1 Introduction

Ether glycerophospholipids are major components of neuronal and glial cell membranes. Physicochemical properties of ether glycerophospholipids are similar to ester-bonded glycerophospholipids except for differences in the phase transition temperature from gel to liquid crystalline, from lamellar to hexagonal phases, and ability to reduce the surface tension. These differences not only account for changes in bilayer thickness, area per molecule, side-chain packing, free volume, and lateral domains, but also cause a decrease in membrane dipole potential, alterations in thermotropic phase behavior, ion permeability, and side-chain mobility (Paltauf, 1994; Lohner, 1996). Studies on model membranes indicate that high ether glycerophospholipid contents provide membranes with a unique microenvironment that is necessary for maintaining activities of membrane-bound enzymes, regulation of permeability, and optimal function of receptors and ion channels (Lohner, 1996). Perturbation of an ether lipid-rich microenvironment in membranes produces significantly more derangements in membrane dynamics than the perturbation of model membranes composed of diacyl glycerophospholipids (Paltauf, 1994).

Depending upon the substituents at carbon-1, ether glycerophospholipids are classified into two groups: (a) alkenylacyl glycerophospholipids and (b) alkylacyl glycerophospholipids. In mammalian tissues, alkenylacyl glycerophophospholipids are represented by plasmalogens, whereas alkylacyl glycerophospholipids are represented by platelet-activating factor and its analogs (Fig. 5.1). In addition, the occurrence of an ether-linked analog of 2-arachidonylglycerol, noladin ether (Fig. 5.1), has been reported in pig and rat brains (Hanuš et al., 2001; Fezza et al., 2002). Noladin ether is an endogenous agonist for CB1 and CB2 cannabinoid receptors. Chronic treatment with noladin ether not only desensitizes and downregulates CB2 receptor, but also inhibits adenylyl cyclase activity in HL-60 cells. It also produces pronounced decrease in μ -opioid receptor activity, which is mediated by CB2 receptor (Páldyová et al., 2008).

Plasmalogens have a vinyl ether (enol ether) linkage at the sn-1 position with 16:0, 18:0, and 18:1 (n-7 and n-9) side chains (alk-1-enyl groups), an ester bond linking arachidonic acid or docosahexaenoic acid at the sn-2 position, and a



Fig. 5.1 Chemical structures of ether lipids. Choline plasmalogen (a); ethanolamine plasmalogen (b); platelet-activating factor (c); and noladin ether lipid (d)

phosphoethanolamine or phosphocholine group at the sn-3 position of the glycerol moiety (Fig. 5.1). Thus, plasmalogens are a reservoir for arachidonic and docosahexaenoic acids in neural membranes (Farooqui and Horrocks, 2001). High levels of ethanolamine plasmalogens are found in brain, lungs, kidney, and testes, whereas high concentrations of choline plasmalogens occur in heart and skeletal muscle. Macrophages and neutrophils also have high levels of ethanolamine plasmalogens not only serve as endogenous anti-oxidants but also play an important role in neural membrane fusion. At the subcellular level, plasmalogens are also found in the nucleus where they may be involved in cellular differentiation (Bichenkov and Ellingson, 1999; Farooqui and Horrocks, 2001). The occurrence of plasmalogens in the synaptic cleft suggests that these phospholipids not only play an important role in synaptogenesis but may also be involved in vesicle formation during neurotransmitter release (Farooqui and Horrocks, 2001).

In contrast, platelet-activating factor (PAF) contains an *O*-alkyl ether linkage at the sn-1 position (fatty alcohol side chain), a short acyl chain (acetyl moiety) at the sn-2 position, and a phosphocholine group at the sn-3 position of the glycerol moiety. PAF stimulates a wide range of biological responses ranging from aggregation and degranulation of platelets and neutrophils to a variety of other cellular effects such as the stimulation of chemotaxis, chemokinesis, superoxide formation, protein phosphorylation, activation of protein kinase C, glycogenolysis, and tumor necrosis factor production (Snyder, 1995). PAF acts by binding to a unique G-protein receptor with seven transmembrane segments. These receptors are linked to intracellular signal transduction pathways, including turnover of phosphatidylinositol, elevation of intracellular Ca^{2+} concentration, and activation of kinases. All these processes are associated with signal transduction processes involved in the modulation of neural cell function (Farooqui and Horrocks, 2004).

5.2 Plasmalogens in Brain

PlsEtn and PlsCho are the two major plasmalogen species found in mammalian cell membranes. In brain, ethanolamine plasmalogen levels are 10-fold higher than the choline plasmalogens. In cardiac and skeletal muscles, choline plasmalogens are dominating glyceroether lipid species. Plasmalogens account for the major portion of the ethanolamine glycerophospholipids in the adult human brain (50%), but brain from newborn babies has low levels, 7%, of total phospholipid mass (Horrocks and Sharma, 1982). Levels of ethanolamine plasmalogen (PlsEtn) increase rapidly during the intense period of myelination, and ethanolamine glycerophospholipids of myelin sheath contain up to 70% PlsEtn (Horrocks, 1972; Horrocks and Sharma, 1982). Factors that modulate levels of plasmalogens in neurons, astrocytes, and oligodendrocytes during myelination and aging remain unknown. In human brain during normal aging, there is an increase in PlsEtn contents up to 30-40 years of age. This is followed by a decline in PlsEtn levels during aging. At 70 years of age, the levels of PlsEtn are 18% less than at 40 years of age (Rouser and Yamamoto, 1968; Horrocks et al., 1981). Similarly, in rat brain, levels of plasmalogens are also decreased between 21 and 24 months in the cerebellum (from 55.9 to $41.9 \,\mu$ mol/g fresh tissue) and in cortex (from 45.3 to 36.9 µmol/g fresh tissue) of docosahexaenoic acid-deficient rats (André et al., 2006a). Hippocampus, a brain structure implicated in memory formation, storage, retrieval, and emotion, is vulnerable to ischemic injury and oxidative stress during aging (Kubota et al., 2001; André et al., 2005). Levels of plasmalogen in the hippocampal CA1 field are 2.3- and 2.7-fold higher than hippocampal CA3 field and cerebral cortex, respectively. Furthermore, the PlsEtn/PtdEtn ratio in the CA1 field is significantly higher than those in the CA3 field and cerebral cortex indicating that presence of plasmalogens make CA1 field more vulnerable than other brain regions. At subcellular level, levels of plasmalogens are high in plasma membrane and synaptic plasma membrane fractions. These membranes undergo rapid membrane fusion and promote synaptic transmission (Kubota et al., 2001; André et al., 2005).

5.3 Biosynthesis of Plasmalogens

Plasmalogen-synthesizing enzymes have not been purified from brain tissue. This may be due to their low activity in brain and time-consuming and laborious assay procedures. The biosynthesis of plasmalogens has been studied in



Fig. 5.2 Biosynthesis of plasmalogens in mammalian tissues. Dihydroxyacetone phosphate (**a**); 1-acyldihydroxyacetone phosphate (**b**); 1-alkyldihydroxyacetone phosphate (**c**); 1-alkyl-glycero-3-phosphate (**d**); 1-alkyl-2-acylglycerol-3-phosphate (**e**); 1-alkyl-2-acyl-glycerophosphoethanolamine (**f**); 1-alkyl-1'-enoyl-2-acyl-glycerophosphoethanolamine or ethanolamine plasmalogen (**g**); dihydroxyacetone phosphate acyltransferase (1); 1-acyl dihydroxyacetone phosphate synthase (2); 1-alkyl-dihydroxyacetone phosphate reductase (3); 1-alkylglycerophosphoethanolamine desaturase (4); CDP-ethanolamine transferase (5); 1-alkyl-2-acyl-glycerophosphoethanolamine desaturase (6); and methyltransferases and base-exchange enzymes (7)

non-neural tissues and cell cultures (Fig. 5.2). The first three enzymes of plasmalogen biosynthesis, dihydroxyacetone phosphate acyltransferase, alkyl-dihydroxyacetone phosphate synthase, and acyl/alkyl-dihydroxyacetone reductase, are located in peroxisomes. The endoplasmic reticulum contains the remaining enzymes, namely, 1-alkyl-*sn*-Gro*P* acyltransferase, 1-alkyl-2-acyl-*sn*-Gro*P* phosphohydrolase, and 1-alkyl-2-acyl-*sn*-Gro: CDP-choline (CDP-ethanolamine) phosphotransferase for plasmalogen biosynthesis (Horrocks and Sharma, 1982; Lee, 1998; Nagan and Zoeller, 2001; Murphy, 2001; Brites et al., 2004; Farooqui et al., 2008). Collectively, these studies suggest that the biosynthesis of plasmalogens starts in peroxisomes and is completed in the endoplasmic reticulum.

The rate-limiting step for plasmalogen biosynthesis in brain has not been identified. However, it is shown that plasmalogen contents may be related to dihydroxyacetone phosphate acyltransferase activity as well as dietary intake of DHA (André et al., 2006b). Based on studies on non-neural cell cultures, it is stated that the rate-limiting step lies downstream from the first three steps (Nagan and Zoeller, 2001). This suggestion is based on the incorporation of 1-O-[9'-(1"-pyrenyl)]nonyl-*sn*-glycerol (pAG), a fluorescent ether lipid with a pyrene moiety covalently attached at the alkyl chain terminus (Zheng et al., 2006). Thus, dihydroxyacetone phosphate acyltransferase may be a crucial enzyme for plasmalogen biosynthesis, but it is not a rate-limiting step for plasmalogen synthesis (Nagan and Zoeller, 2001).

The biosynthesis of plasmalogen starts with dihydroxyacetone phosphate (DHAP), an intermediate generated during glycolysis. Acylation of DHAP results in the generation of 1-acyldihydroxyacetone phosphate (Fig. 5.2). This reaction is catalyzed by DHAP acyltransferase. The acyl group in this metabolite is replaced by a long-chain alcohol that contributes the oxygen for the ether linkage in a reaction catalyzed by alkyl-DHAP synthase resulting in generation of 1-O-alkyl-DHAP. The keto group at the sn-2 position of 1-Oalkyl-DHAP is reduced to an alcohol, followed by acylation resulting in the generation of 1-O-alkyl-2-acylglycero-3-phosphate. A phosphatase dephosphorylates 1-O-alkyl-2-acylglycero-3-phosphate prior to introduction of the phosphocholine or phosphoethanolamine group resulting in the synthesis of 1-alkyl-2-acyl-glycerophosphoethanolamine. A specific desaturase associated with endoplasmic reticulum introduces a double bond at the sn-1 position and converts 1-alkyl-2-acyl-glycerophosphoethanolamine into 1-alkyl-1'-enoyl-2acyl-glycerophosphoethanolamine (ethanolamine plasmalogen) (Horrocks and Sharma, 1982; Lee, 1998; Nagan and Zoeller, 2001; Murphy, 2001; Brites et al., 2004; Gorgas et al., 2006; Farooqui et al., 2008). Choline plasmalogens are synthesized through a base-exchange reaction.

5.4 Degradation of Plasmalogens

As stated above, plasmalogens are enriched in arachidonic and docosahexaenoic acid. The hydrolysis of plasmalogens by plasmalogen-selective phospholipase A_2 results in the release of arachidonic and docosahexaenoic acids and generation of lysoplasmalogens. Arachidonic and docosahexaenoic acids are metabolized to eicosanoids and docosanoids, respectively (Phillis et al., 2006). Lysoplasmalogens are either hydrolyzed by lysoplasmalogenase or undergo CoA-independent transacylation reaction to maintain the levels of plasmalogens in neural membranes (Fig. 5.3) (Farooqui and Horrocks, 2001).

5.4.1 Plasmalogen-Selective Phospholipase A₂ (PlsEtn-PLA₂)

The degradation of plasmalogens is catalyzed by $PlsEtn-PLA_2$ (Hirashima et al., 1992; Farooqui et al., 1995). Brain $PlsEtn-PLA_2$ is a calcium-independent cytosolic enzyme that has a molecular mass of 39 kDa (Hirashima et al., 1992; Farooqui et al., 1995). Low concentrations of ATP and ADP have no effect, but at high concentrations (2 mM or above) of these nucleotides inhibit the enzymic



Fig. 5.3 Lysoplasmalogenase catalyzed and CoA-independent transacylation reaction. Cytosolic phospholipase A_2 (cPLA₂); arachidonic acid (AA)

activity. This is in contrast to the heart PlsCho-PLA₂ that is stimulated by the addition of ATP and other nucleotides and inhibited by DTNB and Triton X-100 (Hazen et al., 1991; Hazen and Gross, 1993). Canine myocardium PlsEtn-PLA₂ has been purified by multiple column chromatographic procedures. This enzyme is associated with phosphofructokinase as a complex with molecular mass of 400 kDa (Hazen and Gross, 1993). Myocardial PlsCho-PLA₂ also binds to calmodulin. The physiological importance of calmodulin binding with PlsCho-PLA₂ is not fully understood. However, it is proposed that this binding may be involved in regulation of PlsCho-PLA₂ activity in heart muscle (Wolf and Gross, 1996). Brain PlsEtn-PLA₂ does not interact with calmodulin.

Rabbit kidney cortex plasmalogen-selective PLA_2 has also been purified to homogeneity by multiple column chromatographic procedure (Portilla et al., 1998). This enzyme has a molecular mass of 28 kDa and hydrolyzes PlsCho >PtdCho. Cloning of a full-length rat cDNA encoding $PlsCho-PLA_2$, using sequence derived from the studies, indicates that rat plasmalogen-selective PLA_2 cDNA encodes a 24 kDa protein and contains the sequence G-F-S-Q-G which fits the active site consensus sequence G-X-S-X-G of carboxylesterase. The expression of rat $PlsCho-PLA_2$ in a baculovirus expression system results in expression of $PlsCho-PLA_2$ and lysophospholipase activities (Portilla et al., 1998).

Brain PlsEtn-PLA₂ is stimulated by Triton X-100 and Tween-20. In contrast, heart PlsCho-PLA₂ is inhibited by these detergents. Octylglucoside, sodium deoxycholate, and sodium taurocholate inhibit the brain PlsEtn-PLA₂ in a dose-dependent manner. Dithio-*bis*-2-nitrobenzoic acid, iodoacetate, and *N*-ethylmaleimide inhibit bovine brain PlsEtn-PLA₂, and this inhibition can be reversed by dithiothreitol. PlsEtn-PLA₂ is also inhibited by polyvalent anions such as citrate > sulfate > phosphate (Farooqui et al., 1995). Quinacrine and nordihydroguaiaretic acid inhibit the PlsEtn-PLA₂ in a dose-dependent manner (Farooqui et al., 1997a).

Immunocytochemical localization studies indicate that in neuronal and astrocytic cultures PlsEtn-PLA₂ is colocalized with glial fibrillary acidic protein (GFAP). This suggests that this PLA₂ is predominantly associated with astrocytes (Farooqui and Horrocks, 2001). In contrast, the 85 kDa cytosolic PLA₂ is localized in neurons as well as astrocytes (Farooqui et al., 2000).

5.4.2 Receptor-Mediated Degradation of Plasmalogens

The release of arachidonic and docosahexaenoic acids in neuronal and astrocytic culture is a receptor-mediated process catalyzed by different isoforms of PLA₂, i.e., cPLA₂ and PlsEtn-PLA₂, respectively (Farooqui et al., 2003; Sergeeva et al., 2005) (Fig. 5.4). Treatment of neuron-enriched cultures with kainic acid results in a concentration-dependent increase in PlsEtn-PLA₂ activity. A kainate/AMPA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), prevents the stimulation of PlsEtn-PLA₂ activity. This suggests that the stimulation of PlsEtn-PLA₂ is a receptor-mediated process and plasmalogen degradation is mediated by KA receptors (Farooqui et al., 2003). Bromoenol lactone (BEL),



Fig. 5.4 Degradation of plasmalogen in brain. Phosphatidylcholine (PtdCho); lysophosphatidylcholine (lyso-PtdCho); ethanolamine plasmalogen (PlsEtn); ethanolamine lysoplasmalogen (lyso-PlsEtn); alkylacyl-glycerophosphocholine (PakCho); platelet-activating factor (PAF); lyso-platelet-activating factor (lyso-PAF); cPLA₂ (1); CoA-independent transacylation reaction (2); plasmalogen-selective PLA₂ (PlsEtn-PLA₂) (3); base-exchange reaction (4); 15-lipoxygenase-like enzyme (5); cyclooxygenase and lipoxygenase (6); acetyltransferase (7); and acyltransferase (8)

a specific PLA₂ inhibitor, retards the stimulation of the 39-kDa PLA₂ activity suggesting that BEL can be used to prevent KA-induced neurodegeneration. Collectively, these studies suggest that receptor-mediated degradation of plasmalogen involves Ca^{2+} -independent PlsEtn-PLA₂.

Based on the effect of incorporation of docosahexaenoic acids in plasmalogens and PLA₂ inhibitors on hippocampal cell cultures, it is reported that the enrichment of DHA in hippocampal tissue has neuroprotective effects (Sergeeva et al., 2005). The neuroprotective effect is substantially higher in dentate gyrus than in CA1 and CA3 areas. Moreover, in astrocytic cultures the release of arachidonic and docosahexaenoic acids is differently regulated through Ca²⁺- and cAMP-dependent signal transduction pathways (Strokin et al., 2003), supporting the view that release of arachidonic and docosahexaenoic acids is differentially modulated under normal and pathological situations (Strokin et al., 2006; Farooqui et al., 2006).

In addition to DHA, kainate receptor-mediated degradation of plasmalogens also results in the generation of lysoplasmalogen (Fig. 5.3). Lysoplasmalogen may act as an acceptor for the transfer of sn-2 fatty acyl group from 1-alkyl-2-acylglycerophosphocholine via CoA-independent transacylase (Uemura et al., 1991). Thus, under normal conditions, lysoplasmalogen is either reacylated or hydrolyzed by a lysoplasmalogenase or utilized for the synthesis of platelet-activating factor, and arachidonic acid and docosahexaenoic acid are metabolized to eicosanoids and docosanoids, respectively. Ethanolamine and choline lysoplasmalogens increase membrane fluidity (Han and Gross, 1991) and modulate activities of various enzymes. Thus, choline lysoplasmalogen activates cAMP-dependent protein kinase (PKA) (Williams and Ford, 1997), suggesting that this lysoplasmalogen may be involved in a signal transduction process. Lysoplasmalogens also inhibit the plasma membrane Na⁺, K⁺-ATPase activity in myocytes and proximal tubule in kidney (McHowat et al., 1998; Schonefeld et al., 1996).

Collective evidence suggests that action of PlsEtn-PLA₂ on plasmalogen generates two classes of second messengers, namely, platelet-activating factors and eicosanoids. During receptor stimulation hydrolysis of plasmalogen by PlsEtn-PLA₂ may constitute the first wave of second messenger generation (the immediate or initial phase of signal transduction) (Turini and Holub, 1994). The generation of lysoplasmalogen may induce changes in membrane permeability and fluidity and allows the influx of external Ca²⁺ via plasma membrane channels. Changes in the Ca²⁺ level result in the translocation of Ca²⁺-dependent enzymes, including 85 kDa cytosolic PLA₂. This process results in the subsequent wave of second messenger generation (the late phase of signal transduction). Thus, plasmalogen-derived second messengers may be involved in earlier stages of signal transduction. In contrast, PtdCho-derived second messengers.

In contrast, under pathological conditions generation of high levels of arachidonic acid and platelet-activating factor initiates many pathophysiological processes. Thus, accumulation of arachidonic acid not only triggers an uncontrolled ROS production and arachidonic acid cascade, but also induces the NF- κ Bmediated cytokine expression. Platelet-activating factor-induced pathological processes include neuroinflammation and apoptotic cell death. These processes are closely associated with brain damage (Farooqui et al., 2007b,a; Sun et al., 2007).

5.5 Roles of Plasmalogens in Brain

Plasmalogens play important roles (Table 5.1) in mammalian brain (Farooqui and Horrocks, 2001; Nagan and Zoeller, 2001; Lee, 1998; Brites et al., 2004; Gorgas et al., 2006). Plasmalogens act as a reservoir for arachidonic and docosahexaenoic acids. These fatty acids are implicated in both physiological (synaptic plasticity) and pathophysiological (neurodegenerative) processes (Katsuki and Okuda, 1995). Arachidonic and docosahexaenoic acids modulate neurotransmission, ion channels, enzymic activities, long-term potentiation, and gene expression (Farooqui et al., 1997b; Zimmer et al., 2000; Högyes et al., 2003; Yehuda et al., 2002; Nishikawa et al., 1994; Xiao and Li, 1999; Fujita et al., 2001; Fernstrom, 1999; Farkas et al., 2000). At high concentrations, arachidonic acid has profound adverse effects on the ATP-producing capacity of mitochondria (Farooqui et al., 1997b). In addition arachidonic acid is metabolized to eicosanoids and lipoxins. These lipid mediators are associated with signal transduction processes (Phillis et al., 2006). In contrast to arachidonic acid, docosahexaenoic acid is metabolized to docosanoids (neuroprotectins and resolvins). These lipid mediators antagonize the effects of eicosanoids (Bazan, 2005a,b; Serhan, 2005, 2004).

Plasmalogens modulate biophysical properties of neural membranes by altering membrane critical temperature. Low transition temperature of plasmalogens may promote initial stages of membrane fusion (Ginsberg et al., 1998; Gorgas et al., 2006). The occurrence of high levels of plasmalogens in the synaptic plasma membrane (Breckenridge et al., 1973) and their interaction

| Role | References |
|---|---|
| Structural component of neural membrane | Paltauf (1994) |
| Reservoir for arachidonic and docosahexaenoic acids | Lee (1998), Farooqui and Horrocks (2001), Gorgas et al. (2006) |
| Involvement in membrane fusion events | Glaser and Gross (1995) |
| Involvement in ion transport | Gross (1985) |
| Involvement in neural cell differentiation | Bichenkov and Ellingson (1999) |
| Modulation of cholesterol efflux | Mandel et al. (1998), Munn et al. (2003) |
| Neuroprotection as antioxidant | Zoeller et al. (1988), Engelmann et al. (1994) |

Table 5.1 Proposed roles of plasmalogens in brain

with a fusion protein suggests that plasmalogens may be involved in membrane fusion events such as endocytosis and exocytosis during neurotransmission, hormone release, and membrane vesicle trafficking (Glaser and Gross, 1995). Interactions between PlsEtn and Ca²⁺-ATPase have been studied in skeletal muscle sarcoplasmic reticulum (Bick et al., 1991). These structures are actively involved in calcium transport (Gross, 1985). Based on reconstitution studies, it is proposed that plasmalogens provide a critical lipid environment in which anionic glycerophospholipids serve as boundary lipids for the regulation of the trans-sarcolemmal sodium–calcium exchanger (Ford and Hale, 1996).

A deficiency of ethanolamine plasmalogens produces alterations in cholesterol transport in the CHO cell mutants, NRel-4 and NZel-1 (Munn et al., 2003). NRel-4 cells have a defect in dihydroxyacetone phosphate acyltransferase (DHAPAT) and NZel-1 cells have altered alkyl-dihydroxyacetone phosphate synthase. In NRel-4 cells, ethanolamine plasmalogen is essential for specific cholesterol transport from the cell surface to acyl-CoA/cholesterol acyltransferase in the endoplasmic reticulum. Defective cholesterol transport can be restored when intermediates of ethanolamine plasmalogen biosynthesis are introduced into the system (Munn et al., 2003). The defect in cholesterol transport was also corrected when NRel-4 cells were transfected with a cDNA encoding the missing enzyme, DHAPAT. This suggests that plasmalogens play a very important role in HDL-mediated reverse cholesterol transport associated with atherosclerosis (Maeba and Ueta, 2003, 2004). Similarly, cellular HDLmediated cholesterol efflux is decreased in plasmalogen-deficient fibroblasts and macrophages. HDL-mediated cholesterol efflux is enhanced when cells are treated with 1-O-hexadecyl-sn-glycerol, a compound that restores the level of plasmalogens (Mandel et al., 1998). These studies support the view that plasmalogens play an important role in cholesterol efflux (Engelmann et al., 1992).

Plasmalogens protect biological structures against free radical attack in Chinese hamster ovary cells as well as in low-density lipoprotein particles (Zoeller et al., 1988; Engelmann et al., 1994; Engelmann, 2004). Plasmalogencontaining liposomes also have a strong ability to chelate copper and iron, which initiate lipid peroxidation by generating peroxyl and alkoxyl radicals, resulting in retardation of lipid peroxidation (Zommara et al., 1995; Sindelar et al., 1999). Loss of plasmalogens from neural membranes following injury and diseases (see below) also supports the view that plasmalogens may act as antioxidants (Farooqui and Horrocks, 2001; Farooqui et al., 2003).

Based on the incorporation of [³H]ethanolamine into progenitor cells, it is suggested that plasmalogens play an important role during differentiation (Bichenkov and Ellingson, 1999). Plasmalogens and their metabolizing enzymes are present in the nucleus (Albi et al., 2004), and plasmalogen-selective PLA₂ is stimulated by retinoic acid (Antony et al., 2001). Retinoic acid treatment produces neuritic outgrowth in LA-N-1 cells. The pan retinoic acid receptor antagonist BMS493 not only inhibits PlsEtn-PLA₂ activity but also blocks the formation of neuritic processes (Antony et al., 2003; Farooqui et al., 2004). Collectively, these studies indicate that besides being the structural component of neural membranes and a major reservoir for arachidonic and docosahexaenoic acids, plasmalogens are also involved in transport of ions across plasma membranes (Gross, 1985), membrane fusion (Lohner, 1996), protection of cellular membranes against oxidative stress (Zoeller et al., 1988; Engelmann et al., 1994), and the efflux of cholesterol from cells mediated by high-density lipoprotein (HDL) (Mandel et al., 1998).

5.6 Platelet-Activating Factor (PAF)

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine) is a potent biologically active ether glycerophospholipid with diverse neurophysiological and neuropathological functions (Fig. 5.1) (Snyder, 1995; Bazan, 2003). Its generation is tightly regulated both at the synthetic and degradative levels. In brain tissue, PAF is released not only from neural cells (neurons, astrocytes, oligodendrocytes, and microglia) but also from macrophages, platelets, endothelial cells, mast cells, and neutrophils. It exerts effects by activating the PAF receptors that consequently activate leukocytes, stimulate platelet aggregation, and induce the release of cytokines and expression of cell adhesion molecules (Maclennan et al., 1996; Blok et al., 2002; Honda et al., 2002). PAF receptors are linked to G-proteins and activate a variety of intracellular messenger systems such as calcium mobilization, arachidonic acid release, polyphosphoinositide turnover, generation of cAMP, and tyrosine phosphorylation (Chao and Olson, 1993; Honda et al., 2002; Ishii and Shimizu, 2000).

Although the synthesizing enzymes have not been purified and fully characterized from brain tissue, reports on the generation of PAF in mammalian brain have been published (Francescangeli et al., 2000). The expression of PAF receptors in brain has been demonstrated by radioligand binding assay and Northern blotting and in situ hybridization in rats and mice (Marcheselli et al., 1990; Ishii et al., 1996). In astrocytes, PAF upregulates nerve growth factor mRNA in a time- and concentration-dependent manner. This increase in nerve growth factor mRNA is suppressed by WEB 2086 and BN52021, potent PAF antagonists (Brodie, 1995; Yoshida et al., 2005). These studies suggest that PAF and PAF receptors occur in neural cells where they perform many important functions.

5.7 Biosynthesis of PAF

Three different pathways of PAF synthesis are known to occur in mammalian tissues (Honda et al., 2002; Snyder, 1995). They include a remodeling pathway, de novo synthesis, and an oxidative fragmentation pathway (Fig. 5.5). The remodeling pathway involves a structural modification of preexisting 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine that serves as structural components



Fig. 5.5 Metabolism of PAF in brain. Acyltransferase (1); cPLA₂ (2); acetyl-CoA: 1-O-alkyl-2-lysophosphatidylcholine acetyltransferase (3); PAF-acetylhydrolase (4); 1-alkyl-2-lyso-*sn*-glycero-3-phosphate (alkyllyso-GP): acetyl-CoA acetyltransferase (5); 1-alkyl-2-acetyl-*sn*-glycero-3-phosphate phosphohydrolase (6); 1-alkyl-2-acetyl-*sn*-glycero-3-phosphate phosphohydrolase (8); and lipoxygenase (9)

of neural membranes (Snyder et al., 1996) and plays a crucial role in inflammatory/hypersensitivity responses of cells. In contrast, the de novo reaction sequence starts with alkyl dihydroxyacetone phosphate (Fig. 5.5). This pathway maintains basal physiological PAF levels in various tissues and blood (Stafforini et al., 1987). The balance between PAF biosynthesis and degradation determines its levels in various tissues. The degradation of PAF is catalyzed by plateletactivating factor hydrolase (PAF-AH), which converts it into inactive lyso-PAF (see below).

5.7.1 Remodeling Pathway

This pathway primarily occurs in inflammatory cells. In the remodeling pathway, action of cytosolic phospholipase A₂ (cPLA₂) on 1-O-alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine generates 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF), which is acetylated by acetyl-CoA: 1-O-alkyl-2-lysophosphatidylcholine acetyltransferase to produce PAF. The released arachidonic acid is oxidized into eicosanoids (Rubin et al., 2005). This reaction is the basis of the interrelationship between the synthesis of PAF and eicosanoids. PAF can also be synthesized from plasmalogens. In neural membranes, the action of PlsEtn-PLA₂ on PlsEtn generates ethanolamine lysoplasmalogen (lyso-PlsEtn). CoA-independent transacylation reaction between alkylacyl-glycerophosphocholine (PakCho) and lyso-PlsEtn results in the synthesis of lyso-PAF, which is then converted to PAF by the enzyme acetyl-CoA: lyso-PAF-acetyltransferase (Prescott et al., 1990; Baker et al., 2002).

5.7.2 De Novo Synthesis of PAF

De novo PAF synthesis involves three enzymes namely 1-alkyl-2-lyso-*sn*-glycero-3-phosphate (alkyllyso-GP): acetyl-CoA acetyltransferase, 1-alkyl-2-acetyl-*sn*glycero-3-phosphate phosphohydrolase, and dithiothreitol (DTT)-insensitive 1-alkyl-2-acetyl-*sn*-glycerol: CDP-choline phosphotransferase (Snyder, 1995). In de novo synthesis pathway, alkylglycerophosphate is converted to alkylacetylglycerophosphate by 1-alkyl-2-lyso-*sn*-glycero-3-phosphate (alkyllyso-GP): acetyl-CoA acetyltransferase (Lee et al., 1986; Baker and Chang, 1993) and alkylacetylglycerophosphate is then dephosphorylated to 1-*O*-alkyl-2-acetyl-*sn*glycerol by 1-alkyl-2-acetyl-*sn*-glycero-3-phosphate phosphohydrolase (Panwala et al., 1998). 1-*O*-Alkyl-2-acetyl-*sn*-glycerol is transformed into PAF by 1-alkyl-2acetyl-*sn*-glycerol: CDP-choline phosphotransferase (Baker and Chang, 1993; Gimenez and Aguilar, 2001).

5.7.3 Oxidative Fragmentation Pathway for PAF Synthesis

The third pathway for the synthesis of PAF requires the oxidative fragmentation of phosphatidylcholines. When exposed to oxidative conditions, 1-O-alkyl-2-arachidonyl-*sn*-glycerophosphocholine breaks down into a variety of species of 1-O-alkyl phospholipids containing different short chain substituents at the sn-2 position. These 1-O-alkyl phospholipids interact with PAF receptors and induce a variety of biological effects (Stafforini et al., 1996; Prescott et al., 2000). These glycerophospholipid stimulates human neutrophils at submicromolar concentrations, and its effects are blocked by specific PAF receptor antagonists (WEB2086, L659989, and CV3988) (Smiley et al., 1991). Based on these observations, the occurrence of oxidative fragmentation pathway of PAF analog production has been proposed.

5.8 Catabolism of PAF

Three PAF-acetyl hydrolases occur in mammalian tissues (Snyder, 1995; Tjoelker and Stafforini, 2000; Arai, 2002; Arai et al., 2002). These enzymes show different biochemical and molecular properties and are encoded by different genes (Table 5.2). Two PAF-acetyl hydrolases, type I and type II, are intracellularly found in brain and other visceral organs. The third PAF-acetyl hydrolase is found in plasma (Arai et al., 2002; Tjoelker and Stafforini, 2000;

| | rear rear | | , , |
|----------------------------|------------|------------|------------|
| | Type I | Type II | Plasma |
| Property | enzyme | enzyme | enzyme |
| pH optimum | 7.4 | 7.4 | 7.4 |
| Km value (µM) | 4.9 | 9.1 | 13.7 |
| Vmax | 20,257 | 50,000 | 170 |
| Localization | Cytosol | Cytosol | Plasma |
| Mol. mass (kDa) | 100 | 40 | 43-67 |
| Diisopropylfluorophosphate | Inhibition | Inhibition | Inhibition |

Table 5.2 Kinetic and physicochemical properties of PAF-acetyl hydrolases

Summarized from Rice et al. (1998), Arai (2002), Prescott et al. (2000), and Karasawa et al. (2003).

Arai, 2002). Although PAF-acetyl hydrolases are serine-dependent enzymes, their ability to hydrolyze PAF is quite different.

PAF-acetyl hydrolases inactivate PAF by removing the acetyl group from the sn-2 position of glycerol moiety and generating lyso-PAF. PAF-acetyl hydrolase selectively hydrolyzes short acyl chains (C_2 to C_9) at the sn-2 position. This enzyme shows no activity with acyl chains longer than C_9 . PAF structural analogs are also degraded by PAF-acetyl hydrolase. These analogs competitively inhibit PAF-acetyl hydrolase activity (Stafforini et al., 1997). PAF receptor antagonists block PAF-acetyl hydrolase activity. In contrast to cPLA₂ and sPLA₂, PAF-acetyl hydrolase is not an interfacial enzyme. It does not require Ca^{2+} , and has broad substrate specificity as an esterase (Gelb et al., 2000).

5.8.1 Mammalian Brain Type I PAF-Acetyl Hydrolases

Bovine brain cytosolic PAF-acetyl hydrolase activity can be separated into three distinct peaks and is unaffected by EDTA (Hattori et al., 1996). Gel filtration chromatography indicates that the purified enzyme has a molecular mass of about 100 kDa. SDS gel electrophoresis shows the occurrence of three distinct bands of 45, 30, and 29 kDa, respectively (Hattori et al., 1996). Brain intracellular PAF-acetyl hydrolase type I is composed of α_1 , α_2 , and β_2 . Type I enzyme subunits (α_1 and α_2) are homologous, both of which account for catalytic activity along interactions with β subunit. Thus, this PAF-acetyl hydrolase is a heterotrimeric enzyme (Manya et al., 1999). The substrate specificity of α_1/α_2 heterodimer is similar to the α_1/α_1 homomer but not to the α_2/α_2 homodimer (Manya et al., 1999). β Subunit interacts with all three catalytic dimmers, but modulates the enzymic activity in a catalytic dimer compositiondependent manner. The β subunit strongly accelerates the enzymic activity of the α_2/α_2 homodimer, but suppresses the activity of the α_1/α_1 homodimer and has little effect on that of the α_1/α_2 heterodimer. Thus, the enzyme activity of type I PAF-acetyl hydrolase may be regulated not only by switching the composition of the catalytic subunit but also by manipulating the β subunit (Manya et al., 1999; Arai, 2002).

5.8.2 Type II PAF-Acetyl Hydrolases in Mammalian Tissues

Mammalian tissue cytosol contains type II PAF-acetyl hydrolase. Purified bovine liver type II PAF-acetyl hydrolase is a 40 kDa monomer. This enzyme shares 43% homology with extracellular, plasma PAF-acetyl hydrolase indicate cDNA cloning studies of bovine and human type II PAF-acetyl hydrolase indicate that both bovine and human enzymes contain a Gly-X-Ser-X-Gly motif that is characteristic of lipases and serine esterases (Hattori et al., 1996). A region surrounding the active site of type II PAF-acetyl hydrolase also shares homology with other lipases. Type II PAF-acetyl hydrolase is myristoylated at the N-terminus, and like other N-myristoylated proteins, it is distributed in both the cytosol and membranes. The amino acid sequence deduced from the cDNA of isoform II shows no homology with any subunit of isoform Ib (Hattori et al., 1996).

Collectively, these studies suggest that type I and type II PAF-acetyl hydrolases are trimeric enzymes consisting of two catalytic subunits and a regulatory subunit with catalytic serine residues (Arai, 2002). The purified enzymes display similar activity against PAF and oxidatively modified phosphatidylcholine, but does not hydrolyze phosphatidylcholine or phosphatidylethanolamine with two long-chain acyl groups (Hattori et al., 1993, 1996; Manya et al., 1998, Manya et al., 1999). The intracellular type II PAF-acetyl hydrolase shares homology with plasma acetyl hydrolase and catalyzes the hydrolysis of oxidized arachidonic acid from oxidized glycerophospholipids. Both the intracellular type II and plasma PAF-acetyl hydrolases have high affinity for esterified F_2 -isoprostanes, but the rate of esterified F_2 -isoprostane hydrolysis is slower than other substrates (Stafforini et al., 2006).

5.8.3 PAF-Acetyl Hydrolases in Mammalian Plasma

Guinea pig plasma PAF-acetyl hydrolase has been purified using multiple column chromatographic procedure. It migrates as a broad band on SDS-PAGE with molecular mass of 58–63 kDa, which is larger than the human enzyme (43–67 kDa) (Karasawa et al., 2003). The binding of plasma enzyme to Concanavalin A indicates its glycoprotein nature. Inhibition of purified enzyme by diisopropyl fluorophosphates supports the view that PAF-acetyl hydrolase is a serine-dependent hydrolase (Karasawa et al., 2003). Substrate specificity studies indicate that plasma PAF-acetyl hydrolase show a broad substrate specificity. The primary structure of human plasma PAF-acetyl hydrolase contains a GXSXG motif. The active site of these esterases contains serine,

aspartate, and histidine. Site-directed mutagenesis studies indicate the presence of Ser²⁷³, Asp²⁹⁶, and His³⁵¹ in a putative catalytic triad (Stafforini et al., 1997).

5.9 Roles of PAF in Brain

PAF activates both neural cells (neurons, astrocytes, oligodendrocytes, and microglia) and non-neural cells (platelets, leukocytes, monocytes, macrophages, endothelial cells, and smooth muscle cells) (Aihara et al., 2000). In normal brain, levels of PAF are low, but levels of lyso-PAF are quite high suggesting that PAF is present in its inactive form in the brain tissue (Tiberghien et al., 1991). As stated above, PAF acts through PAF receptors, which are expressed ubiquitously in neural and non-neural cells. Brain contains a small but significant amount of PAF-R mRNA (Bito et al., 1992; Marcheselli et al., 1990; Ishii et al., 1996). PAF receptor mRNA has been detected in hypothalamus, medulla–pons, olfactory bulb, hippocampus, cerebral cortex, spinal cord, thalamus, and cerebellum in the rat brain. Levels of PAF in the hippocampus are higher than cerebellum and cortex.

In general, PAF-Rs are linked through G-proteins to several intracellular signal transduction pathways, including activation of PLA₂, PLC, and PLD; cyclooxygenases; activation of GTPase activity; turnover of phosphatidylinositol; calcium mobilization; and activation of kinases (Maclennan et al., 1996; Mori et al., 1996; Honda et al., 2002; Kornecki and Ehrlich, 1991; Clark et al., 2000). Activation of PLA₂, PLC, and PLD results in arachidonic acid release, synthesis of eicosanoids, enhancement of polyphosphoinositide turnover, and generation of diacylglycerol, and inositol 1,4,5-trisphosphate (Ins P_3) causing elevation in intracellular calcium concentration. PAF also stimulates phosphatidylinositol 3-kinase and mitogen-activated kinase (MAP) kinase and inhibits adenylate cyclase. Moreover, PAF also acts as an intracellular mediator (Marcheselli and Bazan, 1994). PAF promotes transcriptional activation of a number of genes including immediate-early genes that include c-fos, c-jun, and krox-24; cytokines; enzymes; and growth factors (Table 5.3). In neuroblastoma cells, PAF treatment results in a 7- and 12-fold increase in c-fos mRNA in 15 and 30 min, respectively (Squinto et al., 1989; Bazan et al., 1991). The activation of these genes by PAF can be blocked by PAF antagonist, BN 52021. Similarly, PAF also stimulates c-jun transcription.

Long-term potentiation (LTP) is a long-lasting enhancement of synaptic efficacy due to repeated stimulation of postsynaptic NMDA receptors. LTP depends on gene expression, protein synthesis, calcium influx, arachidonic acid release, and the establishment of new neuronal connections. PAF enhances LTP, and PAF antagonists block its development (del Cerro et al., 1990; Kato and Zorumski, 1996). This observation indicates that PAF modulates LTP.

The exposure of primary neuronal to PAF for 24 h enhances neuronal death in a dose-dependent manner. The PAF-mediated neuronal death is blocked not

| Role | References |
|--|--|
| Modulation of signal transduction and calcium mobilization | Maclennan et al. (1996) |
| Modulation of gene expression | Marcheselli and Bazan (1994), Bazan et al. (1997) |
| Modulation of long-term potentiation | del Cerro et al. (1990), Kato et al. (1994), Bazan (2003) |
| Modulation of excitotoxicity | Xu et al. (2004) |
| Modulation of apoptosis | Hostettler and Carlson (2002) |
| Modulation of enzymic activity | Bazan et al. (1997), Bazan (2003) |
| Modulation of neural cell migration | Tokuoka et al. (2003) |
| Modulation of nociception | Svensson and Yaksh (2002), Teather et al. (2002) |
| Modulation of cerebrovascular function | Kochanek et al. (1988), Kochanek et al. (1990) |
| Modulation of immune function | Müller et al. (1993) |
| Modulation of blood pressure | Wu et al. (1999) |

 Table 5.3 Roles of platelet-activating factor in brain

only by BN52021, a PAF antagonist, but also by MK-801, a NMDA antagonist, suggesting that PAF and glutamate-mediated signal transduction pathways interact with each other and modulate neural cell death (Nogami et al., 1997; Xu et al., 2004; Bazan, 2003).

PAF is an essential component of the intricate mechanisms by which immune cells such as leukocytes are recruited to their targets (Zimmerman et al., 1996). PAF plays an important role in early B-cell activation and enhances IgG and IgA secretion and is an important B-cell immunomodulator which can interact with other leukocyte cell mediators. Although IgE receptors have not been identified, PAF has been shown to increase IgE binding, IgE-dependent adherence, and cytotoxicity of normal human eosinophils (Moqbel et al., 1990). Activation of neutrophils by PAF increases phagocytosis in a calcium-dependent manner suggesting that PAF may contribute to allergic reaction and immune challenge such as infection.

PAF-mediated neuroinflammation is closely associated with short- and long-term responses of cells to stimulation or neural trauma (Bazan et al., 1997; Farooqui et al., 2007b). PAF promotes adhesive interactions between leukocytes and endothelial cells, leading to transendothelial migration of leukocytes. PAF also modulates the migration of cerebellar granule neurons in cultures (Tokuoka et al., 2003).

Intracarotid infusion of PAF decreases cerebral blood flow with a concomitant increase in the global cerebral metabolic rate for oxygen (Kochanek et al., 1988, 1990). PAF administration also produces a dose-dependent decrease in spinal cord blood flow. This decrease in blood flow can be blocked by a PAF receptor antagonist (Faden and Halt, 1992). Generation of PAF following ischemic injury and disturbance in cerebral blood flow also supports the view that PAF modulates blood flow in brain tissue (del Zoppo and Mabuchi, 2003). Treatment of glial cell cultures with PAF induces apoptotic cell death in astrocytes as well as oligodendrocyte cultures, and this effect can be blocked by the PAF receptor antagonists, WEB 2170 and BN 52021 (Hostettler and Carlson, 2002). Suggestion that PAF-mediated cell death involves caspase-3 is based on studies in PAF knockout mice (-/-). These mice are deficient in the caspase-3 gene. Toxic effects of PAF are lost when astrocytes (-/-) are exposed to low concentrations of PAF. Oligodendrocytes derived from knockout mice (-/-) are not susceptible to PAF toxicity. Thus, PAF induces cell apoptotic death in cultured CNS glial cells and this effect is, in part, dependent on caspase-3 activation (Hostettler and Carlson, 2002).

Collective evidence suggests that the physiological activity of PAF is not limited to its pro-inflammatory function and neurotrophic effects in brain. PAF is also involved in a variety of other settings including reproduction, allergic reactions, and circulatory system disturbances in atherosclerosis (Chao and Olson, 1993; Honda et al., 2002; Bazan, 2003).

5.10 Involvement of Plasmalogens in Neurological Disorders

Alterations in plasmalogen levels occur in many neurological disorders (Table 5.4). Changes in plasmalogen levels may be due either to a decrease in their synthesis or to an increase in their degradation. Peroxisomal disorders are characterized by deficiency of plasmalogen-synthesizing enzymes (dihydrox-yacetone phosphate acyltransferase and alkyl-dihydroxyacetone phosphate synthase). In contrast, decreases in plasmalogens in neurotrauma and neurode-generative diseases may by caused by the stimulation of plasmalogen-selective phospholipase A_2 .

| Disorder | Plasmalogen level | References |
|--|----------------------|--|
| Ischemia | Decreased | Zhang and Sun (1995) |
| Alzheimer disease | Decreased | Wells et al. (1995), Guan et al. (1999) |
| Alzheimer disease rat model | Decreased | Hashimoto et al. (2002) |
| Spinal cord injury | Decreased | Demediuk et al. (1985) |
| Zellweger syndrome | Decreased | Datta et al. (1984), Martínez et al. (2000) |
| Zellweger syndrome mouse model | Decreased | Janssen et al. (2000) |
| Experimental allergic encephalomyelitis | Decreased | Jagannatha and Sastry (1981) |
| Rhizomelic chondrodysplasia punctata | Decreased | Poulos et al. (1991), Bams-Mengerink et al. (2006) |

 Table 5.4
 Alterations in plasmalogen levels in neurological disorders

5.10.1 Plasmalogens in Ischemic Injury

Ischemic injury is accompanied by a marked decrease in plasmalogen content of neural membranes and increased PLA₂ activity (Viani et al., 1995; Zhang and Sun, 1995). In rabbit myocardium, microsomal plasmalogen-selective PLA₂ activity is increased 10-fold during ischemic injury (Hazen et al., 1991). The activation of this enzyme is accompanied by an accumulation of lysoplasmalogen in critical subcellular loci, leading to alterations in membrane dynamics. The activation of the plasmalogen-selective PLA₂ can be abolished by pretreatment with bromoenol lactone, a specific inhibitor of plasmalogen-selective PLA_2 (Hazen et al., 1991). In gerbil, ischemic injury is also accompanied by the stimulation of the cPLA₂ hydrolyzing PtdEtn (Edger et al., 1982). It is proposed that the stimulation of PlsEtn-PLA₂ and cPLA₂ induces membrane dysfunction that may lead to cellular injury (Farooqui and Horrocks, 1991; Ray et al., 1994). As mentioned above, the stimulation of plasmalogen-selective PLA_2 -derived lipid mediators may occupy a proximal position in the injury pathway, initiating cell injury, whereas cPLA₂ hydrolyzing PtdCho may participate in amplifying the injury process.

5.10.2 Plasmalogens in Alzheimer Disease

Levels of ethanolamine plasmalogens are markedly decreased in autopsy brain samples from Alzheimer disease patients compared to age-matched controls (Wells et al., 1995; Ginsberg et al., 1995). This reduction in ethanolamine plasmalogen may be due to the stimulation of plasmalogen-selective PLA₂ (Farooqui et al., 1997a, 2003), resulting in marked increases in levels of prostaglandins and lipid peroxides (Iwamoto et al., 1989). The deficiency of ethanolamine plasmalogen in Alzheimer disease may lead to neural membrane destabilization due to changes in the critical temperature necessary for maintaining the stability of the lipid bilayer (Ginsberg et al., 1998). Reduction in plasmalogen levels may also not only cause loss of synapse in Alzheimer disease but also produce impairment of muscarinic cholinergic signals and abnormal amyloid precursor processing. These changes may be closely associated with neural cell death in Alzheimer disease (Périchon et al., 1998; Farooqui et al., 1997a, 2003).

5.10.3 Plasmalogens in Spinal Cord Injury

In spinal cord tissue, plasmalogens account for about one-third of the total glycerophospholipids. Much of the PlsEtn are found in the myelin sheath. Degradation of the PlsEtn is similar in gray and white matter during and after compression of spinal cord trauma (Demediuk et al., 1985). About 10% of these

plasmalogens are lost during the first minute of compression injury, with an overall loss of 18% found 30 min after compression injury. It is proposed that the loss of plasmalogens after compression may be due to the shear stress-mediated stimulation of plasmalogen-selective PLA₂.

5.10.4 Plasmalogens in Peroxisomal Disorders

Zellweger syndrome is an autosomal recessive disease characterized by the deficiency of plasmalogens (Heymans et al., 1983). This deficiency in plasmalogens is caused by the deficiency of dihydroxyacetone phosphate acyltransferase and alkyl-dihydroxyacetone phosphate synthase (Datta et al., 1984). The pathophysiological events associated with peroxisomal disorders may not reflect the effect of plasmalogen insufficiency, but may be the consequences of the loss of other peroxisomal function, such as a defect in the degradation of very long-chain fatty acids (Lee, 1998). However, DHA supplementation has been proposed to restore the levels of plasmalogen (Martínez et al., 2000). A deficiency of ethanolamine plasmalogen is also observed in Niemann–Pick type C disease (Schedin et al., 1997).

5.11 Involvement of Platelet-Activating Factor in Neurological Disorders

High levels of PAF are found in inflammatory syndromes, epileptic seizures, bacterial meningitis, multiple sclerosis, prion diseases, Miller-Dieker lissencephaly, HIV replication associated with AIDS dementia complex, and Alzheimer disease (Table 5.5). Although mechanisms associating neural cell injury with PAF synthesis are fully understood, it is well known that activation of PAF receptors causes the mobilization of calcium through calcium channels and from intracellular stores via inositol 1,4,5-trisphosphate (Clark et al., 2000). At the nuclear level, PAF mediates the stimulation of nuclear factor- κB (NF- κB), resulting in the modulation of gene expression. In general, PAF-mediated neural cell injury is accompanied by the modulation of gene expression for cytokines, $sPLA_2$, nitric oxide synthase, and COX-2 and increase in intracellular calcium. PAF also amplifies excitotoxicity, and PAF antagonists protect against brain damage by blocking calcium mobilization, inhibiting gene expression, and blocking (Clark et al., 1992). PAF and its antagonists interact with other neurotransmitter and their receptors and modulate oxidative stress and neuroinflammation. Thus, PAF synthesis is stimulated by acetylcholine and inhibited by acetylcholine receptor antagonist, atropine (Sogos et al., 1990). Specific PAF antagonist, Y-24180, interacts with low affinity to benzodiazepine receptors in synaptosomal membranes (Takehara et al., 1990). Collectively, these studies suggest that PAF is an important neural cell injury mediator involved in gene expression, inflammation, oxidative stress, and neurodegeneration.

| Neurological | | |
|----------------------------|-----------------------------------|-------------------------------------|
| disorder | Levels of PAF | References |
| Ischemia | Increased | Lindsberg et al. (1991) |
| Spinal cord injury | Increased | Hostettler et al. (2002) |
| Head injury | Increased | Faden and Tzendzalian (1992) |
| Cold injury | Increased | Tokutomi et al. (2001) |
| EAE | Increased | Kihara et al. (2005) |
| Migraine | Increased | Sarchielli et al. (2004) |
| Perinatal aphysia | Increased | Akisu et al. (2003) |
| Subarachnoid hemorrhage | Increased and decreased with time | Hirashima et al. (1993a,b, 1994) |
| Dyslexia | Increased | Kelley et al. (1999) |
| Multiple sclerosis | Increased | Callea et al. (1999) |
| Hemimegalencephaly | Decreased | Hirashima et al. (1999) |
| Human immunodeficiency | Increased | Gelbard et al. (1994) |
| Meningitis | Increased | Arditi et al. (1990) |
| Seizures/convulsions | Increased | Kumar et al. (1988) |

 Table 5.5
 Involvement of PAF in neurological disorders

5.12 Conclusion

Ether glycerophospholipids are important constituents of neural membranes. Major ether glycerophospholipids include plasmalogens and platelet-activating factor. Plasmalogens are major components of myelin sheath. Platelet-activating factor is not stored in neural cells, but it is either generated through de novo synthesis for basal neural cell activities or synthesized in neural cells through remodeling pathway under pathological conditions. In addition to neural membrane constituents, plasmalogens serve as endogenous antioxidants and play an important role in neural membrane fusion. Plasmalogens are also involved in cell differentiation. The occurrence of plasmalogens in the synaptic membrane suggests that they not only play an important role in synaptogenesis, but may also be involved in vesicle formation during neurotransmitter release. Levels of plasmalogens are markedly decreased in ischemia, spinal cord injury, and Alzheimer disease but also in peroxisomal disorders and Niemann-Pick type C disease. In ischemia, spinal cord injury, and Alzheimer disease decrease in plasmalogen levels is due to the stimulation of PlsEtn-selective PLA₂, whereas in peroxisomal disorders decrease in plasmalogen levels is caused by decreased activities of plasmalogen-synthesizing enzymes.

PAF is a potent lipid mediator. It is synthesized through remodeling pathway, de novo synthesis pathway, and oxidative fragmentation pathway. Remodeling pathway is responsible for the generation of PAF in inflammatory cells (astrocytes, microglia, endothelial cells, and leukocytes) while the de novo pathway may be associated with constitutive basal PAF generation in neurons.
PAF acts through PAF receptors that are linked to the activation of phospholipases A_{2} , C, and D: cyclooxygenases: GTPase: stimulation of polyphosphatidylinositide turnover; calcium mobilization; and modulation of transcription of immediate-early genes. Stimulation of PAF receptors plays important roles in modulating neuronal plasticity, memory formation, modulation of neuroinflammation and nociceptive responses, and apoptotic cell death during neuronal injury. PAF-acetyl hydrolases inactivate PAF by removing the acetyl group from the sn-2 position of glycerol moiety. These enzymes play a key role in the degradation of pro-inflammatory oxidized phospholipids and act as general scavenger of oxidized phospholipid species, which may accumulate inappropriately during neural trauma and neurodegenerative diseases. Levels of PAF are elevated in neurological disorders including brain trauma, seizures, stroke, multiple sclerosis, and viral and bacterial infections. PAF is also involved in the pathophysiology of AIDS dementia and Miller-Dieker lissencephaly. Administration of PAF antagonists slows down the progression of the above neurological disorders.

Although ether glycerophospholipids occur in lower amounts in brain tissue, they are involved in multiple neural cell functions. Alterations in their levels have been reported to occur in several neurological disorders that are characterized by neuroinflammation and oxidative stress. It remains to be seen whether changes in ether glycerophospholipids are primary defects in neurological disorders or produced by secondary factors associated with neuroinflammation and oxidative stress.

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Chapter 6 Excitotoxicity-Mediated Neurochemical Changes in Neurological Disorders

6.1 Introduction

Neural cell demise through the overstimulation of glutamate receptors by high glutamate and its analogs is called as excitotoxicity (Olnev et al., 1979; Choi, 1988). Morphologically, excitotoxicity results in neuronal swelling, vacuolization, and eventual neural cell death (Farooqui and Horrocks, 1994). Glutamate and its analogs act by interacting with glutamate receptors (excitatory amino acid receptors). These receptors are classified into *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate (KA), L-2-amino-4-phosphonobutanoate (L-AP4), and trans-1-amino-cyclopentyl-1, 3-dicarboxylate (trans-ACPD) receptors (Wang et al., 2005a; Farooqui et al., 2008). Present interest in excitatory amino acids is due to their involvement in neurological disorders (Farooqui and Horrocks, 1991, 1997; Lipton and Rosenberg, 1994: Whetsell, 1996). Excitotoxicity is accompanied by calcium influx that initiates a cascade of events involving mitochondrial dysfunction, activation of enzymes associated with the release and oxidation of arachidonic acid, and generation of free radicals (Farooqui and Horrocks, 1991, 1994; Farooqui et al., 2001; Wang et al., 2005b). These enzymes include isoforms of PLA₂, cyclooxygenase-2 (COX-2), lipoxygenases (LOX), and epoxygenases (EPOX) (Phillis et al., 2006). An uncontrolled and sustained increase in cytosolic calcium levels also results in stimulation of calpains, nitric oxide synthase, protein phosphatases, and various protein kinases (Bazan et al., 1995; Pavel et al., 2001; Ray et al., 2003; Ellis et al., 2004; Arundine and Tymianski, 2004). Accumulation of oxygenated arachidonic acid metabolites along with abnormal ion homeostasis, changes in redox status, and lack of energy generation is associated with neural cell injury and cell death in acute neural trauma (ischemia, epilepsy, head injury, and spinal cord trauma) and neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), multiple sclerosis (MS), and prion diseases like Creutzfeldt-Jakob disease (CJD) (Farooqui et al., 2008).

Glutamate not only damages neurons but also produces injury to glial cells through mechanisms that do not involve glutamate receptor activation, but rather glutamate uptake (Oka et al., 1993; Matute et al., 2006). Glutamate uptake from the extracellular space by specific glutamate transporters is

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essential for maintaining excitatory postsynaptic currents (Auger and Attwell, 2000) and for blocking excitotoxic death due to overstimulation of glutamate receptors (Rothstein et al., 1996). Five glutamate transporters (EAAT1, EAAT2, EAAT3, EAAT4, and EAAT5) have been cloned from brain tissue. Two transporters, namely excitatory amino acid transporter E1 (EAAT1) and excitatory amino acid transporter E2 (EAAT2), are expressed in astrocytes, oligodendrocytes, and microglial cells (Matute et al., 2006). Exposure of astroglial, oligodendroglial, and microglial cell cultures to glutamate induces glial cell death by a transporter-related mechanism involving the inhibition of cystine uptake, which produces a decrease in glutathione and makes glial cells vulnerable to toxic effects of free radicals (Oka et al., 1993; Matute et al., 2006; Farooqui et al., 2008).

Glutamate-mediated brain damage not only involves interactions among excitotoxicity, oxidative glutamate toxicity, but also mitochondrial dysfunction, decrease in ATP levels, and changes in neural cell redox (Farooqui and Horrocks, 1991, 1994; Wang et al., 2005b; Nicholls, 2004).

6.2 Glutamate-Mediated Neurochemical Changes in Brain

Glutamate and its analogs produce toxic effects through the excessive stimulation of glutamate receptors (Lipton and Rosenberg, 1994; Farooqui and Horrocks, 1994; Matute et al., 2006) (Fig. 6.1). As stated earlier, overstimulation of glutamate receptors causes increased hydrolysis of neuronal membrane glycerophospholipids with the release of arachidonic acid and generation of free radicals and ROS. ROS production induces mitochondrial dysfunction. These processes, along with changes in neural membrane fluidity and permeability and with alterations in energy status and redox status of neuronal cells, are closely associated with glutamate-mediated cell death in acute neural trauma and neurodegenerative diseases (Fig. 6.2) (Farooqui and Horrocks, 1994; Farooqui et al., 2001; Wang et al., 2005b).

6.2.1 Glutamate-Mediated Changes in Arachidonic Acid and Lysophosphatidylcholine Metabolism

Two major enzymic mechanisms are involved in glutamate-mediated release of arachidonic acid (AA) from neural membrane glycerophospholipids. A direct mechanism involves activation of phospholipase A_2 (PLA₂) (Dumuis et al., 1988; Lazarewicz et al., 1990; Farooqui et al., 2003a,b) (Fig. 6.3). The indirect mechanism requires the participation of phospholipase C (PLC)/ diacylglycerol lipase pathway (Farooqui et al., 1989) (Fig. 6.4). Glutamatemediated AA release can be prevented by glutamate receptor antagonist, 2-amino-5-phosphonoverate (APV), and the AMPA receptor antagonist, 6-cyano-



Fig. 6.1 Structures of glutamate and its analogs and antagonists. Glutamate (**a**); kainate (**b**); *N*-methyl-D-aspartate (**c**); ibotenate (**d**); AMPA (**e**); dexoxadrol (**f**); dextrophan (**g**); ketamine (**h**); and MK 801 (**i**)



Fig. 6.2 Excitotoxicity-mediated metabolic alterations in brain tissue



Fig. 6.3 Reaction showing the direct release of arachidonic acid by phospholipase A₂ in brain



Fig. 6.4 Reactions showing the indirect release of arachidonic acid by PLC/DAG-lipase pathway in brain

7-nitroquinoxaline (CNQX), in dose- and time-dependent manner. Quinacrine, a cPLA₂ non-specific inhibitor, as well as arachidonoyl trifluoromethylketone, a potent inhibitor of cPLA₂, also block glutamate-mediated AA release (Sanfeliu et al., 1990; Kim et al., 1995; Farooqui et al., 2003a,b). Similarly, 1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino] hexyl]-1*H*-pyrrole-2,5-dione (U-73122) and 1-*O*-octade-cyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH₃), two agents that inhibit PLC, prevent glutamate-mediated neurotoxicity (Llansola et al., 2000) and NMDA receptor antagonist dextrorphan (Fig. 6.1) and the diacylglycerol lipase inhibitor RHC80267 inhibit the release of AA through phospholipase C (PLC)/diacylglycerol lipase pathway (Farooqui et al., 1993; Farooqui et al., 2003a,b).

The release of AA within brain tissue is an important physiological event because this fatty acid modulates multiple neurochemical processes. At low concentrations, AA acts as a second messenger. It regulates the activity of protein kinase A (PKA), protein kinase C (PKC), NADPH oxidase, choline acetyltransferase, and caspase-3. In addition, AA modulates ion channels, neurotransmitter release, induction and potentiation of long-term potentiation, long-term depression, and neural cell differentiation (Farooqui et al., 2002). AA may act as a facilitatory retrograde neuromodulator in glutamatergic synapses (Katsuki and Okuda, 1995), because it is released upon activation of glutamate receptors. In the nucleus, AA interacts with elements of gene structure, such as promoters, enhancers, suppressors, etc., to modulate gene expression in a specific manner that is not shared by eicosanoids or other fatty acids (Farooqui et al., 1997a; Farooqui and Horrocks, 2007).

Under pathological conditions high concentrations of AA produce considerable brain damage through multiple neurochemical processes. Thus, AA produces intracellular acidosis and uncouples oxidative phosphorylation resulting in mitochondrial dysfunction (Schapira, 1996). AA produces changes in membrane permeability by modulating ion channels. This results in mitochondrial swelling in neurons (Farooqui et al., 1997a,b,c). High levels of AA have a profound adverse effect on the ATP-producing capacity of mitochondria. Arachidonic acid activates nuclear factor- κ B (NF- κ B) and decreases neuronal viability (Toborek et al., 1999; Farooqui and Horrocks, 1994, 2006, 2008).

AA is also metabolized to 4-hydroxynonenal (4-HNE). This metabolite reacts with nucleophilic sites of proteins on lysine, cysteine, and histidine residues. 4-HNE impairs the activities of key metabolic enzymes, including Na⁺, K⁺-ATPase, glucose 6-phosphate dehydrogenase, and several kinases. It stimulates stress-activated protein kinases (Camandola et al., 2000) such as c-jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase. 4-HNE also disrupts transmembrane signaling and the glucose and glutamate transporters in astrocytes (Mark et al., 1997). The inhibition of Na⁺, K⁺-ATPase depolarizes neuronal membranes leading to the opening of NMDA receptor channels and influx of additional Ca²⁺ into neurons. In cortical neurons, 4-HNE is known to alter G-protein-linked muscarinic cholinergic receptors (mAChR) and metabotropic glutamate receptors (mGluRs), indicating that 4-HNE can disrupt signal transduction processes in brain tissue.

Lysophospholipid, the other product of PLA₂ catalyzed reaction, also produces many neurochemical effects in brain tissue through its neurotropic and neurotoxic effects. Morphologically, lyso-PtdCho produces de-ramification of murine microglia and other immune cells (Schilling et al., 2004). The de-ramification of microglial cells facilitates complete retraction of cell extensions and increased size of microglial cell bodies with amoeboid morphology. Although the molecular mechanism associated with de-ramification is not understood, this process can be retarded by the inhibition of non-selective cation channels and K^+-Cl^- cotransporters. Lyso-PtdCho also promotes cell motility and releases pro-inflammatory cytokines. It modulates ion channel permeability in various brain preparations by perturbing the orderly packing of glycerophospholipid bilayers (Maingret et al., 2000). Lyso-PtdCho modulates protein kinases such as protein kinase C, protein kinase A, and c-jun terminal kinase. Lyso-PtdCho stimulates phospholipase D and inhibits CTP:phosphocholine cytidylyltransferase (Gómez-Muñoz et al., 1999).

Under pathological conditions, glutamate-mediated elevation in lyso-PtdCho levels increases dopamine release, inhibits dopamine uptake, and decreases mitochondrial potential in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated PC12 cells (Lee et al., 2004, 2005a,b). Injections of lyso-PtdCho into the lateral ventricle of rat brain induce alterations in locomotor activity and produce changes in biogenic amine levels. Quinacrine, a nonspecific PLA₂ inhibitor, protects against changes induced by lyso-PtdCho in this PD model (Lee et al., 2005a,b). This suggests that PLA₂ activation and lyso-PtdCho generation may be involved in MPTP-mediated neurotoxicity.

6.2.2 Glutamate-Mediated Changes in Platelet-Activating Factor Metabolism

Glutamate-mediated activation of PLA₂ promotes the synthesis of 1-alkyl-2lyso-sn-glycero-3-phosphocholine (lyso-platelet-activating factor, lyso-PAF), and its acetylation at the sn-2 position results in the generation of plateletactivating factor (PAF), a lipid mediator that modulates a variety of neural cell functions including upregulation in activities of mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinases, c-jun N-terminal kinase, and p38 kinases in primary hippocampal neurons in vitro (Mukherjee et al., 1999; DeCoster et al., 1998). The PAF receptor antagonist BN 50730 can prevent this activation. The PAF receptor antagonist BN 50730 also retards activation of kainate receptors (DeCoster et al., 1998). In contrast, CNOX has no effect on PAF activation of the kinases, indicating that PAF is downstream to kainate activation (Mukherjee et al., 1999; DeCoster et al., 1998). Collectively, these studies suggest that glutamate-mediated enhancement of PAF metabolism may be involved in neuronal plasticity and long-term potentiation. In rats, injections of PAF antagonists impair spatial learning and inhibitory avoidance tests, while treatment with a synthetic non-hydrolysable analog of PAF, 1-O-hexadecyl-2-methylcarbamoyl-sn-glycerol-3-phosphocholine, enhances memory (Packard et al., 1996).

Pathophysiologically, PAF is involved in inflammation, allergic reactions, and immune responses. It is a potent inducer of gene expression in CNS. High levels of PAF induce the release of cytokines and expression of cell adhesion molecules (Snyder, 1995; Maclennan et al., 1996; Ishii et al., 2002; Honda et al., 2002). The NMDA receptor/nitric oxide (NO) signaling pathway is involved in PAF-induced neurotoxicity. The degree of neuronal death in cultures increased in a dose-dependent manner during exposure to PAF for 24 h (Xu and Tao, 2004). The PAF antagonist (BN52021), and also MK-801, an NMDA antagonist, and L-NAME, a nitric oxide synthase (NOS) inhibitor, retarded the

neurodegenerative effect of PAF significantly. Moreover, BN52021 and MK-801 dramatically block the increases in NOS activity and neuronal NOS expression induced by chronic exposure of the cultured neurons to PAF. Collective evidence suggests that the NMDA receptor-mediated NO signaling is closely associated with PAF-induced cell death (Xu and Tao, 2004). Glutamate-mediated elevation in PAF has been implicated in mitochondrial swelling, membrane permeability transition (MPT), and release of cytochrome c (Parker et al., 2002) in rat brain mitochondrial preparations. The PAF antagonist BN50730 can block this process. This further supports the view that glutamate-mediated neural cell injury is associated with PAF elevation.

6.2.3 Glutamate-Mediated Alterations in Eicosanoid Metabolism

Glutamate and its analogs modulate the generation of prostaglandins, leukotrienes, and thromboxanes from AA. These metabolites are known collectively as eicosanoids (Phillis et al., 2006). Neurons and glia generate prostaglandins, whereas cerebral microvessels and the choroid plexus mainly produce thromboxanes. Eicosanoids act through their receptors and modulate signal transduction pathways and gene transcription. Thus, PGD₂ activates the DP receptors, PGE₂ activates the EP receptors, and PGF_{2 α}, PGI₂, and TXA₂, respectively, stimulate the FP, IP, and TP receptors (Coleman et al., 1994). These receptors are linked to the generation of cyclic AMP, diacylglycerol, and phosphatidylinositol 1,4,5-trisphosphate and modulation of Ca^{2+} influx. Eicosanoid and their receptors may be involved in regulation of glutamate release in the synaptic cleft (Bazan, 2003). Glutamate release modulates neuronal excitability and synaptic transmission at the presynaptic level. In contrast, the uptake of glutamate by astrocytes prevents its neurotoxic accumulation in the synaptic cleft. Eicosanoids also modulate glutamate receptors in the hippocampus (Chabot et al., 1998). This is tempting to speculate that a cross talk may be taking place among glutamate, prostaglandin, leukotriene, and thromboxane receptors. Under normal conditions, this cross talk refines the communication among receptors, but under pathological situations, this cross talk may promote neuronal injury, which depends upon the levels of glutamate, magnitude of PLA₂ expression, levels of eicosanoids and the intensity of oxidative stress (Farooqui and Horrocks, 2006, 2008). Collectively, these studies suggest that in neural cells, glutamate-mediated modulation of eicosanoid generation, activities of glutamate transports, depletion of glutathione, and decreased ATP levels are closely associated with initiation, maintenance, and modulation of oxidative stress, neuroinflammation, and apoptotic cell death (Gilroy et al., 2004; Uz et al., 1998; Chabot et al., 1998; Manev et al., 2000).

6.2.4 Glutamate-Mediated Generation of Reactive Oxygen Species

During excitotoxic insult, the release and accumulation of arachidonic acid triggers an uncontrolled "arachidonic acid cascade" setting the stage for elevated generation of reactive oxygen species (ROS). ROS include oxygen superoxide radicals, hydroxyl and alkoxyl radicals, lipid peroxy radicals, and peroxides (hydrogen peroxide and glycerophospholipid hydroperoxide) (Phillis et al., 2006; Farooqui et al., 2008). At low levels, ROS can function as signaling intermediates in the regulation of fundamental cell activities such as growth and adaptation responses. At higher concentrations, ROS contribute to neural membrane damage when the balance between reducing and oxidizing (redox) forces shifts toward oxidative stress resulting in an irreversible damage to neural membrane lipids, proteins, and nucleic acids (Wang et al., 2005b; Farooqui and Horrocks, 1994, 2006).

The major sources of ROS are the mitochondrial respiratory chain and arachidonic acid cascade. In the early phase of excitotoxicity, cytochrome c is released from the mitochondrial membrane as a defense mechanism against oxidative stress. At high glutamate concentrations, the release of high levels of cytochrome c is associated with either permeability transition pore opening or collapse of the mitochondrial membrane potential (Atlante et al., 2000). In neural membranes, ROS-mediated chemical changes can lead to depletion of unsaturated glycerophospholipids. This depletion may be associated with an alteration in membrane fluidity (Farooqui and Horrocks, 2006). Glutamatemediated generation of glycerophospholipid hydroperoxides also inhibits the reacylation of lyso-glycerophospholipids in neuronal membranes, and therefore prevents the restoration of native glycerophospholipids in neural membranes (Zaleska and Wilson, 1989; Farooqui et al., 2000). This inhibition may constitute another mechanism whereby peroxidative process contributes to irreversible neuronal injury and death. ROS also attack DNA bases causing damage through hydroxylation, ring opening, and fragmentation. This reaction generates 8-hydroxy-2'-deoxyguanosine (8-OHdGua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (Jenkinson et al., 1999).

6.2.5 Glutamate-Mediated Depletion of Reduced Glutathione

Glutathione (γ -glutamyl-cysteinylglycine, GSH) protects brain tissue from ROS-mediated oxidative stress (Weber, 1999). Glutathione binds to its own receptors and modulates glutamatergic excitatory neurotransmission by displacing glutamate from its ionotropic receptors (Janaky et al., 1999). It increases the response of NMDA receptors by interacting with its redox sites. A decreased glutathione level, through inhibition of its synthesis, is accompanied by increased excitotoxic response to NMDA, degeneration of mitochondria, and larger infarct areas in stroke models (Janaky et al., 1999).

Collectively, these studies suggest that although GSH and GSH-related enzymes are abundant in cytosolic compartments of astrocytes, the mitochondrial pools are relatively small. Since brain mitochondria are sites of significant ROS generation, the mitochondrial localization of GSH and its associated enzymes in neural cells provides important defenses against toxic oxygen species in the brain tissue. Collectively, these studies suggest that the glutathione not only works as a peroxide scavenger, but also regulates the redox state of the neural cells under pathological situations. Thus, the maintenance of appropriate intracellular glutathione (GSH) levels is crucial for cellular defense against excitotoxicity and oxidative damage (Weber, 1999).

6.2.6 Glutamate-Mediated Alterations in Nuclear Transcription Factor κB (NF-κB)

NF- κ B functions as key regulators of neuronal death as well as survival. It has been implicated in excitotoxicity (Furukawa and Mattson, 1998; Oin et al., 1999; Djebaili et al., 2000; Cruise et al., 2000; Pizzi et al., 2005) (Table 6.1). In response to glutamate and NMDA, NF- κB translocates to the nucleus (Ko et al., 1998) (Fig. 6.5). The mechanism by which NF- κ B mediates cell death during excitotoxicity remains unknown. It is proposed that glutamate interacts and activates the p50 and p65 protein subunits of NF- κ B and promotes the translocation of NF- κB from the cytoplasm to the nucleus where it binds to target sequences in the genome and facilitates the expression of a number of proteins including many enzymes (sPLA₂, COX-2, NADPH oxidase, and inducible nitric oxide synthase) and cytokines (TNF- α , IL-1 β , and IL-6) (Fig. 6.6). Interactions of ROS with NF- κ B also promote the translocation of NF- κ B to the nucleus (McInnis et al., 2002). Antioxidant, M40403, not only blocks excitotoxicity but also prevents NF- κ B translocation to the nucleus (McInnis et al., 2002). NMDA receptor-mediated activation of NF- κ B involves I κ B- α degradation by a caspase-3-like cysteine protease. Based on these studies, it is proposed that caspase-3 may contribute to the excitotoxin-induced apoptosis in rat striatal neurons (Qin et al., 2000) (see below).

| | 1 | 5 5 |
|----------------------|-----------------------|---|
| Transcription factor | Effect | References |
| NF- <i>k</i> B | Increased | Cruise et al., 2000; Pizzi et al., 2005 |
| AP1 | Increased | Lidwell and Griffiths, 2000 |
| Nrf2 | Increased | Li et al., 2007 |
| OCT1 | Decreased | Qin et al., 1998 |
| F2F-1 | Transiently increased | Qin et al., 1998 |

 Table 6.1 Transcription factors associated with excitotoxic cell injury



Fig. 6.5 Glutamate-mediated degradation of glycerophospholipids, generation of ROS, and activation of NF-*κ*B in brain tissue. Glutamate and its analogs (A₁ and A₂); NMDA receptor (NMDA-R); α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPA-R); phosphatidylcholine (PtdCho); lysophosphatidylcholine (lyso-PtdCho); inositol 4,5-bisphosphate (PtdIns(4,5)*P*₂); inositol 1,4,5-trisphosphate (Ins*P*₃); diacylglycerol (DAG); endoplasmic reticulum (ER); arachidonic acid (AA); platelet-activating factor (PAF); cytosolic phospholipase A₂ (cPLA₂); phospholipase C (PLC); cyclooxygenase-2 (COX-2); reactive oxygen species (ROS); nuclear factor *κ*B (NF-*κ*B); nuclear factor *κ*B-response element (NF-*κ*B-RE); inhibitory subunit of NF-*κ*B (I-*κ*B); I-*κ*B kinase (I-*κ*K); tumor necrosis factor-α(TNF-α); interleukin-1β (IL-1β); interleukin-6 (IL-6); inducible nitric oxide synthase (iNOS); matrix metalloproteinases (MMPs); vascular adhesion molecule-1 (VCAM-1); and secretory phospholipase A₂ (sPLA₂). Excessive stimulation of glutamate receptors contributes to inflammation and oxidative stress-mediated neural cell injury

6.2.7 Glutamate-Mediated Changes in Enzymic Activities

Intracerebellar or systemic injections of the glutamate and its analogs induce changes in activities of a number of enzymes (Fig. 6.7) including isoforms of PLA₂, protein kinases, calpains, nitric oxide synthases, cyclooxygenases, lipoxygenases, calcineurin, and endonucleases. Activation of isoforms of PLA₂, cyclooxygenase-2, and lipoxygenase contributes to inflammation through the production of eicosanoids (Table 6.2). Activation of calpains not only causes a breakdown of the cytoskeleton proteins (spectrin and MAP2) but also mediates the conversion of xanthine dehydrogenase to xanthine oxidase. This helps in production of more free radicals (Farooqui and Horrocks, 1994). Activation of isoforms of protein kinase C not only results in phosphorylation of GAP-43,



Fig. 6.6 NF-κB-mediated modulation of enzymes and genes associated with inflammation, oxidative stress, and immune function. Secretory phospholipase A₂ (sPLA₂); cyclooxygenase-2 (COX-2); superoxide dismutase (SOD); inducible nitric oxide synthase (iNOS); matrix metalloproteinases (MMPs); vascular adhesion molecule-1 (VCAM-1); cell adhesion molecule 1 (ICAM-1); and cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)



Fig. 6.7 Excitotoxicity-mediated alterations in enzymic activities

| Proteins | Effect | References | |
|---------------------------------------|-----------|--|--|
| cPLA ₂ , sPLA ₂ | Increased | Sandhya et al., 1998 | |
| Lipoprotein lipase | Increased | Paradis et al., 2004 | |
| DAG/MAG-lipase | No effect | Farooqui et al., 1993 | |
| COX-2 | Increased | Sandhya et al., 1998 | |
| NOS | Increased | Milatovic et al., 2002 | |
| Calpain II | Increased | Ong et al., 1997 | |
| Acetylcholine esterase | Decreased | Cohen et al., 1987 | |
| SOD | Increased | Block and Hong, 2005 | |
| NADPH oxidase | Increased | Qin et al., 2004 | |
| Caspase-3 | Increased | Qin et al., 2000; Pizzi et al., 2005; Faherty et al., 1999 | |
| Caspase-7 | Increased | Delgado-Rubin de Celix et al., 2006 | |
| Caspase-9 | Increased | Delgado-Rubin de Celix et al., 2006 | |
| Endonuclease | Increased | Rothstein and Kuncl, 1995 | |
| Protein disulfide isomerase | _ | Lipton, 2007; Lipton et al., 2007 | |
| E3 ubiquitin ligase | - | Lipton, 2007; Lipton et al., 2007 | |
| MMP | Increased | Block and Hong, 2005 | |
| VCAM-1 | Increased | Block and Hong, 2005 | |
| TNF- α | Increased | Block and Hong, 2005 | |
| IL-1 β | Increased | Block and Hong, 2005 | |
| IL-6 | Increased | Block and Hong, 2005 | |

 Table 6.2 Induction of inflammation and oxidative stress-related proteins in excitotoxicity

RC3, MARCKS, and MLP at early and late phases of NMDA- and KAmediated limbic motor seizures and during the early and late phases of hippocampal neuronal degeneration (Wang et al., 2005a, 2006), but phosphorylates a broad range of effectors such as cell surface receptors, ion channels, transcription factors, as well as cytoskeletal proteins. Glutamate-mediated elevation of both Ca²⁺ and DAG is necessary for sustained translocation and activation of EGFP (enhanced green fluorescent protein)-PKC γ . The translocation and activation of PKC γ is a localized process, typically observed within discrete microdomains along the dendritic branches. It contributes to long-term potentiation and synaptic plasticity (Codazzi et al., 2006; Wang et al., 2007).

Nitric oxide (NO) is formed from L-arginine by Ca²⁺/calmodulin-dependent nitric oxide synthase (NOS). In brain, glutamate and its analogs increase NO generation (Bolanos et al., 1997) (Table 6.2). At low levels, NO is an important signaling molecule but glutamate-mediated excessive NO production is associated with neurotoxicity. Nitric oxide synthase (NOS) inhibitor *N*-omeganitro-L-arginine methyl ester (NAME) or the NMDA receptor antagonist 2-amino-5-phosphonopentanoate (APV) retards the neurotoxic effects of NO (Almeida et al., 1998). Excitotoxicity-mediated neuronal death occurs through a mechanism involving NO and superoxide production and the generation of peroxynitrite, ONOO⁻. ONOO⁻ not only interacts with sulfhydryl groups of enzymes, but also *S*-nitrosylates (transfer of NO to a critical thiol group) a number of proteins. Recently, S-nitrosylation-mediated post-translational protein misfolding has also been implicated in excitotoxicity (Lipton, 2007; Lipton et al., 2007). Protein disulfide isomerase (PDI), the enzyme responsible for normal protein folding, is associated with endoplasmic reticulum (ER). S-nitrosylation of PDI during excitotoxicity compromises the function of this enzyme, leading to misfolded proteins and contributing to neuronal cell injury and loss. Another enzyme whose S-nitrosylation leads to abnormal protein misfolding is the E3 ubiquitin ligase (Lipton, 2007; Lipton et al., 2007). In addition, ONOO⁻ reduces mitochondrial respiration, inhibits membrane pumps, depletes cellular glutathione, and damages DNA, thus activating poly (ADP-ribose) synthase, an enzyme that leads to cellular energy depletion (Pryor and Squadrito, 1995; Radi et al., 1991; Qi et al., 2000). All these processes are associated with neuronal energy deficiency and neurotoxicity caused by glutamate and its analogs.

6.2.8 Glutamate-Mediated Expression of Cytokines

Injections of kainate mediate the expression of cytokines such as interleukin-1 β (IL-1 β), interleukin-6, tumor necrosis factor (TNF)- α , and leukemia inhibitory factor in different regions of rat brain (Minami et al., 1991; Yabuuchi et al., 1993). In brain tissue, cytokines mediate cellular intercommunication through autocrine, paracrine, or endocrine mechanisms (Wilson et al., 2002). Their actions involve a complex network linked to feedback loops and cascades. Like glutamate, cytokines produce their effects by binding to specific membrane-associated receptors that are composed of an extracellular ligand-binding region, a membrane-spanning region, and an intracellular region that is activated by binding of cytokines, and hence delivering a signal to the nucleus (Rothwell and Relton, 1993). Their overall response depends on the synergistic or antagonistic actions of various components. Thus, cytokines play an important role in neuronal development, maturation, survival, regeneration, and recovery following neural insult (Yabuuchi et al., 1993).

The molecular mechanism associated with cytokine mRNA expression in neurons is not fully understood. However, it is becoming increasingly evident that cytokines play an important role in neuroinflammation in acute neural trauma (ischemia and traumatic brain and spinal cord injuries) and neurodegenerative diseases (Farooqui et al., 2007). In brain, inflammation is promoted by eicosanoids, which are generated through PLA₂/cyclooxygenase cascade reactions. Several mechanisms of cPLA₂ stimulation by cytokines are possible. One molecular mechanism of cPLA₂ stimulation by TNF- α and IL-1 β involves the phosphorylation of cPLA₂ by mitogen-activated protein kinase in the presence of agents that mobilize intracellular Ca²⁺ (Clark et al., 1995). Another mechanism involves TNF- α -mediated activation of caspase-3 and the proteolytic cleavage of cPLA₂ by caspase-3 (Wissing et al., 1997; Beer et al., 2000). Acetyl-Asp-Glu-ValAsp-aldehyde, a tetrapeptide inhibitor of caspase-3, prevents the proteolytic cleavage and activation of cPLA₂, indicating that caspase-3-mediated cPLA₂ proteolysis retards cell death. Arachidonoyl trifluoromethyl ketone, a potent inhibitor of cPLA₂ activity, also blocks neural cell death (Wissing et al., 1997). Thus, the stimulation of cPLA₂ and caspase-3 along with induction of cyclooxygenase results in the oxidative stress, mitochondrial dysfunction, and calcium ion overload along with the release of cytochrome c and the activation of downstream caspase-9 and -3 resulting in cell death. Glutamate-mediated activation of microglia also involves the expression of cytokines (Minami et al., 1991; Farooqui et al., 2004; Phillis et al., 2006). These cytokines may mediate cellular intercommunication through autocrine, paracrine, or endocrine mechanisms (Wilson et al., 2002).

6.2.9 Glutamate-Mediated Changes in Growth Factors

In rat brain, excitotoxic insult produces a marked increase in the expression of mRNA for many growth factors including nerve growth factor (NGF), brainderived growth factor (BDNF), insulin-like growth factor (IGF)-I, and pigment epithelium-derived factor (PEDF), while neurotrophin-3 mRNA is decreased (Sperk, 1994; McCusker et al., 2006). Induction of growth factors in neurons may be region and subcellular structure specific. Thus, kainate injections produce a rapid increase in BDNF mRNA in hippocampus and other cortical regions, but the increase in GDNF mRNA is slow and mainly restricted to the hippocampal formation. Kainate injections in rats also upregulate the immunoreactivity of basic fibroblast growth factor like in nuclei of astrocytes in several forebrain areas. KA injections into the right cellebellum produce severe loss of calbindin-positive Purkinje neurons and an increased number of GFAPpositive astrocytes in the ipsilateral side of the lesioned cerebellum. Although the PEDF levels on the ipsilateral side of the cerebellum are dramatically decreased 2 days after KA treatment, significant elevation of PEDF levels is observed at 7 days. The molecular mechanism associated with PEDF effect remains unclear. However, the increase in PEDF expression in injured brain suggests the occurrence of a compensation mechanism against excitotoxicity (Sanagi et al., 2007). Collective evidence suggests that following excitotoxic insult, glutamate-mediated alterations in growth factor mRNA expression is associated with the maintenance of neuronal circuitry, modulate synaptic efficacy, and consolidate information in developing and adult brain (Zafra et al., 1991). Growth factors also protect hippocampal neurons from death induced by serum deprivation. Possible mechanisms of neuronal protection include activation of phosphatidylinositol 3-kinase (PtdIns 3-K)/Akt (protein kinase B) kinase, the mitogen-activated protein kinase (MAPK) pathways by IGF-1 and BDNF (Zheng and Quirion, 2004), and enhancement of mitogen-activated kinase (MEK)/extracellular signal regulated kinase (ERK) pathways. The phosphorylation of Akt and its downstream target, FKHRL1, a winged-helix family transcription factor induced by IGF-1, is rapid and sustained, whereas that of MAPK is transient. Furthermore, IGF-1 also mediates the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and its association with PtdIns 3-kinase.

6.2.10 Glutamate-Mediated Changes in Heat Shock Protein Expression

Neural cells respond to glutamate toxicity by inducing the expression of heat shock proteins and their mRNA (HSPs). A number of heat shock proteins (HSP27, HSP32, and HSP47) are known to occur in brain tissue (Acarin et al., 2002; Hashimoto et al., 1998; Akbar et al., 2001; Sato and Matsuki, 2002). In post-excitotoxic damaged brain, the expression of HSP32, HSP27, and HSP47 in glial cells provides neuroprotection through different mechanisms. Thus, HSP32 may promote antioxidant protective mechanisms to microglia/macrophages, whereas HSP47 is associated with extracellular matrix remodeling, and HSP27 may stabilize the astroglial cytoskeleton and participate in astroglial antioxidant mechanisms (Acarin et al., 2002). Furthermore, in brain, astrocytes and microglia in various brain regions respond differently to excitotoxicity. Thus, intracortical injections of NMDA in 9-day-old rat brain produce primary damage in cortical region and secondary damage in the corresponding thalamus. The expression of HSP32 in reactive microglia/macrophages starts at 10 h and reaches maximal value at 3-5 days. In contrast, reactive astrocytes in the cortex express HSP47 from 10 h until 14 days post-lesion. Some astrocytes also induce the expression of HSP27 from 1 day post-lesion. In addition, some cortical reactive astrocytes display a temporary expression of HSP32 at day 1. In general, the expression of HSP in astrocytes in the cortex reaches maximal levels at days 3-5 post-lesion. In the damaged thalamus, HSP32 is not significantly induced, but reactive astrocytes express HSP47 and some of them also express HSP27. HSP induction in thalamic astroglia is transient, peaks at 5 days post-lesion and returns to basal levels by day 14 (Acarin et al., 2002).

Like NMDA, KA injections induce the expression of HSP. Thus, a moderate expression of HSP-70 mRNA occurs in pyramidal cell layers of CA1-3, dentate gyrus of the hippocampus, amygdala, piriform cortex, and the central medial thalamic nucleus of rat brain (Hashimoto et al., 1998; Akbar et al., 2001; Sato and Matsuki, 2002). It is proposed that the behavioral changes, seizure intensity and body temperature, may be associated with early expression of various HSP mRNAs by glial cells and that HSP protects CA1 and CA3 hippocampal neurons from excitotoxic insult (Hashimoto et al., 1998; Akbar et al., 2001; Sato and Matsuki, 2002).

An increased expression of the inducible form of heme oxygenase-1 (HO-1) also occurs in hippocampal neurons and astrocytes after kainate injection

(Huang et al., 2005). No changes are observed in heme oxygenase-2. HO-1 immunoreactivity first appears in neurons after KA injection and then astrocytes show marked induction at later time interval. Maximal induction of HO-1 is observed 1 day after KA treatment and then there is a gradual decrease in HO-1 levels. The neuroprotective effect of HO-1 is determined by injecting tin protoporphyrin (SnPP), a blood-brain barrier permeable inhibitor of HO. SnPP treatment does not improve neuronal survival. Instead, this treatment increases the mortality. The surviving tin protoporphyrin-treated rats show significantly less Nissl or MAP2 staining in hippocampal field compared to the saline-injected rats, suggesting that induction of HO-1 has neuroprotective effect. These results indicate that HO-1 induction has a net protective effect following excitotoxic insult (Huang et al., 2005).

6.2.11 Glutamate-Mediated Upregulation of Genes

Injections of glutamate and its analog in rats upregulate immediate-early genes (Table 6.3) through the expression of transcription factor AP1, which contains jun and fos proteins or jun dimers (Ogita and Yoneda, 1994; Yoneda et al., 1999). Gene transcription through AP1 involves participation of CREB (Yoneda et al., 1999). Other genes that are upregulated include genes for Metenkephalin and other neuropeptides (Gall, 1988) and neuropeptide Y (Vezzani et al., 1994). The molecular mechanism and significance of upregulation of these genes are not understood. However, changes in intracellular Ca²⁺ play an important role in modulating these genes promote synaptic reorganization, recapitulation of hippocampal development, and synaptogenesis. KA injections also upregulate p53, a tumor suppressor gene that encodes a nuclear phosphoprotein, which is associated with the regulation of cell proliferation and DNA damage repair (Sakhi et al., 1994). It is interesting to note that mice deficient in

| Tuble ole Contes usseenated with energy contest injury | | | |
|--|---|--|--|
| Gene | References | | |
| c-Fos | Fernandez et al., 2005 | | |
| c-Jun | Fernandez et al., 2005 | | |
| FosB | Fernandez et al., 2005 | | |
| Jun D | Fernandez et al., 2005 | | |
| Bcl-2 | Schelman et al., 2004; Lopez et al., 1999 | | |
| Bcl-x | Lopez et al., 1999 | | |
| Bax | Lopez et al., 1999 | | |
| c-Myc | Nakai et al., 2000 | | |
| p53 | Nakai et al., 2000; Sakhi et al., 1994 | | |
| Heat shock proteins | Acarin et al., 2002; Hashimoto et al., 1998 | | |
| Cyclin D1 | Liang et al., 2007; Cruise et al., 2000 | | |

| Table 6.3 | Genes associated | with excitor | toxic cell injury |
|-----------|------------------|--------------|-------------------|
|-----------|------------------|--------------|-------------------|

p53 are protected from excitotoxic cell death (Morrison et al., 1996). Involvement of p53 in excitotoxicity suggests that cell cycle components may also contribute to cell death.

6.2.12 Glutamate and Apoptotic Neural Cell Death

Excitotoxicity mediates apoptotic cell death in neural cultures (Oin et al., 1998; Nakai et al., 2000a,b). Thus, treatment of neuronal cultures with glutamate agonists (NMDA and KA) induces the release of cytochrome c, DNA fragmentation, and translocation of NF- κ B from cytosol to the nucleus in striatal neuronal cultures (Nakai et al., 2000a,b). Similarly, intrastriatally infused KA produces substantial neuronal loss as indicated by the caspase-3 activation, fragmentation of internucleosomal DNA, and the presence of terminal transferase-mediated dUTP digoxigenin nick end labeling-positive nuclei (Qin et al., 1998). These processes are blocked by the AMPA/KA receptor antagonist NBQX (1,2,3,4-tetrahydro-6-nitro-2,3-dibenzo[f]quinoxaline-7-sulfonamide) but not by the NMDA receptor antagonist MK-801 (Nakai et al., 2000a,b). In addition, there is a 5- to 7-fold increase in c-Myc and p53 after KA administration (Table 6.3) (Nakai et al., 2000a,b). Immunolocalization studies indicate high levels of c-Myc and p53 immunoreactivity in medium-sized striatal neurons. Pretreatment with the cell-permeable recombinant peptide NF- κB SN50 retards NF- κ B nuclear translocation but has no effect on AP-1 binding. NF- κ B SN50 also blocks the KA-induced upregulation of c-Myc and p53 as well as internucleosomal DNA fragmentation, suggesting that apoptotic cell death in striatal neuronal cultures involves excitotoxicity through NMDA receptor (Nakai et al., 2000a,b). Intrastriatally injected N-methyl-D-aspartate receptor agonist quinolinic acid (OA) causes an increase in cyclin D1 mRNA and protein levels (Liang et al., 2007). QA not only facilitates translocation of NF- κ B to the nucleus, and increases cyclin D1 immunoreactivity, but also promotes DNA replication as evidenced by BrdU incorporation. This suggests that NF- κ B activation stimulates cyclin D1 expression and triggers DNA replication in striatal neurons, suggesting the involvement of cell cycle components in glutamate and its agonist-mediated neuronal apoptosis (Liang et al., 2007).

6.3 Mechanism of Glutamate-Mediated Neural Cell Injury in Neurological Disorders

At the molecular level, neurotoxic concentrations of glutamate and its analogs interact with glutamate receptors and mediate Ca^{2+} influx through NMDA receptor Ca^{2+} channel. Sustained Ca^{2+} levels have many harmful consequences for neural cell survival: (a) It enhances loss of neural membrane

glycerophospholipid metabolism through the activation of isoforms of PLA₂, cvclooxygenases (COX), and lipoxygenases (LOX). Stimulation of these enzymes releases arachidonic acid and lyso-glycerophospholipids from neural membrane glycerophospholipids. Lyso-glycerophospholipid is either reacylated to the native glycerophospholipid or acetylated to pro-inflammatory platelet-activating factor (PAF). Arachidonic acid is metabolized to eicosanoids. The non-enzymic oxidation of arachidonic acid generates reactive oxygen species (ROS) (Phillis et al., 2006); (b) Sustained Ca^{2+} levels induce the generation of ROS. ROS formation is also facilitated by NADPH oxidase. ROS stimulates NF- κ B activity. NF- κ B is present in the cytoplasm in an inhibitory form attached to its inhibitory protein $I-\kappa B$. This protein contains ankyrin repeat motif, which is important for the maintenance of NF- κ B in the cytoplasm. NF- κ B activity is tightly controlled by the I- κ B kinase complex, consisting of I- κ B kinases I- κ K α , I- κ K β , and I- κ K γ . I- κ K β is essential for glutamate-mediated activation of NF- κ B (Yamamoto and Gaynor, 2004) (Fig. 6.5). Upon glutamate-mediated stimulation, $I-\kappa B$ is rapidly phosphorylated, ubiquinated, and then degraded by proteasomes resulting in the release and translocation of active NF- κ B to the nucleus where it mediates the transcription of more than 150 genes that not only influence the survival of neural cells but also maintain their normal functional integrity. NF- κ B also induces many genes implicated in inflammation, oxidative stress, and immune responses (Figs. 6.5 and 6.6). These genes code for enzymes, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, cytokines, and matrix metalloproteinases (MMP) (Table 6.3). Activation of NF- κ B also leads to the local generation of more cytokines and chemokines, which in turn promulgate glutamate-mediated signals and potentiates the activation of NF- κ B activity (Block and Hong, 2005).

The molecular mechanism associated with glutamate toxicity depends on the type of neural cells involved, neuron versus glia. The factors that initiate, propagate, and maintain glutamate toxicity include multiple forms of PLA₂, cyclooxygenases (COX), and lipoxygenases (LOX), inducing the release of arachidonic from neural membrane phospholipids and generating lyso-glycer-ophospholipids, platelet-activating factor (PAF), pro-inflammatory prostaglandins, and reactive oxygen species (ROS) (Phillis et al., 2006). In addition excitotoxic insult also stimulates proteases, protein kinases, protein phosphatases, inducible nitric oxide synthases, and endonucleases (Fig. 6.7). In glutamate-mediated toxicity, the stimulation of nitric oxide synthase by Ca²⁺ generates nitric oxide, which reacts with superoxide to form peroxynitrite. Peroxynitrite produces single-stranded breaks in DNA, which activate poly (adenosine diphosphate ribose) polymerase leading to NAD and ATP depletion. This may be another mechanism that may contribute to glutamate-mediated neural cell death.

Eicosanoids and platelet-activating factor interact and modulate glutamate receptors in the hippocampus (Chabot et al., 1998; Phillis et al., 2006) through cross talk among glutamate, eicosanoids, platelet-activating factors, and

thromboxane receptors. Under normal conditions, this cross talk refines their communication among neurons, macroglial cells, microglial cells, and vascular cells. Under pathological situations, this cross talk initiates and promotes neuronal injury depending on the magnitude of PLA₂, COX, and LOX expression; production of arachidonic acid metabolites; synthesis of platelet-activating factor; and generation of ROS. It is proposed that increased activities of PLA₂ isoforms, COX-2/LOX enzymes, and high levels of their reaction products initiate, maintain, and extend excitotoxicity and oxidative stress during pathological conditions (Farooqui and Horrocks, 2007).

6.4 Involvement of Excitotoxicity in Neurological Disorders

As stated earlier, excitotoxic insult promotes neurodegeneration in acute neural trauma (ischemia, spinal cord trauma, and head injury) and neurodegenerative diseases (Alzheimer disease, amyotrophic lateral sclerosis, and Huntington disease) (Fig. 6.8). Following excitotoxic insult, the enhanced glutamate efflux and its impaired cellular reuptake result in a rapid accumulation of glutamate in the extracellular space (Matute et al., 2006). This prolonged and excessive accumulation of glutamate causes overstimulation of glutamate receptors, and a sustained increase in intracellular calcium occurs not only through



Fig. 6.8 Association of excitotoxicity with neurological disorders

NMDA receptor channels but also through glutamate transporters operating in the reverse mode. These processes are sufficient to trigger downstream pathophysiological mechanisms associated with neural cell death (Farooqui et al., 2008; Farooqui and Horrocks, 2008).

6.4.1 Glutamate in Ischemic Injury

Ischemic insult is accompanied by a marked accumulation of glutamate (Phillis and O'Regan, 1996). In vitro a brief exposure of neurons to glutamate and its analogs causes widespread neuronal death that can be attenuated by the removal of extracellular Ca^{2+} (Choi, 1988). This finding suggests that Ca^{2+} entry via the NMDA receptor channel is responsible for neuronal cell death. Glutamate-mediated ischemic injury causes immediate as well as delayed neuronal cell death in hippocampal region. The immediate neuronal death occurs in the core of infarct while delayed cell death occurs in the surrounding penumbra. Neurons continue to die in stroke patients up to 10 days after infarction (Saunders et al., 1995). Neurons within the core undergo necrosis, whereas neurons in the penumbra may undergo either necrotic or apoptotic cell death (Hill et al., 1995). The extent of ischemic injury varies according to the age of animals. Thus, 10- and 21-day-old rats develop greater damage from ischemic insult than 6-week, 9-week, and 6-month-old rats (Yager et al., 1996; Yager and Thornhill, 1997). Younger rats may be more susceptible to ischemic insult because of unbalanced maturation of excitatory versus inhibitory neurotransmitter systems (Hattori and Wasterlain, 1990).

Physicochemical and neurochemical consequences of glutamate-mediated injury include alterations in membrane fluidity and permeability, alterations in ion homeostasis, and changes in activities of membrane-bound enzymes, receptors, and ion channels, increased oxidative stress, and neuroinflammation. Collective evidence suggests that increased glycerophospholipid and protein degradation through the activation of PLA₂ isoforms, generation of nitric oxide through the activation of nitric oxide synthase (NOS), and elevation in calpain activity lead to changes in membrane permeability and stimulation of many enzymes associated with lipolysis, proteolysis (calpains), and disaggregation of microtubules with a disruption of cytoskeleton and membrane structure and sets the stage for increased production of reactive oxygen and nitrogen species (ROS and NOS) through the arachidonic acid cascade and generation of peroxynitrite (Farooqui and Horrocks, 1991; Farooqui et al., 1997a,c; Ray et al., 2003; Farooqui et al., 2006; Crocker et al., 1991; Samdani et al., 1997). At low levels, under normal conditions ROS and NOS function as signaling intermediates in the regulation of fundamental cell activities such as growth and adaptation responses. At higher concentrations, ROS and NOS contribute to neural membrane damage when the balance between reducing and oxidizing (redox) forces shifts toward oxidative stress (Juranek and Bezek, 2005; Farooqui and Horrocks, 1994). ROS also upregulate the gene expression of cytokines through transcription factors in the nucleus (Gabriel et al., 1999; Schneider et al., 1999). This process leads to the intensification of glutamate-mediated neural injury in ischemia.

6.4.2 Glutamate in Spinal Cord Injury

The traumatic injury to spinal cord tissue causes a rapid release of glutamate from intracellular stores (Demediuk et al., 1988; Panter et al., 1990; Sundström and Mo, 2002; Klussmann and Martin-Villalba, 2005). Like ischemic injury, the released glutamate interacts with its receptors and mediates not only the calcium influx and stimulation of PLA₂, nitric oxide synthases, and calpains, but also the overexpression of cytokines (Hayes et al., 2002; Ahn et al., 2004) and uncoupling of mitochondrial electron transport (Bazan et al., 1995; Ellis et al., 2004; Arundine and Tymianski, 2004; Xu et al., 2006; Farooqui and Horrocks, 2009). Stimulation of these enzymes, along with a rapid decrease in ATP levels, changes in ion homeostasis, alterations in cellular redox, and induction of inflammation, results in neural cell death in spinal cord trauma. Glutamate also causes delayed post-traumatic white matter degeneration (Park et al., 2004). This process is associated with glutamate release by the reversal of Na⁺-dependent glutamate transport with subsequent activation of AMPA receptors and oligodendrocyte death (Li and Stys, 2000). Collectively, these studies suggest that the general response of spinal cord tissue to traumatic injury is blood-brain barrier disruption, infiltration and activation of inflammatory cells, death of local neurons and glial cells caused by ischemia and hypoxia along with release of excitatory amino acids, gliosis, recruitment of endothelial cells, angiogenesis, compensatory responses in the surroundings of the most severely affected area, and formation of a glial scar (Bazan et al., 1995; Ellis et al., 2004; Arundine and Tymianski, 2004; Xu et al., 2006).

6.4.3 Glutamate in Head Injury

Like spinal cord trauma, traumatic head injury is accompanied by breakdown of the blood-brain barrier, glutamate release, stimulation of PLA₂, nitric oxide synthases and calpains, overexpression of cytokines, increased levels of arachidonic acid and eicosanoids as well as leukotrienes, and glial cell reactions involving both activated microglia and astroglia and demyelination involving oligodendroglia (Dhillon et al., 1994; Homayoun et al., 1997; McIntosh et al., 1998; Hoffman et al., 2000). These processes mediate gliosis, decrease in ATP levels, altered ion homeostasis, inflammation and oxidative stress in head injury (Pan et al., 1999).

At the molecular level, head injury-mediated inflammation and oxidative stress is accompanied by the activation of NF- κ B and AP-1, synthesis of TNF- α and IL-1 β , and chemokines, induction of nitric oxide synthase (NOS) and cyclooxygenase-2 (COX-2), and the coordinated infiltration of leukocytes (McIntosh et al., 1998). Generation of NO and its reaction with superoxide anion produces peroxynitrite. This results in a rapid increase in cortical mitochondrial 3-nitrotyrosine levels as early as 30 min following head injury (Singh et al., 2006). Furthermore, peroxynitrite not only induces the activation of proto-oncogenes (Kamat, 2006), but also decomposes rapidly to toxic hydroxyl radicals. These processes are closely associated with neural cell death in traumatic head injury.

6.4.4 Glutamate in Epilepsy

Epileptic seizures in rat and human brain produce marked increase in glutamate levels in the focal, compared to nonfocal, regions of the cerebral cortex (Bazan et al., 2002). The mechanism by which persistent seizure activity results in neuro-degeneration and brain damage is not fully understood (Spencer, 2007). However, increase in glutamate levels during seizure activity is known to increase Ca²⁺ influx and release cytokines such as TNF- α and interleukin-6. These processes may result in stimulation of isoforms of PLA₂ and NOS (Visioli et al., 1994; De Bock et al., 1996; Kajiwara et al., 1996; Penkowa et al., 2001). Based on these studies, it has been proposed that release of glutamate following epileptic seizures may be associated with neuronal cell death in epilepsy (Bazan et al., 2002).

6.4.5 Glutamate in Alzheimer Disease

Alzheimer disease (AD) is characterized by the accumulation of amyloid neuritic plaques, neurofibrillary tangles, inflammation, oxidative stress, loss of synapses, impairment of memory, and severe dementia. The molecular mechanism associated with the pathogenesis of AD is not fully understood. However, interactions among glutamate-mediated excitotoxicity, cytokine-mediated inflammation, and amyloid β peptide-mediated oxidative stress along with alterations in energy and redox status of degenerating neurons may contribute to the pathogenesis of AD (Farooqui and Horrocks, 2007).

Decrease in the number of NMDA and AMPA receptors and alterations in glutamate transporter activity have been observed in several brain regions from AD patients compared to brain tissue from age-matched control subjects (Greenamyre and Young, 1989; Penney et al., 1990; Dewar et al., 1990; Masliah et al., 1996). Detailed investigations on the involvement of NMDA receptor have indicated a marked reduction in the expression of NR2A and NR2B subunit mRNA in the hippocampus and entorhinal cortex in brain from AD

patients (Bi and Sze, 2002). This may induce changes in glutamate homeostasis in AD causing a major disturbance in Ca^{2+} homeostasis (Mattson, 2002). Abnormal Ca^{2+} homeostasis modulates alterations in the activities of protein kinases C (Shimohama et al., 1990) and phospholipases A₂ (Farooqui et al., 2003a; Farooqui et al., 2003b). Collective evidence suggests that anomalous glutamatergic activity and changes in Ca^{2+} homeostasis are associated with alterations in postsynaptic receptor function and downstream defects that lead not only to neuronal injury and death but also to cognitive deficits associated with dementia (Wenk et al., 2006).

The relationship between abnormal glutamate levels and the neuropathological changes in AD is not fully understood. Neurofibrillary tangles, the hallmark of AD, are composed of paired helical filaments (Terry and Katzman, 1983). Treatment of cultured human neurons with glutamate causes the formation of paired helical filaments (Mattson, 1990). Furthermore, the incubation of hippocampal neurons with glutamate in the presence of Ca^{2+} not only induces their degeneration but also increases τ and ubiquitin immunostaining (Mattson, 1990). It is proposed that glutamate-mediated Ca^{2+} influx may lead to modifications of cytoskeleton-associated proteins similar to those seen in the neurofibrillary tangles of patients with AD. The formation of neurofibrillary tangles may also be promoted by calpains and protein kinases (Mattson, 1990; Haddad, 2004). The β -amyloid protein increases the vulnerability of cortical cultures to glutamate and its analogs (Koh et al., 1990), supporting the view that excitotoxicity may play an important role in the pathogenesis of AD. The mechanism underlying the compromised function of glutamatergic neurons in AD has not vet been elucidated. However, abnormalities in glycerophospholipid metabolism are known to induce inflammation and β -amyloid deposition, which may impair glutamatergic receptor function in such a way that the ability of these receptors to prevent the influx of Ca^{2+} in the absence of an appropriate presynaptic signal is compromised. If this hypothesis is correct, then glutamate-mediated neuroinflammation, abnormalities in glycerophospholipid metabolism, oxidative stress, and β -amyloid deposition will start long before the symptomatic onset of AD (Mattson, 2002; Zafrilla et al., 2006; Farooqui and Horrocks, 2007).

6.4.6 Glutamate in Amyotrophic Lateral Sclerosis (ALS)

ALS is a neurological disease characterized by the loss of anterior horn motor neurons as well as degeneration of the corticospinal tracts (Shaw and Ince, 1997). The molecular mechanism associated with ALS remains elusive, but excitotoxicity and oxidative stress may contribute to the pathogenesis of AD (Rao and Weiss, 2004). Underediting of RNA in AMPA receptor subunit GluR2 at the Q/R site in motor neurons is known to enhance the Ca²⁺ permeability of AMPA receptors (Hume et al., 1991; Burnashev et al., 1992).

This may cause neuronal cell demise in ALS (Kawahara et al., 2004; Kwak and Kawahara, 2005; Kawahara et al., 2006). This view is supported by studies on mice with RNA editing abnormalities at the GluR2 Q/R site. These mice die young (Kwak and Kawahara, 2005), and mice transgenic with abnormal Ca²⁺ permeability develop motor neuron disease 12 months after birth (Kuner et al., 2005; Kawahara et al., 2006).

Association of oxidative stress with pathophysiology of ALS involves mitochondrial dysfunction (Bacman et al., 2006). In a mouse model of ALS, glutamate induces mitochondrial dysfunction that causes mitochondrial swelling, release of cytochrome c, and apoptotic cell death in ALS. An increase in p38 mitogen-activated protein kinase (p38MAPK) is reported in motor neurons of spinal cord from familial ALS transgenic mice (SOD1G93A), but no changes are observed in its mRNA levels (Tortarolo et al., 2003). Progression of ALS results in activation and accumulation of p38MARK in hypertrophic astrocytes and reactive microglia. These studies suggest that the activation of p38MARK in motor neurons and then in reactive glial cells may phosphorylate cytoskeletal proteins, activate cytokine release, and upregulate nitric oxide synthase. All these processes may promote motor neuron pathology in SOD1G93A mice (Tortarolo et al., 2003).

ROS-mediated inhibition of high affinity glutamate uptake in cell culture is known to increase the synaptic glutamate concentration (Volterra et al., 1994). A sustained exposure of neurons to glutamate induces excitotoxicity and inhibits cystine uptake via the glutamate/cystine anti-porter (Murphy et al., 1989; Ye et al., 1999) inducing oxidative stress. The sporadic form of ALS is characterized by a prominent neuroinflammatory component and upregulation of COX-2 mRNA (Yasojima et al., 2001), and mutations in the Cu/Zn superoxide dismutase (SOD1) gene have been reported to occur in the familial form of ALS (Beal, 1998). Upregulation of COX-2 mRNA also occurs in SOD1 transgenic mice at the onset of ALS (Almer et al., 2001). Treatment of organotypic cell culture in an ALS model with SC236, a COX-2 inhibitor, retards the degeneration of motor neurons (Drachman and Rothstein, 2000), supporting the view that COX-2 may play an important role in inflammatory processes in ALS. Collectively, these studies suggest that interactions and interplay among excitotoxicity, oxidative stress, and inflammation may be a major contributing factor in the pathogenesis of ALS.

6.4.7 Glutamate in Huntington Disease

Huntington disease (HD) is caused by an autosomal dominant mutation in a gene located on chromosome 4 (Cepeda et al., 2001). This gene encodes a novel protein called huntingtin. The function of huntingtin remains unknown. Since huntingtin is associated with synaptic vesicles and/or microtubules, it is proposed that it plays an important role in vesicular transport and/or the binding to

the cytoskeleton (Bonilla, 2000). Association of glutamate toxicity in HD has been studied in HD mutants. Thus, in YAC transgenic mice model of HD (YAC46 or YAC72) NMDA receptor-mediated calcium influx and mitochondrial membrane depolarization are significantly increased compared to wildtype mice MSN (Zeron et al., 2004; Cepeda et al., 2001; Li et al., 2004). Inhibitors of the mitochondrial permeability transition (mPT), cyclosporin A and bongkrekic acid, and coenzyme Q10, an antioxidant involved in bioenergetic metabolism, block NMDA receptor-mediated cell death. In YAC46 MSN, NMDA stimulates caspase-3 and caspase-9 but not caspase-8. Cyclosporin can block activation of caspase-3 and -9 mediated through a NMDA receptor, suggesting a link among NMDA receptors, mitochondrial dysfunction, and apoptotic cell death.

6.4.8 Glutamate in AIDS Dementia Complex

Levels of glutamate are elevated in blood and cerebrospinal fluid from subjects with AIDS dementia complex (Famularo et al., 1999; Ferrarese et al., 2001). Chronic overactivation of glutamate receptors in brains of patients infected with HIV-1 may contribute to the pathogenesis of the HIV-1-associated dementia complex. HIV-1 enters the brain very soon after peripheral infection and can induce severe and debilitating neurological problems that include behavioral abnormalities, motor dysfunction, and frank dementia (Kaul and Lipton, 2006). Neural injury in HIV infection may involve microglial activation, glutamate toxicity, oxidative stress, and apoptosis (Gras et al., 2003). The microglial cells interact with viral proteins, tat and gp120, and facilitate not only viral replication, but also the expression of pro-inflammatory factors. gp120 like glutamate increases the intracellular Ca^{2+} concentration and produces neurotoxicity in neuronal cultures at picomolar concentration (Sucher et al., 1991). It is proposed that gp120 interacts with the chemokine receptors and facilitates the arachidonic acid release through PLA₂ activation. Arachidonic acid interferes with the high-affinity uptake of glutamate in astrocytes resulting in an elevation of the extracellular glutamate concentration that overstimulates glutamate receptors, increases Ca²⁺ influx, and causes excitotoxic brain damage in patients with AIDS dementia complex (Dreyer and Lipton, 1995). In addition, glutamate inhibits cystine uptake and produces glutathione depletion-mediated oxidative stress. These processes activate numerous downstream signaling pathways and disturb neuronal and glial functions (Gras et al., 2003; Porcheray et al., 2006). These include generation of nitric oxide, quinolinic acid, and platelet-activating factor (Lipton, 1998). It still remains unknown whether the glutamatemediated neurochemical changes in AIDS dementia complex are the primary event or a secondary effect of an abnormal signal transduction process related to inflammation and oxidative stress in degenerating neurons.
6.4.9 Glutamate in Creutzfeldt–Jakob Disease (CJD)

CJD is the most prevalent form of prion disease in humans. CJD is characterized by neuronal loss, astrocytic gliosis, and accumulation of an abnormal extracellular β -helix-rich prion protein (PrP^{sc}). This abnormal protein is an isoform of a normally occurring α -helix-rich prion protein (PrP^c) (Prusiner, 2001; Brown, 1999). The function of PrP^c is not known. However, it is proposed that PrP^{c} functions as a copper chaperone, regulates Cu^{2+}/Zn^{2+} superoxide dismutase, alters cellular redox, and induces oxidative stress. Thus, the oxidative stress may be closely associated with the pathogenesis of CJD (Capellari et al., 1999; Brown, 2005). There are many similarities between brain pathological changes caused by glutamate toxicity and by prion infection. Like glutamate-mediated neurotoxicity, prion infection leads to oxidative stress (Brown, 2005). Furthermore, at the end stages prion infection-mediated neuropathological changes have many of the characteristics of glutamate-mediated neuronal necrosis. The NMDA receptorchannel antagonists such as memantine (1-amino-3,5-dimethyladamantane; MEM), MD-ADA (1-N-methylamino-3,5-dimethyladamantane), or dizocilpine (MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine malate) have cytoprotective effects in prion diseases (Scallet et al., 2003).

Upregulation of arachidonic acid metabolism also occurs in a cell culture model of prion diseases. Thus, the treatment of primary cerebellar granule neuronal cultures with prion peptide (PrP106-126) stimulates PLA₂ (Bate and Williams, 2004) and release of arachidonic acid (Stewart et al., 2001). COX-1, COX-2, and 5-LOX activities are also increased in brain and CSF samples from CJD patients compared to age-matched controls, implicating both enzymes in the pathogenesis of CJD (Deininger et al., 2003; Minghetti et al., 2000).

The antimalarial drug quinacrine and some phenothiazine derivatives, acepromazine, chlorpromazine, and promazine, and the 5-LOX inhibitors, nordihydroguaiaretic acid and caffeic acid, can block PrP106-126-mediated neurodegeneration (Stewart et al., 2001). Some of these drugs have been used for the treatment of prion diseases (Doh-ura et al., 2000; Korth et al., 2001; May et al., 2003). The molecular mechanism associated with the inhibition of PrP^{sc} formation by quinacrine remains unknown. However, it is proposed that quinacrine binds with human prion protein at the Tyr-225, Tyr-226, and Gln-227 residues of helix α 3 (Vogtherr et al., 2003) and provides neuroprotection. Quinacrine may also act as an antioxidant and reduce the toxicity of prP^{sc} (Turnbull et al., 2003). It still remains unknown whether PLA₂/COX/LOX-mediated enhancement of arachidonic acid metabolism in CJD is the primary event or a secondary effect of an abnormal signal transduction process related to inflammation and oxidative stress in degenerating neurons in CJD.

6.4.10 Glutamate in Multiple Sclerosis (MS)

MS is the most prevalent inflammation-mediated demyelinating disease. In MS, the immune system attacks the white matter of the brain and spinal cord, leading to disability and/or paralysis. Myelin, oligodendrocytes, and neurons are lost due to the release by immune cells of cytotoxic cytokines, autoantibodies, and toxic amounts of the excitatory neurotransmitter, glutamate. Oligodendroglial cells are highly vulnerable to glutamate-mediated neurotoxicity at various stages, preoligodendroglial cells and mature oligodendroglial cells, of their development in cell culture (McDonald et al., 1998). Glutamate treatment increases [Ca²⁺]i, and this effect can be totally abolished by the non-NMDA receptor antagonist CNQX or by removal of Ca^{2+} from the culture medium (Alberdi et al., 2002). NBQX treatment can prevent oligodendroglial cell death, suggesting that oligodendrocytic cell death is a glutamate receptor-mediated process. Treatment of mice with NBQX sensitizes them to experimental autoimmune encephalomyelitis (EAE), a demyelinating model that mimics many of the characteristics of MS. NBOX treatment causes substantial amelioration of the disease, promotes oligodendroglial cell survival, and downregulates dephosphorylation of neurofilament H, an indicator of axonal damage (Pitt et al., 2000).

Complement stimulates glutamate-mediated toxicity in oligodendroglial cell cultures and in freshly isolated optic nerves (Alberdi et al., 2006). This process is mediated by KA receptors and is blocked by removing Ca^{2+} from the medium during glutamate priming. Dose-response experiments indicate that sensitization to complement attack is mediated by two distinct KA receptor populations. One has a high and the other a low affinity for glutamate (Alberdi et al., 2006). Oligodendrocyte death by complement requires the formation of the membrane attack complex, which in turn increases membrane conductance and induces Ca²⁺ overload and mitochondrial dysfunction as well as elevations in ROS levels. Glutamate sensitization of oligodendrocytes to complement attack may contribute to white matter damage in acute and chronic neurological disorders (Alberdi et al., 2006). Collective evidence suggests that glutamatemediated neuroinflammation and oxidative stress may be important mechanisms associated with autoimmune demyelination in MS, and neuroinflammation and oxidative stress can be treated with AMPA/KA antagonists and antioxidants (Pitt et al., 2000; Alberdi et al., 2006).

MRI and PET studies indicate that in MS lesions the activation of microglial cells is accompanied by increase in COX-2 (Rose et al., 2004) and iNOS expression and generation of PGE₂ and nitric oxide, respectively (Banati et al., 2000; Rose et al., 2004; Hill et al., 2004). The production of nitric oxide through iNOS results in the generation of peroxynitrite, which has been implicated in the pathogenesis of white matter injury that occurs in MS (Zhang et al., 2006). These studies indicate that excitotoxicity contributes to the MS pathogenesis. It remains unknown whether these changes are primary to the pathophysiology of MS or caused by secondary factors involved in MS.

6.4.11 Domoic Acid Neurotoxicity

Domoic acid is a phytoplankton-derived excitatory amino acid. Its injections damage the hippocampus of rodents and marine mammals. Consumption of domoic acid-contaminated sea food by humans causes symptoms similar to glutamate toxicity. In domoic acid toxicity, neurodegeneration is paralleled by reduced glutamate receptor NR1, GluR1, and GluR6/7 immunolabeling throughout the whole hippocampal formation. Pre-injections of the AMPA/ KA receptor antagonist, NBOX, retard DOM-mediated neurodegeneration as well as behavioral symptoms (Qiu et al., 2006; Qiu and Curras-Collazo, 2006). No changes are observed at low DOM concentrations, but increases in immunolabeling of Ca²⁺-calmodulin-dependent kinase II (CaMKII, p-Thr286) and phosphorylated cAMP response element-binding protein (CREB, p-Ser133) are observed at higher concentration 24 h post-injection. In vitro, 24 h exposure of neuronal cultures to DOM produces concentration-dependent neuronal cell death. The CNOX and AMPA receptor-selective antagonist LY293558 ((3S,4aR,6R,8aR)-6-[2-(1H-tetrazol-5-vl)-ethyl]-1,2,3, 4,4a,5,6,7,8,8adecahydroisoquinoline-3-carboxylic acid) prevents cell death (Larm et al., 1997). DOM-induced cell death can also be blocked by melatonin (Chandrasekaran et al., 2004)

The mechanism underlying domoic acid-mediated neuronal damage is not fully understood. However, upregulation of nNOS expression and downregulation of NMDAR1 expression at 5 days after DOM administration have been shown to occur (Ananth et al., 2003a,b; Chandrasekaran et al., 2004). This suggests that nNOS and alterations in NMDAR1 may contribute to domoic acid neurotoxicity. In addition in neuronal cultures, domoic acid induces oxidative stress through its effects on cellular glutathione metabolism. Thus, cerebellar granule neurons (CGN) from mice lacking the modifier subunit of glutamate–cysteine ligase [Gclm^{-/-}] have very low levels of GSH and are 10-fold more sensitive to domoic acid-mediated neurotoxicity than CGN from Gclm^{+/+} mice. Antagonists of AMPA/KA receptors and NMDA receptors block domoic acid-mediated neurotoxicity (Walser et al., 2006). Ca²⁺ chelators and antioxidants also attenuate domoic acid-induced neurotoxicity.

Domoic acid treatment not only decreases cellular GSH but also increases oxidative stress as indicated by increases in ROS. In this system, astrocytes from both genotypes are resistant to domoic acid-induced neurotoxicity (Walser et al., 2006). Exposure of neonatal rat microglia to domoic acid triggers the release of TNF- α and matrix metalloproteinase-9 (MMP-9) (Mayer et al., 2001). Both these molecules are involved in the modulation of neuroinflammation in brain (Farooqui et al., 2007). Collective evidence suggests that DOM-mediated neurodegeneration involves changes in cellular redox, oxidative stress, and increased expression of cytokines, nitric oxide synthase, NADPH diaphorase, and matrix metalloproteinase-9 (Walser et al., 2006; Chandrase-karan et al., 2004; Ananth et al., 2003a,b).

6.5 Conclusion

Glutamate is a major excitatory transmitter in mammalian CNS. It interacts with glutamate receptors. Glutamate toxicity is mediated through two distinct processes: an excitotoxic pathway, in which glutamate receptors are stimulated, and an oxidative pathway, in which cystine uptake is inhibited. This pathway causes neural injury through glutathione depletion and oxidative stress. Hyperstimulation of glutamate receptors due to glutamate elevations is associated with acute neural trauma in ischemia, spinal cord injury, and head trauma. Alterations in glutamate receptor density and activity occur in neurodegenerative disorders such as AD, PD, HD, ALS, AIDS complex, and domoic acid neurotoxicity. Glutamate-mediated toxicity is accompanied by a rapid and sustained increase in intracellular Ca²⁺ that stimulates and regulates phospholipases, proteases, protein kinases, and endonucleases. Under normal conditions, action of these enzymes produces lipid mediators that are necessary for physiological cell functions. However, under pathological situations, increase in extracellular glutamate levels overstimulates glutamate receptors and induces a persistent increase in intracellular Ca²⁺. Sustained increase in intracellular Ca^{2+} mediates abnormal neural membrane glycerophospholipid metabolism, with generation of high levels of eicosanoids, lipid peroxides, and ROS. These metabolites, along with abnormal ion homeostasis, altered redox status, and lack of energy generation, may be responsible for neural cell death in acute neural trauma and neurodegenerative diseases.

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Chapter 7 Recent Developments on Kainate-Mediated Neurotoxicity and Their Association with Generation of Lipid Mediators

7.1 Introduction

Kainate (KA) is a cyclic and nondegradable analog of glutamate (Fig.7.1). It is more potent neuronal excitant than glutamate. Systemic administration of KA in adult rats induces persistent seizures and seizure-mediated brain damage syndrome (Coyle, 1983). KA interacts with KA receptors and produces selective degeneration of neurons, especially in striatal and hippocampal areas of brain after intraventricular and intracerebral injections (Coyle, 1983; Lerma, 1997). At a presynaptic level, KA receptors modulate short- and long-term synaptic plasticity while at the postsynaptic level KA receptors contribute and regulate neuronal excitability by the inhibition of K^+ channels. KA receptors allow the influx of Na^+ and the efflux of K^+ . They are composed of five different subunits designated as GluR5, GluR6, GluR7, KA1, and KA2. Channels formed by the expression of GluR6 alone are stimulated by KA. but not by AMPA. Homomers of GluR5 form channels that are activated by domoate, KA, glutamate, and AMPA. GluR7 and KA1 and KA2 do not form channels when each protein is expressed alone, but contribute to the formation of heteromeric assemblies of KA receptors when expressed with GluR5 and GluR6. Coexpression of the GluR5-7 and KA1 and KA2 proteins in different combinations leads to the formation of channels similar to KA receptors (Mulle et al., 2000; Chittajallu et al., 1999). Thus, GluR5, GluR6, and GluR7 correspond to the lowaffinity kainate receptors, whereas KA1 and KA2 correspond to high-affinity KA receptors. The cDNAs for GluR5, GluR6, and GluR7 have 35-40% homology to the AMPA receptor subunits and they are component of high-affinity KA receptors. It is difficult to distinguish AMPA from KA receptors on the basis of electrophysiological and pharmacological studies (Paternain et al., 2000; Michaelis, 1998). However, synthesis of series of 2,3-benzodiazepines compounds that specifically block AMPA receptors has now made it easier to study the pharmacological and functional aspects of KA receptors in brain. Selective agonists of KA receptor are SYM 2081 ((2S.4R)-4-methylglutamic acid and 5-I-Will (5-iodowillardiine)) (Fig. 7.1). Selective antagonists of KA receptor include AMOA ((RS)-2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionic acid), ((RS)-2-amino-3-[2-(3-hydroxy-5methylisoxazol-4-yl)-methyl-5-methyl-3-oxoisoxazolin-4-yl)]propionic acid),



Fig. 7.1 Structures of KA receptors agonists and antagonists. Kainic acid (**a**); glutamate (**b**); 2S,AR isomer of 4-cinnamylglutamic acid (**c**); 5-iodowillarddiine (5-Will,) (**d**); (2S,4R)-methylglutamate (SYM 2081) (**e**); (3SR,4aRS,6SR,8aRS)-6-[(1H-tetrazol-5-yl)methoxy-methyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid (Ly294486) (**f**); (RS-2-amino-3-[2-(3-hydroxy-5-methylisoxazol-4-yl)-methyl-5-methyl-3-oxoisoxazolin-4-yl)] propionic acid (AMNH) (**g**); and ((RS)-2-amino-3-[3-(carboxymethoxy)-5-methylisoxazol-4-yl]propionic acid) (AMOA) (**h**)

and LY294486 (3SR,4aRS,6SR,8aRS-6-[(1H-tetrazol-5-yl)methoxymethyl]-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid) (Frandsen et al., 1990). KA receptor is partially inhibited by KA/AMPA antagonists, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), DNQX (6,7-dinitroquinoxaline-2,3-dione), and NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl quinoxaline 2,3-dione and GYK1 53655 ((\pm)-1-(4-aminophenyl) 3-methylcarbamyl-4methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine). KA receptors mediate rapid excitatory transmission at most glutamatergic synapses (Farooqui et al., 2001; Farooqui et al., 2008).

7.2 KA Receptor-Mediated Ion Fluxes in Neural Cells

KA generates large influx of Na⁺ ion through receptor-gated ion channels. This is accompanied by the passive movement of Cl^- and water molecules into neural cells resulting in the osmotic overloading of water and neural cell damage in KA-mediated neurotoxicity (Rothman, 1985; Olney et al., 1986). KA

treatment also causes focal swelling on the dendrites of hippocampal interneuron and primary cortical cultures (Hasbani et al., 1998; Al Noori and Swann, 2000). The dendritic beading is inhibited by 6-cyano-7-nitroquinoxaline-2,3-dione, an AMPA/KA receptor antagonist. The bead formation is not dependent on extracellular Ca^{2+} . Instead, the retardation of bead formation by tetrodotoxin and veratridine indicates the involvement of sodium ions (Al Noori and Swann, 2000). Finally, the inhibition of KA-mediated beading when chloride is removed from artificial cerebrospinal fluid further suggests that KA-mediated dendritic beading may be produced by the water movement in order to maintain osmotic balance following the movement of sodium and chloride ions into dendrite (Al Noori and Swann, 2000).

KA receptor stimulation also induces membrane depolarization and this may produce alteration in intracellular Ca^{2+} (Meldrum and Garthwaite, 1990). In neuronal and glial cell, KA-mediated cell death can be blocked though the removal of calcium from the culture medium indicating that KA receptormediated calcium influx triggers the excitotoxic cascade that may be associated with neural injury (Choi, 1988). The delayed Ca^{2+} influx may be produced by KA-induced Na⁺ influx, loading, and reversal of membrane Na⁺/Ca²⁺ exchanger following prolonged KA exposure. In addition, KA-mediated neural cell death is greatly reduced in the absence of sodium. Thus, it appears that calcium and sodium influx is required to trigger neural cell damage in KA neurotoxicity (Brorson et al., 1994). Nuclear microscopic studies also indicate a steady increase in Ca²⁺ in the KA-injected side from 1 day post-injection (Ong et al., 1999). This increase peaks at 3 weeks post-injection, reaching a concentration that is 18 times higher than normal. Large numbers of glial cells but no neurons are seen in the lesioned CA fields at this time, suggesting that elevation in calcium level is due to glial cells. This is confirmed by high-resolution elemental maps of lesioned areas. These maps show very high intracellular calcium concentrations in almost all glial cells. Collective evidence suggests that KA-mediated neural cell injury may be initiated by Ca²⁺ influx through voltagedependent Ca²⁺ channels due to membrane depolarization, maintained by failure of Ca²⁺ extrusion because of ATP depletion, and intensified by increase in intracellular Ca²⁺ leading to mitochondrial dysfunction resulting in further depletion of ATP (Coyle, 1983; Choi, 1988; Farooqui et al., 2001). These processes also result in the loss of Mg^{2+} blockade of the NMDA receptor, which allows further Ca²⁺ influx through NMDA receptor channel. Thus, elevation in intracellular Ca2+ is a major component of neural cell death in KA-mediated neurotoxicity (Choi, 1988; Farooqui et al., 2001). In KA-mediated neurotoxicity, the stimulation of nitric oxide synthase by Ca^{2+} generates nitric oxide, which reacts with superoxide to form peroxynitrite. This metabolite reduces mitochondrial respiration, inhibits membrane pumps, depletes cellular glutathione, and damages DNA by causing the activation of poly (ADP-ribose) synthase. This enzyme leads to NAD and ATP depletion (Pryer and Squadrite, 1995). Benzamide, a poly (ADP-ribose) synthase inhibitor, prevents NAD and ATP depletion and protects from KA-mediated neurotoxicity (Cosi et al., 2004). This may be another mechanism that contributes to KA-mediated neural cell damage and death. The relative contribution of Ca^{2+} and peroxynitrite in KA-mediated cell death still remains unknown.

The high intracellular calcium level activates calcium-dependent enzymes, including cPLA₂, nitric oxide synthase, and calpain II, observed in reactive glial cells after KA injections (Table 7.1) (Sandhya et al., 1998; Ong et al., 1997, 1999; Farooqui et al., 2001, 2008). KA receptors regulate mitogen-activated protein kinase (MARK) through increase in the calcium levels. MARK modulates gene expression by phosphorylating transcription factors (Fig. 7.2). Thus, in cytosol MARK phosphorylates NF κ B, whereas its translocation in the nucleus results in the phosphorylation of AP1 transcription factor. NF- κ B mediates and modulates a number of genes and processes, and transporters that promote the onset of inflammation and oxidative stress, and modulates immune function responses(Fig. 7.3).

In addition to calcium, an increase in iron content is also observed at 4 weeks after KA injection. The increase in iron level is accompanied by increased expression of ferritin and divalent metal transporter-1 (DMT1) in KA-treated hippocampus (see below), suggesting that the transporter may be partially responsible for the iron accumulation in KA-induced neurotoxicity (Ong et al., 2006). In KA-mediated neurotoxicity a gradual disregulation in the expression of the iron-binding protein ferritin with time after neuronal injury may result in increased levels of non-ferritin-bound iron that is present in the ferrous form (Huang and Ong, 2005). The increase in ferrous form of iron may account for alterations in cellular redox and an increase in oxidative stress in the hippocampus with time after KA-mediated injury (Ong et al., 1999; Farooqui et al., 2001; Ong and Farooqui, 2005). In addition, significantly higher lead and cadmium levels are detected in the hippocampus and other brain areas of

| Neurochemical alteration | Reference |
|---|---|
| Isoforms of PLA ₂ , PLC, and PLD | Farooqui et al. (2008); Kim et al. (2004) |
| Isoforms of PKC | McNamara et al. (1999); Guglielmetti et al. (1997) |
| Ca ²⁺ /calmodulin kinase II | Lee et al. (2001a) |
| P44/p42 MAP kinase | Liu et al. (1999) |
| Caspase-3 | Djebaili et al. (2002) |
| Proto-oncogene Bcl-2 | Lee et al. (2001b) |
| c-FOS | Lerea et al. (1996) |
| GABA transporter | Zhu and Ong (2004) |
| Divalent metal ion transporter-1 (DMT-1) | Wang et al. (2002a, b) |
| Growth factors | Aquado et al. (2007); Delgado-Rubin de Celix et al. (2006); Sanagi et al. (2007) |
| Cytokines | Yabuuchi et al. (1993) |

Table 7.1 Effects of KA-mediated neurotoxicity on enzymes, genes, and transporters



Fig. 7.2 Kainic acid-mediated stimulation of PLA₂ and generation of ROS along with the activation of NF-*κ*B activation in brain tissue. Kainic acid (KA); phospholipase A₂ (PLA₂); arachidonic acid (AA); reactive oxygen species (ROS); nuclear factor *κ*B (NF-*κ*B); nuclear factor *κ*B-response element (NF-*κ*B-RE); inhibitory subunit of NF-*κ*B (I*κ*B); I*κ*B kinase (I*κ*K); tumor necrosis factor-*α* (TNF-*α*); interleukin-1*β* (IL-1*β*); interleukin-6 (IL-6); cyclooxygenase-2 (COX-2); inducible nitric oxide synthase (iNOS); matrix metalloproteinases (MMPs); cytosolic phospholipase A₂ (cPLA₂); secretory phospholipase A₂ (sPLA₂); positive sign (+) indicates stimulation (pro-inflammatory)

intracerebroventricular KA-injected rats treated with lead and cadmium in the drinking water, compared to intracerebroventricular saline-injected rats treated with lead and cadmium in the drinking water. Since very low levels of lead and cadmium are present in the normal animal, these results indicate increased uptake of lead and cadmium into brain areas as a result of the KA (Ong et al., 2006).

7.3 KA-Mediated Alterations in Neural Membrane Glycerophospholipids

As stated in Chapter 6, two enzymic mechanisms release arachidonic acid (AA) from neural membrane glycerophospholipids in brain tissue. Direct mechanism involves phospholipase A_2 (PLA₂)-mediated degradation of glycerophospholipids, whereas indirect mechanism utilizes phospholipase C (PLC)/



Fig. 7.3 Enzymic and non-enzymic glycerophospholipid metabolism and generation of ROS in kainic acid neurotoxicity. Ferrous ions(Fe^{2+}); ferric ions (Fe^{3+}); phospholipase A_2 (PLA₂); nitric oxide synthase (NOS); cyclooxygenase (COX-2); peroxynitrite (ONOO–); platelet activating factor (PAF); and 4-hydroxynonenal (4-HNE)

diacylglycerol lipase pathway (Sanfeliu et al., 1990; Kim et al., 1995; Farooqui et al., 1989). Detailed description of glutamate and KA-mediated alterations in glycerophospholipid metabolism and other neurochemical changes has been provided in Chapter 6. Thus, loss of essential glycerophospholipids, as a result of activation of PLA_2 isoforms, and generation of high levels of eicosanoids and ROS, mitochondrial dysfunction, decrease in ATP level, alteration in redox status along with changes in neural membrane permeability, and loss of ion homeostasis result in neuronal injury in KA-mediated neurotoxicity (Farooqui and Horrocks, 2006, 2007).

 F_2 -IsoPs are a novel class of prostaglandin F_2 (PGF₂)-like compounds, generated in vivo by a non-cyclooxygenase and free radical-catalyzed mechanism involving the peroxidation of AA (Fig. 7.4) (Fam and Morrow, 2003; Roberts II et al., 2005). In KA-mediated neurotoxicity, marked changes have been observed in F_2 isoprostane (F_2 -IsoP) (Patel et al., 2001; Farooqui et al., 2007b). Determination of F_2 -IsoPs in micro-dissected hippocampal CA1, CA3, and dentate gyrus regions at various times following subcutaneous KA administration (10 mg/kg) indicates that KA causes a large increase in F_2 -IsoP levels in the highly vulnerable CA3 region 16 h post-injection (Table 7.2) (Patel et al., 2001). The CA1 region shows small but statistically insignificant increase in F_2 -IsoP levels. Interestingly, the dentate gyrus, a region resistant to kainate-mediated



Fig. 7.4 Scheme showing the generation of non-enzymic AA-derived isoprostanes. F2-isoprostane (F_2 -IsoP); E_2 -isoprostane (E_2 -IsoP); D_2 -isoprostane (D_2 -IsoP); A_2 -isoprostane (A_2 -IsoP); and J_2 -Isoprostane (J_2 -IsoP)

| Table 7.2 | Alterations | in levels of | glyceropho | spholipid, | sphingolipid, | and choleste | rol-derived |
|------------|--------------|--------------|--------------|------------|---------------|--------------|-------------|
| lipid medi | ators in KA- | -induced ne | eurotoxicity | and ische | mia | | |

| | KA- mediated | | |
|-----------------------------------|-----------------|-----------|---|
| Lipid mediator | toxicity | Ischemia | Reference |
| Arachidonic acid | Increased | Increased | Farooqui et al. (2007a); Farooqui and Horrocks (2007) |
| Prostaglandins | Increased | Increased | Farooqui et al. (2007a); Farooqui and Horrocks (2006) |
| 4-HNE | Increased | Increased | Farooqui et al. (2001); McKrachen et al. (2001) |
| Isoprostanes | Increased | Increased | Musiek et al. (2005); Patel et al. (2001) |
| Ceramide | Increased | Increased | Guan et al. (2006); He et al. (2007); Yu et al. (2000) |
| 7 - β -Hydroxycholesterol | Increased | _ | Ong et al. (2003); He et al. (2006) |
| 7-Ketocholesterol | Increased | _ | He et al. (2006) |
| 24-Hydroxycholesterol | Increased | No effect | Holdenrieder et al. (2004); He et al. (2006) |

Modified from Farooqui et al. (2007).

neuronal death, also shows marked (2.5- to 5-fold) increase in F₂-IsoP levels 8, 16, and 24 h post-injection. The increase in F₂-IsoP levels in CA3 and dentate gyrus is accompanied by inactivation of mitochondrial aconitase, increase in superoxide production, and neuronal vulnerability in these regions. This marked sub-region-specific increase in F₂-IsoP post-KA administration not only is associated with oxidative lipid damage but also promotes seizure-induced death of vulnerable neurons (Patel et al., 2001). In contrast, KA injections (1 μ l of a 1 mg/ml solution) into the right lateral ventricle (coordinates: 1.0 mm caudal to bregma, 1.5 mm lateral to the midline, 4.5 mm from the surface of the cortex) produce no changes in F₂-IsoPs levels at 3 days, 1 week, and 2 weeks after KA administration. However, there was a significant increase (~134%) in F₂-IsoP levels at 4 weeks after kainic acid injection compared to controls. At 8 weeks after injection, the F₂-IsoP levels were increased (~180%) compared to those in the 4-week post-KA injected rats (Fig. 7.5) (Farooqui et al., 2007b).

KA treatment has no effect on iron levels in hippocampi at 3 days after kainic acid injection into the right lateral ventricle, but produces significant increase in iron levels at 2 weeks (~42%), 4 weeks (~76%), and 8 weeks (~88%) after KA injection when compared to the controls (Fig.7.6). There is a significant correlation between the iron and F₂-IsoP levels in the hippocampus produced by the KA-mediated neurotoxicity. As stated above, a marked accumulation of iron occurs in the hippocampus of KA-injected rats. This increase in iron is not due to the diffusion and redistribution of iron, but as a result of increased iron uptake into brain tissue. Although, detailed investigations on the molecular mechanism of iron accumulation have not been performed, a significant increase in density ratios of divalent metal ion transporter-1 (DMT-1)/ β -actin



Fig. 7.5 F₂-isoprostane levels in the rat hippocampus. CONT, K3D, K1W, K2W, K4W, and K8W indicate control, 3 days, 1 week, 2 weeks, 4 weeks, and 8 weeks post-kainate injection. The mean and standard error are indicated. P < 0.05 was considered significant. * Significant difference compared to control group. n = 4 rats at each time point. Reproduced with kind permission from Elsevier, Farooqui et al. (2007b)



bands has been observed on Western blots in the 1-week, 1-month, and 2-month post-KA-injected hippocampus compared to uninjected and 1-day post-KAinjected hippocampus. The increase in DMT-1 protein is paralleled by an increase in DMT-1 immunoreactivity in astrocytes. Light staining for DMT-1 is seen in the uninjected, saline-injected, and 1 day post-KA-injected rat hippocampus. In contrast, an upregulation of DMT-1 has been observed in reactive glial cells at 1 week, 1 month, and 2 months post-KA injection. Electron microscopic studies confirm that the glial cells have morphological features of astrocytes. The observation that DMT-1 is present on astrocytic end feet in contact with blood vessels suggests that these cells may be involved in the uptake of iron from endothelial cells. In addition, it is also reported that there occurs a shift in the oxidation state of the accumulated iron, with more cells becoming Fe^{2+} than Fe^{3+} at the late stages. A possible consequence of the high amounts of Fe^{2+} in the hippocampus after kainate injection is that Fe^{2+} may facilitate iron-mediated damage in the lesioned areas (Wang et al., 2002a, b). Slight increases in the expression of iron transport protein, ferroprotein 1, and copper-containing protein, ceruloplasmin, are also observed in KA-treated rat brain after 2 weeks. This is followed by a decrease to unlesioned levels by 4 weeks after KA injections, indicating that there are differences in the expression of DMT1 and ferroprotein 1. This may be due to differences in iron influx and efflux in areas undergoing inflammatory reactions and oxidative stress (Wang et al., 2002a, b).

Treatment of hippocampal slices with KA results in a time-dependent increase in nuclear NF- κ B levels in CA3 and CA1 areas of hippocampus, but not dentate glyrus, compared with controls. Detailed investigation on hippocampal area CA3 indicates increased NF- κ B DNA-binding activity in response to KA stimulation. Based on inhibition studies, it is proposed that extracellular signal-regulated protein kinase (ERK) and phosphatidylinositol-3 kinase (PtdIns-3 K) pathways play an important role in KA-mediated NF- κ B activation in hippocampal area CA3 area. It is likely that these kinases may target the NF- κ B pathway at different loci (Lubin et al., 2005).

7.4 KA-Mediated Alterations in Sphingolipid Metabolism

Ceramide or *N*-acylsphingosine is an important sphingolipid associated with the extracellular leaflet of neural membranes. It is generated either by the activity of acid, neutral, or alkaline sphingomyelinases or by the de novo synthesis. A concept unifying the diverse biological functions of ceramide indicates that ceramide forms distinct membrane domains, called ceramideenriched membrane domains or platforms. These domains are associated not only with the clustering of receptor molecules and reorganization of signaling proteins, but also with the exclusion of inhibitory signals and initiation and amplification of a primary signal (Grassmé et al., 2007). In addition, ceramide directly interacts with and stimulates intracellular enzymes that may act together with signals initiated in ceramide-enriched membrane domains to transmit signals into a cell (Marchesini and Hannun, 2004). Furthermore, ceramide serves as a precursor to complex sphingolipids. It is also involved in the induction of cell cycle arrest and promotion of apoptotic cell death.

Intracerebroventricular injections of KA in rats produce a significant increase in ceramide immunoreactivity and levels in the hippocampus, at 1 day and 3 days post-injection compared to controls (Fig. 7.7). This increase in ceramide may be due to either increased ceramide synthesis or increased degradation of sphingomyelin. No immediate increase in serine palmitoyltransferase (SPT) immunolabeling is seen in degenerating neurons, but occasional increase is observed in glial cells in the KA-injected rat brain CA fields. In KAtreated rat brains, increase in ceramide occurs in several ceramide molecular species including 16:0, 18:0, 20:0, 22:0, and 24:1 fatty acids (Guan et al., 2006). This increase in ceramide levels is mainly due to increased expression and activity of SPT, after 1-3 weeks intracerebroventricular KA injections (He et al., 2007). Immunohistochemical analyses indicate low expression (baseline expression) of SPT in neurons and gradual increase in immunoreactivity in astrocyte post-KA treatment. In glial expression of SPT peaks at 2 weeks post-KA injections. Electron microscopic studies confirm that degenerating neurons show dense ceramide immunoreactivity in hippocampus, at 3 days and 1 week after kainate injections (Fig. 7.8). Ceramide and SPT immunoreactivities are observed in reactive glial cells at 1 week and 2 weeks after kainate injection. The nucleus contained fine heterochromatin clumps and the cytoplasm contained dense bundles of glial filaments characteristic of astrocytes. Immunoreactivity is also present in end feet around blood vessels.

In vitro treatment of rat brain slices with KA also elevates ceramide levels with increase in 16:0, 18:0, 20:0 molecular species, at 1 day after KA treatment. Addition of SPT inhibitors (myriocin, or L-cycloserine) after KA treatment not



Fig. 7.7 A, B: light micrographs of sections of hippocampal CA1 field from a saline-injected rat. A: Section immunostained for ceramide, showing light or no immunolabeled pyramidal neurons or glial cells (asterisk). B: Section immunostained for SPT, showing light immunolabeled pyramidal neuronal cell bodies (arrowheads) and the neuropil. C, D: Adjacent sections through the affected CA1 field of the lesioned right hippocampus, from a rat that had been injected with kainate 3 days earlier. C: Section immunostained for ceramide, showing immunolabeled pyramidal neuronal cell bodies (arrowheads) and diffuse labeling of the neuropil; this is associated with occasional immunolabeled glial cells (arrows). D: Section immunostained for SPT, showing occasional immunolabeled glial cells (arrows) but not neurons. E. F: Adjacesnt sections through the affected CA1 field of the lesioned right hippocampus, from a rat that had been injected with kainate 1 week earlier. E: Section immunostained for ceramide, showing immunolabeled pyramidal neuronal cell bodies (arrowheads) and diffuse labeling of the neuropil; this is associated with immunolabeled glial cells (arrows). F: Section immunostained for SPT, showing immunolabeled glial cells (arrows). CTRL: saline-injected control rat; 3D: 3 days after kainate injection; 1 W: 1 week after kainate injection. Scale bar = $50 \,\mu$ M. Reproduced with kind permission from Willey-Liss, He et al. (2007) J. Neurosci. Res. 85: 423-432

only reduces total ceramide levels, but also partially reduces cell death caused by KA in vitro. This reduction is most pronounced in 16:0 ceramide molecular species. In contrast, treatment with sphingomyelinase inhibitor (GW4869) has no effect. These observations indicate that a large proportion of the ceramide is generated at 1–4 weeks after kainate treatment and is due to the increased expression of SPT (Guan et al., 2006; He et al., 2007). Based on GC/mass analysis of ceramide molecular species, it is stated that some decrease in the



Fig. 7.8 Electron micrographs of ceramide or SPT immunoreactive cells in the kainateinduced degenerating CA1 field of hippocampus. A: Section immunostained for ceramide from a rat that had been injected with kainate 1 week earlier, showing an immunoreactive neuron with morphological features of degenerating neuron. The cell and nuclear outlines are indistinct. N: neuronal cell body. **B**: Section immunostained for ceramide from a rat that had been injected with kainate 1 week earlier, showing an immunoreactive glial cell with morphological features of astrocytes. The nuclei of the glial cells (AS) contain evenly dispersed fine heterochromatin clumps. The cytoplasm is light and contained dense bundles of glial filaments (F). **C**, **D**: Sections immunostained for SPT from a rat that had been injected with kainate 2 weeks earlier, showing an immunoreactive glial cell with morphological features of astrocytes (C) and labeled astrocytic end feet (D) around blood vessel. Label is absent from endothelial cells (E). L: lumen of the blood vessel. *Arrows* indicate immunoreaction product. Scale (A) = 2 μ M; scale (B, C, D) = 1 μ M. Reproduced with kind permission from Willey-Liss, He et al. (2007)

sphingomyelin content of the hippocampus may also account for the increase in ceramide, but relative contribution of these reactions on ceramide accumulation in KA-mediated toxicity still remains unknown (Guan et al., 2006; He et al., 2007).

The involvement of ceramide biosynthesis in neuronal injury is also supported by other studies in slice cultures (Guan et al., 2006; He et al., 2006). Treatment of rat brain hippocampal slices with KA produces a decrease in MAP2 staining and an increase in LDH release into the culture media

indicating neural injury. Addition of SPT inhibitors (L-cycloserine or myriocin) to hippocampal slices, after KA treatment, reduces MAP2 staining and increases LDH release. In contrast to inhibition of ceramide biosynthesis by L-cycloserine or myriocin, the incubation of slices with an inhibitor of sphingomyelinase has no significant protective effect. Thus, inhibition of ceramide biosynthesis has a significant protective effect after KA-mediated neural cell injury. It should be noted that light SPT immunoreactivity is observed in neurons up to 3 days after KA treatment, whereas astrocytic immunoreactivity to SPT does not peak until 2 weeks after kainate injection (Guan et al., 2006; He et al., 2006). These observations are consistent with studies, which indicate that myriocin blocks $A\beta$ -mediated death of hippocampal neurons (Cutler et al., 2004). Myriocin also attenuates ethanol-mediated cell death in SK-N-SH neuroblastoma cells (Saito et al., 2005). Collective evidence suggests that inhibition of ceramide biosynthesis has a significant neuroprotective effect for a short time after kainate toxicity and ceramide biosynthesis inhibitors most likely act by inhibiting ceramide biosynthesis in neurons rather than glial cells. This suggestion is supported by the observation that light SPT immunoreactivity is observed in neurons up to 3 days after kainate lesions, whereas astrocytic immunoreactivity to SPT does not peak till 2 weeks after kainate injection (Guan et al., 2006; He et al., 2006) indicating that neuron and glial cell respond to KA toxicity differently, and late response of glial cells may contribute to gliosis in the injured brain.

7.5 Cholesterol Metabolism in Brain

Cholesterol is an integral component of neural membranes. It is crucial for the function of neuronal and glial cells in brain (Dietschy and Turley, 2001; Pfrieger 2003). Cholesterol affects the physicochemical properties of neural membranes and regulates endocytosis, antigen expression, and activities of membrane-bound enzymes, receptors, and ion channels (Dietschy and Turley, 2001; Pfrieger 2003). Neurons synthesize sufficient cholesterol to survive and grow, but at the time of spermatogenesis cholesterol is provided by glial cells through cholesterol transporting proteins (Pfrieger, 2003). In brain, cholesterol is metabolized to oxycholesterols through hydroxylation reactions, and occurrence of 24-, 27-, 7-hydroxycholesterol has been well established in brain tissue and CSF samples (Gylys et al., 2007).

7.5.1 KA-Mediated Changes in Cholesterol and Its Metabolites

Intraventricular injections of KA increase cholesterol immunostaining with filipin in the CA1 region of rat hippocampus (Fig. 7.9) (Ong et al., 2003). Hippocampal sections also show increase in staining of cholesterol in cell bodies



Fig. 7.9 Light micrographs of hippocampal sections stained with filipin stain. Section through field CA3 of a saline injected rat, showing little staining to cholesterol (A, arrow); section through field CA3 of a rat which has been injected with kainate 1 day earlier, showing intense filipin staining in the affected hippocampal neurons (B, arrows); CA1 fields of hippocampal slices that have been treated with ethanol and immunolabeled with antibody to a neuronal marker, GluR1. Dense staining for GluR1 is observed in the cell bodies and dendrites of pyramidal neurons (arrow) in the slices treated with ethanol (C, arrow). Dense staining for GluR1 is observed in the cell bodies and dendrites of pyramidal neurons (arrow) in the slices treated with cholesterol (**D**, *arrow*); CA1 field of hippocampal slices that have been treated with COPs 7-ketocholesterol and immunolabeled with antibody to a neuronal marker, GluR1. Staining for GluR1 is absent (E); and CA1 field of hippocampal slices that have been treated with glutathione, followed by cholesterol oxidation product 7-ketocholesterol and immunolableled for GluR1. Dense staining for GluR1 is observed in the cell bodies and dendrites of pyramidal neurons indicating a protective effect of reduced glutathione on loss of the neuronal marker (F). Scale $= 250 \ \mu m$. Reproduced with kind permission from Blackwell Publishing, Ong et al. (2003)

and dendritic fields of neurons. No increase in cholesterol staining is seen in glial cells. The maximal increase in both cholesterol (from 23.0 ± 15.8 to $98.6 \pm 16.7 \ \mu g/mg$) and oxysterols is observed at 2 weeks post-KA injection. At this time, significant increases are observed in both 24-hydroxycholesterol (from 7.2 ± 5.4 to $30.1 \pm 4.9 \ ng/mg$) and 7-ketocholesterol (from 1.4 ± 0.4 to $4.2 \pm 0.9 \ ng/mg$) (He et al., 2006). Treatment of hippocampal slices with KA also produces increased staining with filipin in neurons of CA1 fields. In neuronal cultures, addition of lovastatin, an inhibitor of cholesterol synthesis, attenuates the increase in cholesterol may be due to an increase in cholesterol synthesis (Ong et al., 2003). The molecular mechanism involved in the upregulation of cholesterol synthesis in KA-mediated neurotoxicity is not fully understood. However, it may be caused by the oxidative stress of those pyramidal neurons



Fig. 7.10 Levels of cholesterol, 24-hydroxycholesterol, and 7-ketocholesterol in KA-mediated neurotoxicity. Modified from He et al. (2006)

that escape cell death. These neurons participate in compensatory reorganization of local networks and upregulate their cholesterol synthesizing machinery, not only for maintaining their own membrane integrity but also for synthesizing new membranes (Vance et al., 1996; Perez et al., 1996; Dietschy and Turley, 2001). Gas chromatographic mass spectrometry analyses of the hippocampus indicate a significant increase in cholesterol and the oxysterols (24-hydroxycholesterol and 7-ketocholesterol) at 2 weeks after neuronal injury (Ong et al., 2003) (Fig. 7.10). Cholesterol oxidation products are known to have toxic effect on neuronal cultures through decrease in staining of the AMPA receptor subunit GluR1 in the CA field. The addition of glutathione to hippocampal cultures and slices blocks the decrease in GluR1 immunoreactivity (Fig 7.9 e,f). This observation suggests that KA-induced toxicity is mediated through cholesterol oxidation products (Table 7.2) (Park et al., 2000).

7.5.2 KA-Mediated Alterations in Oxycholesterols

KA treatment produces a marked increase in cholesterol 24-hydroxylase immunoreactivity in glial cells of the affected CA fields. The number of cholesterol 24hydroxylase positive glial cells increases significantly from 1 week (1312 ± 195 cells/mm²) to 2 weeks (1815 ± 225 cells/mm²) after KA injection, when compared to saline-injected rats (0 ± 0 cells/mm²). The cholesterol 24-hydroxylase positive glial cells are GFAP positive with large diameter processes that tapered gradually from the cell bodies. No increase in cholesterol 24-hydroxylase immunoreactivity in glial cells is observed in other brain regions that are not affected by the KA injections.

Organotypic slices from the hippocampus of rats injected with kainate plus lovastatin show significantly lower levels of cholesterol, 24-hydroxycholesterol,

and 7-ketocholesterol, compared to those that have been treated with KA alone, suggesting that lovastatin modulated the loss of hippocampal neurons after KA treatment. The levels of 24-hydroxycholesterol detected in vivo after KA treatment, $>50 \mu$ M, are neurotoxic in hippocampal slice cultures. Brainpermeable statins such as lovastatin may have a neuroprotective effect by limiting the levels of oxysterols in brain areas undergoing neurodegeneration (Ong et al., 2003; He et al., 2006). In hippocampal slices, glutathione protects neurons from cholesterol oxidation products (COPS)-mediated toxicity. Collectively, these studies suggest that cholesterol and COPS in particular may be a factor in aggravating oxidative damage to neurons following neuronal injury induced by KA (Ong et al., 2003).

Oxysterols including 7-ketocholesterol and 24-hydroxycholesterol induce exocytosis when applied externally to PC12 cells, as determined by capacitance measurements under patch clamp conditions and total internal reflection fluorescence microscopy (TIRFM) of labeled vesicles (Ong et al., unpublished). The effect of 7-ketocholesterol is dependent on the integrity of lipid rafts. Enhanced exocytosis induced by the oxysterol can be abolished by pretreatment of cells with methyl cyclodextrin, which chelates cholesterol and disrupts rafts. Similar effects on enhanced exocytosis are observed after external application of several ceramide species, including C18:0 ceramide, and as with oxysterols, the effect is raft dependent. These results indicate that increased oxysterol (Park et al., 2000) and ceramide (Guan et al., 2006; He et al., 2007) levels in the hippocampus following KA-mediated neurotoxicity may enhance neurotransmitter release and exocytosis and further propagate excitotoxic brain injury (Park et al., 2000; Guan et al., 2006; He et al., 2007). 7-Ketocholesterol has a reactive keto group and may form adducts with amino group of other lipid and protein components of neural membranes. The nature and effect of such interactions remain to be elucidated.

Both cholesterol and oxysterol levels in the degenerating hippocampus are affected by intraperitoneal injections of a blood-brain barrier-permeable statin, lovastatin. As stated above, similar effects are observed in hippocampal slice cultures in vitro. These effects are independent of cholesterol transport across the blood-brain barrier (Park et al., 2000). Modulation of excessive increase in cholesterol and oxysterol levels in brain tissue after statin treatment correlates with increased survival of hippocampal pyramidal neurons, suggesting that reduction in oxysterols may be an important mechanism for the neuroprotective effect of statins. Once again detailed investigations are required on this aspect of oxycholesterol metabolism.

7.5.3 KA-Mediated Changes in Steroid Hormones

In normal brain, cholesterol can also be converted into steroid hormones, the neurosteroids. Their formation involves cholesterol side-chain cleavage enzyme

P450 scc and the enzyme 3β -hydroxysteroid dehydrogenase/ δ 5- δ 4-isomerase (3β -HSD) (Kohchi et al., 1998). Cholesterol side-chain cleavage enzyme P450 scc is localized in neurons, and it converts cholesterol to pregnenolone. The latter is then metabolized to other hormones such as DHEA and estradiol. After kainate injections, a loss of immunoreactivity to P450 scc is observed in pyramidal neurons, and induction of P450 immunoreactivity is observed in astrocytes (Fig. 7.11). Western blots show a net decrease in the level of P450 scc enzyme protein in the hippocampus of rats after kainate injections (Chia et al., 2008). A slight, non-significant decrease in pregnenolone, but a significant 40% decrease in estradiol levels is observed in the hippocampus after kainate lesions. DHEA levels are very low, less than a tenth of estradiol and a



Fig. 7.11 P450_{scc} immunolabeled sections of the hippocampal field CA1 and CA3. **A** and **B**: Fields CA1 (A) and CA3 (B) from a saline-injected rat showing dense labeling in pyramidal neurons (*arrows*). **C**: Field CA3 from a rat which had been injected with kainate 3 days earlier showing loss of immunoreactivity in pyramidal neurons (*arrows*). **D**: Field CA1 from a rat which had been injected with kainate 2 weeks earlier, showing induction of expression a small number of astrocytes (*arrows*). **E**; Electron microscopy from CA1 of the same rat as D, showing light immunoreactivity (*arrows*) in cell with dense glial filament (F) characteristic of astrocyte (AS). Scale: A 200 μ m, B–D 70 μ m and E 1 μ m. Reproduced with kind permission from Springer, Chia et al. (2008)

hundredth that of pregnenolone in comparison with the above two hormones in both normal and kainate-lesioned hippocampus. DHEA levels are below the detection limit in individual rat hippocampal samples (Chia et al., 2008; Ong et al., unpublished results). These results indicate that there is no increase, or even a decrease in neurosteroid levels in the degenerating brain, despite the increase in cholesterol content. Catalytic efficiencies of cholesterol-metabolizing P450 s vary significantly and probably reflect physiological requirements of different organs for the rate of cholesterol turnover. These enzymes represent a unique system for elucidation of how different enzymes have adapted to fit their specific roles in cholesterol elimination. Neurosteroids mostly control the CNS activity through allosteric modulation of neurotransmitter receptors within concentration ranges used by neurotransmitters themselves (Shibuya et al., 2003; Pikuleva, 2006).

Together these findings indicate that most of the increased cholesterol in the hippocampus after kainate injury is converted to oxysterols rather than metabolized to neurosteroids. Furthermore, since estradiol is commonly thought to be neuroprotective steroid, both the decrease in neurosteroids and the increase in oxysterols together may both contribute to neurodegeneration. Thus kainic acid-mediated neurotoxicity is accompanied by changes in the composition of neural membrane glycerophospholipids, sphingolipids, cholesterol, and its oxidation products but not in sterols. These alterations in membrane constituents produce changes in membrane permeability and fluidity resulting in kainic acid-mediated neurodegeneration (Farooqui and Horrocks, 1991; Farooqui et al., 2000, 2002, 2004; Park et al., 2000; He et al., 2007; Guan et al., 2006; Farooqui and Horrocks, 2007; Farooqui et al., 2007a, b).

7.6 Consequences of Interactions Among Glycerophospholipid, Sphingolipid, and Cholesterol-Derived Lipid Mediators in KA-Mediated Neurotoxicity

Biophysical properties of neural membranes are maintained by specific glycerophospholipid, sphingolipid, and cholesterol composition (Farooqui et al., 2007a). Both external and internal stimuli can alter levels of neural cell glycerophospholipid, sphingolipid, and cholesterol levels by regulating enzyme activities associated with their metabolism. It is becoming increasingly evident that KA-mediated neurotoxicity enhances glycerophospholipid, sphingolipid, and cholesterol metabolism (Farooqui et al., 2008). This enhancement results in increased generation of glycerophospholipid, sphingolipid, and cholesterolderived lipid mediators (Fig. 7.12). KA-mediated alterations in levels of lipid mediators may disturb normal signal transduction homeostasis and threaten neural cell survival due to increase in the intensity of specific signal transduction in the brain tissue. Interactions among glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators occur at several sites (Fig. 7.12) (Farooqui



Fig. 7.12 Kainic acid-mediated changes in neural membrane glycerophospholipid, sphingolipid, and cholesterol metabolism. Kainic acid (KA); phosphatidylcholine (PtdCho); cytosolic phospholipase A_2 (cPLA₂); arachidonic acid (AA); cyclooxygenase-2 (COX-2); prostaglandin E_2 (PGE₂); reactive oxygen species (ROS); sphingomyelin (SM); sphingomyelinase (SMase); serine palmitoyltransferase (SPT); note that AA stimulates sphingomyelinase (SMase) and ceramide and ceramide 1-phosphate stimulate PLA₂ activity. ROS and lipid peroxides may also play an important role in apoptosis. Sphingosine 1-phosphate stimulates COX-2. KA also stimulates SPT and cholesterol 24-hydroxylase. The symbols (+) indicate stimulatory action of metabolites

et al., 2008). Thus, in KA-mediated neurotoxicity, increased synthesis of ceramide may induce a spontaneous association of ceramide molecules to form ceramide-enriched microdomains, which fuse to large ceramide-enriched membrane platforms. These large ceramide-enriched membrane platforms may be associated with a transmembrane signaling mechanism for a subset of cell surface receptors (Bollinger et al., 2005). In neural membranes, both ceramide and its metabolites (ceramide 1-phosphate and sphingosine 1-phosphate) stimulate the generation of arachidonic acid and ROS through the stimulation of PLA₂ isoforms and arachidonic acid cascade (Farooqui and Horrocks, 2009). Similarly, cPLA₂-derived arachidonic acid enhances the generation of ceramide via sphingomyelinase stimulation. Simultaneous intensification of these processes following KA-mediated toxicity not only indicates that glycerophospholipid and sphingolipid metabolism support each other for the generation of lipid mediators, but are also involved in an interplay (cross talk) that lowers the levels of essential glycerophospholipid and ceramide molecular species in neural membranes and bring about cellular demise (Farooqui et al., 2008). It should

be noted here that like ceramide metabolizing enzymes, enzymes mediating release of ROS are also localized in membrane rafts, and the integrity of ceramide-enriched rafts may be required for continuous cellular ROS release during KA-mediated neurotoxicity (Dumitru et al., 2007; Farooqui et al., 2008; Farooqui and Horrocks, 2009). In a feed-forward mechanism, ROS stimulate ceramide-generating enzyme, acid sphingomyelinase, which results in the formation of more ceramide-enriched membrane platforms that may alter optimal sphingolipid to cholesterol ratio, and mediate neural cell demise via KA-mediated oxidative stress.

Similarly, KA stimulates the hydrolysis of plasmalogens, the vinylether containing glycerophospholipids, by plasmalogen-selective PLA₂ (PlsEtn-PLA₂) in a dose- and time-dependent manner (Farooqui et al., 2003) and ceramide decreases the levels of plasmalogens by inhibiting PlsEtn-PLA₂ in rat brain slices (Latorre et al., 1999). The decrease in plasmalogen levels by ceramide can be blocked by quinacrine, ganglioside, and bromoenol lactone. These compounds inhibit PlsEtn-PLA₂ activity. Thus, it is likely that interplay between plasmalogen and sphingomyelin-derived lipid mediators may modulate inflammation and oxidative stress, processes that are closely associated with KA neurotoxicity (Farooqui et al., 2003; Latorre et al., 1999).

7.7 Interactions Between Ceramide and Cholesterol Metabolism in KA-Mediated Toxicity

Very little information is available on in vivo interactions between ceramide and cholesterol in brain (Farooqui et al., 2007a). In the artificial membrane system, ceramide-enrichment facilitates the displacement of cholesterol. This results in tight lipid packing of membrane glycerophospholipids. Minimizing the exposure of cholesterol and ceramide to water may be a strong driving force for the association of ceramide with lipid rafts. Thus, the displacement of cholesterol by ceramide produces marked changes in molecular composition, liquid ordered properties, and function of lipid rafts (Megha and London, 2004; Megha et al., 2007). As stated above, in KA-mediated neurotoxicity activities and immunoreactivity of cholesterol hydroxylase are markedly increased, and free cholesterol is converted into hydroxycholesterol (He et al., 2006). These enzymes convert cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol. Except 24-hydroxycholesterol, 27- and 25-hydroxycholesterol also undergo 7 α -hydroxylation with subsequent oxidation to 7 α -hydroxy-3-oxo-delta 4 steroids in neurons, astrocytes, and Schwann cells (Zhang et al., 1997). The conversion of cholesterol into hydroxycholesterol is an important mechanism for the maintenance of brain cholesterol homeostasis (Kolsch et al., 2001).

Hydroxycholesterols are cytotoxic to neural and endothelial cells. They induce apoptotic cell death. In human neuroblastoma cells, SH-SYSY 24-hydroxycholesterol increases caspase-3 and decreases the number of viable cells (Kolsch et al., 2001).
24-Hydroxycholesterol modulates caspase-3, a cysteine-dependent endoprotease with specificity for aspartate residues in proteins. It mediates apoptotic cell death by cleaving a number of enzymes such as protein kinase C, cytosolic phospholipase A₂, calcium-independent phospholipase A₂, phospholipase C, cytoskeletal proteins such as α -spectrin, β -spectrin, actin, vimentin, Bcl-2 family of apoptosis-related proteins, and DNA-modulating enzymes, poly (ADP-ribose) polymerase (Kolsch et al., 2001). Although molecular mechanism associated with hydroxyl- and ketocholesterol-mediated toxic effect is not fully understood, 7-ketocholesterol is known to trigger the stimulation of NADPH oxidase, generation of superoxide anions, loss of mitochondrial transmembrane potential ($\Delta \Psi m$), the release of cytochrome c, and activation of caspase-3. These processes are closely associated with the apoptotic cell death (Lizard et al., 2000). 7-Oxycholesterol not only modulates Ca²⁺ signals, but also prevents the phosphorylation of endothelial nitric oxide synthase and cPLA₂ (Millanvoye-Van Brussel et al., 2004).

Furthermore, in retinal pigment epithelial cells oxysterols are also known to induce slight mitochondrial dysfunctions, but with a significant increase (2- to 4-fold) in reactive oxygen species (ROS) production compared with the control. They also enhanced IL-8 gene expression and IL-8 protein secretion in the following decreasing order: 25-hydroxycholesterol > 24-hydroxycholesterol > 7-ketocholesterol (Joffre et al., 2007).

Collective evidence suggests that oxysterols have neurotoxic effects on neural cell cultures and among them; 24-hydroxycholesterol can be used as a marker for neurodegeneration (Rojo et al., 2006). Levels of hydroxycholesterols are markedly increased in patients with neurological disorders (Teunissen et al., 2007).

7.8 Interactions Between Glycerophospholipid and Cholesterol Metabolism in KA-Mediated Neurotoxicity

The curvature, cholesterol content, and transbilayer distribution of glycerophospholipids significantly modulate the physicochemical and functional properties of cellular membranes. It is interesting to note that differences in membrane curvature and cholesterol content alter the array of PtdCho molecules present on the surfaces of phospholipid bilayers (Williams et al., 2000). In neural membranes, these differences may have profound effects on a number of critical membrane functions and signal processes (Farooqui et al., 2007a, 2008). Furthermore, cholesterol has also been implicated in the assembly and maintenance of sphingolipid-rich rafts (Barenholz, 2004). Collective evidence suggests that KA-mediated changes in cholesterol may markedly affect neural membrane function regulating fluidity and permeability (Farooqui et al., 2008; Farooqui and Horrocks, 2009). The generation of high levels of oxy- and hydroxycholesterol in KA-mediated neurotoxicity may have serious consequences for neural cell survival. Oxysterols also exert tight control over neural cell cholesterol trafficking by altering cholesterol influx/efflux (Koudinov and Koudinova, 2003). Oxysterols modulate Ca²⁺ signals, and inhibit the phosphorylation of endothelial nitric oxide synthase and cPLA₂ and interact with lipid metabolites of glycerophospholipid and sphingolipid metabolism (Millanyove-Van Brussel et al., 2004; Farooqui et al., 2007a). A β and APP oxidize cholesterol to form 7 β -hydroxycholesterol, a proapoptotic oxysterol, which is neurotoxic at nanomolar concentrations. 7β-Hydroxycholesterol not only retards secretion of soluble APP from cultured rat hippocampal H19-7/IGF-IR neuronal cells and inhibits tumor necrosis factor- α -converting enzyme α -secretase activity, but has no effect on β -site APP-cleaving enzyme 1 activity (Nelson and Alkon, 2005). 7β -Hydroxycholesterol also inhibits α -protein kinase C, an enzyme critical in memory consolidation and synaptic plasticity. Oxidation of cholesterol is accompanied by stoichiometric production of hydrogen peroxide and requires divalent copper. These results suggest that a function of APP may be to produce low levels of 7-hydroxycholesterol. It is proposed that higher hydroxycholesterol levels produced by $A\beta$ may contribute to the oxidative stress and neural cell loss observed in AD (Nelson and Alkon, 2005). Similarly, 7-ketocholesterol also induces apoptosis through the production of superoxide anions (Lizard et al., 2000).

7.9 Conclusion

Neural membranes are composed of glycerophospholipids, sphingolipids, cholesterol, and proteins. Existence and maintenance of cholesterol-ceramide rafts are dependent on the level of membrane cholesterol and ceramide. This explains why reduction of cholesterol level (either through reverse cholesterol transport using cholesterol acceptors such as β -cyclodextrin or through cholesterol biosynthesis inhibition using stating) interferes with many processes which involve rafts. KA-mediated toxicity markedly enhances glycerophospholipids, sphingolipids, and cholesterol metabolism that produces elevations in glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators. The increased interaction among glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators intensifies degradation of neural membrane components and loss of essential glycerophospholipids, sphingolipids, and cholesterol causing changes in neural membrane fluidity and permeability. These processes allow a sustained Ca²⁺ influx and result in stimulation of Ca²⁺-dependent enzymes including PLA₂, PLC, PLD, nitric oxide synthase, calpains, and endonucleases. Stimulation of these enzymes along with mitochondrial dysfunction, decrease in ATP levels, and changes in redox status of neural cells may be responsible for neural cell death in KA-mediated neurotoxicity.

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Chapter 8 Beneficial Effects of Docosahexaenoic Acid on Health of the Human Brain

8.1 Introduction

Docosahexaenoic acid (22:6 n-3, DHA) is an important essential polyunsaturated fatty acid (PUFA) that contains 6 cis double bonds located at positions 4, 7, 10, 13, 16, and 19 (Fig. 8.1). It is highly enriched in neural membranes of the cerebral cortex and retina where it accounts for approximately 30–40% acyl groups in glycerophospholipids (Lauritzen et al., 2001). The concentration of DHA is particularly high in gray matter of cerebral cortex and in photoreceptor cell. DHA constitutes >17% by weight of total fatty acids in the brain of adult rats and >33% of total fatty acids in the retina. DHA is mainly found on the sn-2 position of amino glycerophospholipids such as phosphatidylethanolamine (PtdEtn), plasmenylethanolamine (PlsEtn), and phosphatidylserine (PtdSer). In contrast, neural membrane PtdCho mainly contains esterified arachidonic and oleic acids (Farooqui et al., 2000a,b). Other neural membrane glycerophospholipids and sphingomyelin have amide-linked stearic acid. At present, full implications of this unique distribution of glycerophospholipid head groups, esterified fatty acids, and amide-linked fatty acids are not fully understood. However, it is well known that a correct balance between arachidonic acid (n-6) and docosahexaenoic acid (n-3 fatty acids) in neural membranes is important for cerebral and cardiac health (Simopoulos, 2002, 2004; Farooqui et al., 2008). In brain, DHA is particularly concentrated in retinal membranes of the rod outer segments as well as synaptosomal membranes. Morphological and neurochemical consequences of DHA deficiency produce a decrease in neuronal cell bodies in several regions of the rat brain, deficit in serotonin and dopamine neurotransmission, decrease in glucose uptake, reduction in cytochrome oxidase activity, and decrease in G-protein coupled signaling efficiency in photoreceptor outer segment and reduction in levels of PlsEtn and PtdSer (Ahmad et al., 2002; Hamilton et al., 2000; Horrocks and Farooqui, 2004). These neurochemical changes in brain tissue result in reduction in learning and memory, abnormal electro-retinogram, impaired vision, elevated behavioral indices of anxiety, aggression, and depression (Chalon, 2006; McNamara and Carlson, 2006; Fedorova and Salem, 2006). In contrast, AA deficiency produces



Fig. 8.1 Chemical structures of α -linolenic (a), eicosapentaenoic (b), docosapentaenoic (c), and docosahexaenoic (d) acids

several non-neural abnormalities including reduced growth, skin lesions, fatty liver, and polydipsia (excessive thirst). Diet rich in n-6 polyunsaturated fatty acids produces inflammation, oxidative stress, and compromised vasoregulation due to the generation of eicosanoids and platelet-activating factor.

DHA not only modulates physical properties of the lipid bilayer (Siddiqui et al., 2007), but also provides second messengers (resolvins and protectins) for biological signaling (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Phillis et al., 2006; Farooqui and Horrocks, 2007). In addition, DHA provides neural membranes with an appropriate physical environment for the activity of integral membrane proteins such as membrane-bound enzymes. It modulates ion channels and neurotransmitter receptors (Yehuda et al., 2002), and stabilizes neuronal membranes by suppressing voltage-gated Ca^{2+} currents and Na⁺ channels (Young et al., 2000).

The turnover of DHA involves deacylation and reacylation cycle. This cycle utilizes DHA-selective phospholipase A_2 (plasmalogen-selective phospholipase A_2) and acyltransferase reactions for maintaining the phospholipid composition necessary for the normal function of neural membranes (Farooqui et al., 2000a). Nearly, all of the DHA is recycled. DHA is used continuously for the biogenesis and maintenance of neuronal and retinal membranes throughout human life.

8.2 Synthesis of DHA in Brain

Neurons lack the ability to synthesis DHA from its precursor, α -linolenic acid (18:3*n*-3, ALA) (Fig. 8.2). DHA is obtained either directly from the diet or synthesized from ALA in liver, and transported to the brain through plasma lipoproteins (Scott and Bazan, 1989). Cerebral endothelium synthesizes DHA from dietary precursors via Δ^6 -desaturation and retroconversion steps. Astrocytes have the ability to synthesize DHA either from 18-, 20-, and 22-carbon *n*-3 precursors (via elongation and desaturation steps) or from 24-carbon precursors (Innis and Dyer, 2002), but this is a minor pathway in quantitative terms as compared to DHA supplied to brain tissue from plasma. About 2–8% of DHA in rat brain glycerophospholipids is replaced daily with DHA from the unesterified fatty acid pool in the plasma (Rapoport et al., 2001).



Fig. 8.2 Metabolism of docosahexenoic, eicosapentaenoic, and arachidonic acids and interactions among their metabolites. The action of a 15-lipoxygenase-like enzyme on docosahexaenoic acid produces docosanoids. These metabolites inhibit neuroinflammation and apoptosis. Eicosapentaenoic acid-derived eicosanoids are less potent than arachidonic acid-derived eicosanoids. Minus sign (-) indicates inhibition

8.3 Transport and Incorporation of Docosahexaenoic Acid in Brain

Injected [³H]DHA binds to plasma albumin in the blood. Two factors control the extent of DHA incorporation in brain: (a) the rate of its dissociation from plasma albumin and (b) the esterification of DHA with co-enzyme A by longchain acyl-CoA synthetase (Rapoport, 1999, 2003). Lysophosphatidylcholine (lyso-PtdCho) is a preferred carrier for DHA to the brain tissue (Lagarde et al., 2001). It is becoming increasingly evident that endothelial phospholipase A_1 that plays an important role in the metabolism of high-density lipoproteins may be responsible for the synthesis of DHA lyso-PtdCho in plasma (Chen and Subbaiah, 2007). Substrate specificity studies indicate that the polar head group specificity varies considerably among glycerophospholipids. The kinetic studies indicate that PtdEtn is a better substrate than PtdCho followed by PtdSer (Chen and Subbaiah, 2007). Within the same phospholipid class, the enzyme shows preference for species containing DHA at the sn-2 position. The enzyme shows no activity with glycerophospholipids containing an ether linkage at the sn-1 position. Endothelial lipase is secreted by the endothelial cells of blood-brain barrier, therefore, it is proposed that it may play an important role in the delivery of DHA lysophospholipid carriers to the brain.

Turnover studies have indicated that apparent half-lives of DHA in individual glycerophospholipids of DHA-adequate rat brain ranges from 23 to 56 days (DeMar et al., 2004). These results do not account for the recycling of DHA. About 5% of the brain DHA is lost daily with replacement from the diet (Rapoport, 1999, 2003). When recycling is accounted for, half-lives in some regions of the brain are measured in minutes (Rapoport, 2003). Therefore, some unknown mechanism must exist in the adult rat brain to minimize a metabolic loss of DHA from neural membrane glycerophospholipids (DeMar et al., 2004). Mice deficient in n-3 fatty acids in their diet are quite slow to recover a more normal fatty acid composition in different areas of their brains (Carrie et al., 2000). Rats deprived of n-3 fatty acids for 15 weeks have 37% less DHA in their brains compared with 89% less DHA in their plasma, and have apparent half-lives for DHA at least twofold greater than in controls (DeMar et al., 2004).

Labeled DHA preferentially incorporates in nerve growth cone membranes and photoreceptors (Martin, 1998), indicating that it is important for development and function of the central nervous system. The supplementation of serum-free medium with hormones and DHA permits a full development of synapses of cultured mouse fetal hypothalamic cells. This is manifested by the increase in number and the regular shape and diameter of synaptic vesicles. In PC12 cell cultures, [³H]DHA preferentially incorporates into the ethanolamine glycerophospholipid fraction from cell bodies and nerve growth cones (Martin et al., 2000). Based on turnover studies, it is suggested that [³H]DHA-labeled glycerophospholipids are exclusively synthesized in the cell body and then trafficked to nerve growth cones (Martin, 1998; Martin et al., 2000). The trafficking of DHA from cell bodies to growth cones may be involved in synaptogenesis.

The reincorporation of DHA into membrane glycerophospholipids is catalyzed by acyl-CoA synthetases and acyl-CoA: lyso-glycerophospholipid acyltransferases. These enzymes are localized in microsomes (Farooqui et al., 2000a). Acyl-CoA synthetase converts docosahexaenoic acid to docosahexaenoyl-CoA. This enzyme requires ATP and CoA. The same acyl-CoA synthetase and lyso-glycerophospholipid acyl-CoA transferase utilize and facilitate the incorporation of arachidonic acid (AA) and DHA in brain glycerophospholipids (Reddy et al., 1984; Reddy and Bazan, 1984).

8.4 Release and Catabolism of DHA in Brain

DHA is released from PlsEtn by plasmalogen-selective phospholipase A_2 (PlsEtn-PLA₂). This release is a receptor-mediated process (Strokin et al., 2003; Farooqui et al., 2003; Sergeeva et al., 2005). Thus, the treatment of neuron-enriched cultures with kainate and other glutamate analogs results in a dose- and time-dependent stimulation of PlsEtn-selective PLA₂ (Table 8.1). A kainate/AMPA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), inhibits enzymic activity and also prevents KA-mediated cell death in neural cell cultures (Table 8.2). This suggests that the stimulation of PlsEtn-PLA₂ activity is a KA receptor-mediated process. Similarly, bromoenol lactone (BEL), a potent inhibitor of iPLA₂ (Farooqui et al., 2003), also blocks both the stimulation of PlsEtn-PLA₂ and neural cell death in neuron-enriched cultures (Lu et al., 2001). Collectively, these studies suggest that the release of DHA from PlsEtn is a receptor-mediated process catalyzed by PlsEtn-PLA₂. This enzyme has been purified and characterized from bovine brain (Hirashima et al., 1992; Yang et al., 1994).

Purified brain PlsEtn-PLA₂ has a molecular mass of 39 kDa, and does not require Ca^{2+} (Hirashima et al., 1992; Farooqui et al., 1995). It is inhibited by

| Table 8.1 | Effect of | f kainic acid | on plas- |
|------------|-------------|---------------|---------------------|
| malogen- | selective | phospholip | base A ₂ |
| activity | of neuro | on-enriched | cultures |
| from rat o | cerebral co | ortex | |

| KA (μM) | Specific activity (pmol/min/mg protein) | | |
|---------|--|--|--|
| Control | 2.5±0.62 | | |
| 10 | $5.2{\pm}0.8$ | | |
| 20 | $7.6{\pm}1.2$ | | |
| 30 | 11.3 ± 1.4 | | |
| 40 | 15.3±1.3 | | |
| 50 | 16.8±1.2 | | |
| 60 | 17.5±1.0 | | |
| 70 | 17.8 ± 1.8 | | |
| | | | |

Results are the means \pm SEM for five determinations. Data modified from Farooqui et al. (2003).

| Treatment | Specific activity (pmol/ min/mg protein) |
|--|---|
| Control | 2.7±0.43 |
| Kainic acid (50 µM) | 17.53±2.3 |
| CNQX (25 µM) | $2.93 {\pm} 0.36$ |
| Kainic acid + CNQX ($50 \mu\text{M} + 25 \mu\text{M}$) | 11.3 ± 1.4 |
| Control | $2.73 {\pm} 0.53$ |
| Kainic acid (50 µM) | 17.53±2.3 |
| BEL (10 µM) | $2.63 {\pm} 0.57$ |
| Kainic acid + BEL $(50 \mu M + 10 \mu M)$ | 3.57 ± 0.68 |

Table 8.2 Effect of CNQX and BEL on kainic acid-stimulated plasmalogen-selective phospholipase A_2 activity of neuron-enriched cultures from rat cerebral cortex

Results are the means \pm SEM for five determinations. Data modified from Farooqui et al. (2003). CNQX (6-cyano-7-nitroquinoxaline) and BEL (bromoenol lactone).

ATP at high concentration (2 mM or above) (Table 8.3). Non-ionic detergents, Triton X-100 and Tween-20, stimulate the enzymic activity of brain PlsEtn-PLA₂. These detergents inhibit the activity of heart PlsCho-PLA₂. Other detergents, such as octylglucoside, sodium deoxycholate, and sodium taurocholate inhibit the PlsEtn-PLA₂ in a dose-dependent manner. PlsEtn-PLA₂ is inhibited by SH-group blocking agents. This inhibition can be reversed by dithiothreitol and glutathione (Farooqui et al., 1995). The purified PlsEtn-PLA₂ is inhibited by polyvalent anions such as citrate > sulfate > phosphate and metal ions such as Ag⁺ and Hg²⁺ > Fe³⁺. Quinacrine and nordihydroguariaretic acid inhibit the PlsEtn-PLA₂ in a dose-dependent manner.

DHA-enriched PtdSer is metabolized to PtdEtn or PtdCho by baseexchange reactions, or to PtdEtn by PtdSer decarboxylation (Porcellati, 1983; Farooqui and Horrocks, 1985; Farooqui et al., 2000b; Mozzi et al., 2003; Akbar et al., 2005). DHA is also hydrolyzed from PtdSer by the action of PLA₂ (Garcia and Kim, 1997). Incubation of C6 glioma cells with [³H]DHA results in about 40% incorporation of DHA in 24 h. Treatment with ROI, a 5-HT_{2A} receptor agonist, causes the release of [³H]DHA, suggesting the presence of a

Table 8.3 Physicochemical and kinetic properties of various isoforms of brain PLA2 activities

| Property | $PlsEtn-PLA_2$ | Reference |
|-------------------------------|----------------|------------------------------|
| Localization | Cytosol | Farooqui and Horrocks (2001) |
| Mol. mass | 39 kDa | Farooqui and Horrocks (2001) |
| Effect of calcium | No effect | Farooqui and Horrocks (2001) |
| Substrate | PlsEtn | Farooqui and Horrocks (2001) |
| Fatty acid specificity | AA DHA | Farooqui and Horrocks (2001) |
| Effect of AACOCF ₃ | Inhibited | Farooqui and Horrocks (2001) |
| Effect of BEL | Inhibited | Farooqui and Horrocks (2001) |
| ATP | Inhibited | Farooqui and Horrocks (2001) |

PLA₂ activity hydrolyzing PtdSer in C6 glioma cells. *Naja naja* PLA₂ also hydrolyzes DHA from synaptosomal preparations in a dose- and time-dependent manner (Garcia and Kim, 1997; Kim et al., 1996). The released fatty acids also include AA and oleic acid. However, the rate of DHA release is faster than that of AA or oleic acid.

Action of lipoxygenase-like enzyme on DHA produces 10, 17S-docosatrienes, 17S-resolvins, and neuroprotectins (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Phillis et al., 2006). These lipid mediators are called as docosanoids (Fig. 8.3). They act on leukocyte trafficking as well as downregulating expression of cytokines. Docosanoids inhibit both interleukin-1- β -mediated NF- κ B activation and cyclooxygenase activation, and downregulate pro-inflammatory gene induction. Thus, docosanoids slow down the inflammatory cycle induced and maintained by cytokines on astrocytes. The infusion of docosanoid, neuroprotectin D1 (NPD1), following ischemic reperfusion injury or during oxidative stress in cell culture, downregulates oxidative



Fig. 8.3 Chemical structures of n-3 fatty acids and docosanoids. Docosahexaenoic acid (**a**); eicosapentaenoic acid (**b**); 16, 17*S*-docosatriene (**c**); 10, 17*S*-docosatrienes (**d**); 4*S*, 5, 17*S*-resolvin (**e**); and 7*S*, 16, 17*S*-resolvin (**f**)



Fig. 8.4 Role of docosanoids in neural and non-neural tissues

stress and apoptotic DNA damage. NPD1 also upregulates the anti-apoptotic Bcl-2 proteins, Bcl-2 and bclxL, and decreases the expression of the proapoptotic proteins (Fig. 8.4) such as Bax and Bad (Mukherjee et al., 2004; Bazan, 2005b). Furthermore, NPD1 inhibits caspase-3 activity and blocks IL-1--mediated expression of cyclooxygenase-2. Collective evidence suggests that inclusion of DHA in the diet suppresses the production of both tumor necrosis factor- α (TNF- α) and IL-1 β and protects brain tissue by generating docosanoids (Bazan, 2005b). In contrast, AA and eicosanoids are pro-inflammatory (Fig. 8.5). As stated in Chapters 1 and 2, these metabolites are associated with fever,



Fig. 8.5 Roles of eicosanoids in neural and non-neural tissues

sensitivity to pain, sleep, inflammation, and oxidative stress, and are the target of aspirin-like drugs. Eicosanoids are not stored in neural cells, but are synthesized in response to physiological and non-physiological stimuli. Because of their amphiphilic nature, eicosanoids can cross cell membranes and leave the cell in which they are synthesized to act on neighboring cells. Eicosanoids act through specific superficial or intracellular receptors called eicosanoid receptors. These receptors modulate signal transduction and gene transcription.

8.5 Role of DHA in Brain Tissue

Dietary supplementation of DHA has many effects on neural and non-neural tissues. Thus, incorporation of DHA changes physicochemical properties of neural membranes. These include bilayer thickness, acyl chain packing, free volume, and phase transition temperature (Mitchell et al., 1998; Wassall et al., 2004). DHA is specifically involved in inducing lateral phase separations into DHA-rich/cholesterol-poor and DHA-poor/cholesterol-rich lipid microdomains. The relatively low affinity between the DHA acyl chain and cholesterol may promote phase separations (Wassall et al., 2004; Shaikh et al., 2003, 2004). This process may be involved in microdomain (raft) formation in neural membranes. These lipid microdomains play an important role in the compartmentalization and modulation of cell signaling. In non-neural cells, DHA supplementation alters membrane fluidity, but also improves fatty acid unsaturation index (Hashimoto et al., 1999). Collective evidence suggests that DHA incorporation in neural membranes induces changes in neural membrane fatty acid composition that may lead to restoration of many membrane properties such as permeability, receptor affinities, ion fluxes, and activities of membranebound enzymes.

Changes caused by the incorporation of DHA in neural membrane are not limited to physicochemical properties. In brain tissue, dietary intake of DHA also modulates dopaminergic, noradrenergic, glutamatergic, and serotonergic neurotransmission (Fig. 8.6) (Zimmer et al., 2000; Chalon et al., 1998; Högyes et al., 2003), activities of cytosolic and membrane-bound enzymes (Fig. 8.7), ion channels (Yehuda et al., 2002; Nishikawa et al., 1994; Xiao and Li, 1999; Salem et al., 1988), learning and memory processes (Fujimoto et al., 1989; Fujita et al., 2001), and inflammation and immune function (James et al., 2000; Calder and Grimble, 2002).

In rat retinal neuronal cultures, oxidative stress increases levels of ceramide, which mediates photoreceptor cell apoptosis. DHA blocks oxidative stress and ceramide-mediated damage by upregulating Bcl-2 expression and glucosylating ceramide. Both these processes decrease intracellular concentrations of ceramide (German et al., 2006b). Detailed investigations on DHA-mediated signaling indicate that 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophynyltio) butadiene (U0126), a specific MEK inhibitor, completely prevents the DHA-mediated



Fig. 8.6 Roles of DHA in brain tissue



Fig. 8.7 Effect of DHA on enzyme activities

anti-apoptotic effect. DHA rapidly increases ERK phosphorylation in photoreceptors, whereas U0126 retards this increase. U0126 not only hinders DHA-mediated mitochondrial depolarization, but also retards the DHA-induced increase in opsin expression. DHA upregulates the early expression of Bcl-2 and downregulates Bax expression. At the same time, DHA also decreases caspase-3 activation in photoreceptors. Collectively, these studies suggest that DHA exclusively activates the ERK/

MAPK pathway to promote photoreceptor survival during early development in vitro and upon oxidative stress. This leads to the regulation of Bcl-2 and Bax expression. These processes not only preserve mitochondrial membrane integrity and potential, but also blocks caspase activation (German et al., 2006a).

A variety of biological effects of DHA in non-neural tissues have been observed when fish oil is supplemented in humans and animals. These include effects on triacylglycerol, high-density lipoprotein cholesterol, platelet function, endothelial and vascular function, blood pressure, cardiac excitability, measures of oxidative stress, pro- and anti-inflammatory cytokines, and immune function (Lauritzen et al., 2001; Horrocks and Yeo, 1999). Collective evidence supports the view that DHA not only prevents arrhythmias and has anti-inflammatory immunoprotective effects on heart, but also stabilizes plaques in cardiovascular and cerebrovascular systems (Massaro et al., 2006).

8.5.1 Modulation of Gene Expression by DHA

Polyunsaturated fatty acids interact with the genome through several mechanisms (Jump, 2004). They regulate the activity of several transcription factors, including PPAR, LXR, HNF-4, NF-kB, and SREBP. Fatty acids and their metabolites bind directly to specific transcription factors to regulate gene transcription. Polyunsaturated fatty acids indirectly modulate gene expression not only through their effects on enzymic activities of cyclooxygenase, lipoxygenase, protein kinase C, and sphingomyelinase, but also by producing changes in membrane lipid/lipid raft composition, a process that affects G-protein receptor or tyrosine kinase-linked receptor signaling (Jump, 2004). Supplementation of DHA in animal produces the overexpression of numerous genes in the brain. These genes are associated with signal transduction, synaptic plasticity, energy metabolism, and membrane trafficking (Kitajka et al., 2002; Puskás et al., 2003). DHA interacts with transcription factors associated with upregulation of β -oxidation enzymes. DHA downregulates the expression of desaturases. These enzymes modulate the synthesis of DHA from α -linolenic acid (Price et al., 2000; Nakamura and Nara, 2003).

DHA is a ligand for the retinoid X receptor (RXR) in brain (Lengqvist et al., 2004; Farooqui et al., 2004; de Urquiza et al., 2000). RXR activation is closely associated with nuclear signaling and gene expression. RXR serves as a fatty acid sensor in vivo. A number of proteins act as co-activators. They interact with nuclear receptors and play a role in the regulation of transcriptional activity (Jump, 2002b). DHA not only modulates gene expression, but also induces changes in the stability of the mRNA for several lipogenic enzymes. A deficiency of DHA results in downregulation of brain glucose transporter expression resulting in a decrease in glucose utilization in the cerebral cortex of DHA-deficient rats (Pifferi et al., 2005). DHA-mediated gene expression changes are critical for adaptive responses for neural cell survival. Key

transcription factors associated with these processes include peroxisome proliferator-activated receptors (PPAR), sterol regulatory element-binding protein (SREBP), and carbohydrate response element-binding protein (ChREBP) (Clarke, 2000; Jump, 2002b; Nakamura et al., 2004). In addition, DHA and EPA affect the expression of genes that modulate cytokines and their receptors, cell adhesion molecules, cytoskeleton proteins, and hormone receptors. The effect of DHA is sustained as long as the DHA remains in the diet. Thus, DHA acts like a hormone to control the activity of key transcription factors and modulate many signal transduction processes associated with a variety of neural cell functions (Jump, 2002a; Valentine and Valentine, 2004). Collective evidence suggests that dietary consumption of DHA has numerous beneficial effects on the health of human brain through the modulation of genes associated with synaptic plasticity, neuroinflammation, and energy metabolism (Horrocks and Yeo, 1999; Horrocks and Farooqui, 2004).

8.5.2 Modulation of Enzymic Activities by DHA

DHA modulates the expression and activities of a number of enzymes (Fig. 8.7) including cyclooxygenases, which are responsible for the overproduction of prostaglandins (PG) at inflammatory sites. DHA effects are dependent on the NF- κ B-binding site in the COX-2 promoter. Cytokine/NF- κ B-mediated signaling involves MAPK, PKC, and NADPH oxidase. Western blotting studies show that DHA inhibits nuclear p65 NF- κ B subunit translocation by decreasing cytokine-stimulated reactive oxygen species and ERK1/2 activation by effects on both NADPH oxidase and PKCepsilon activities (Massaro et al., 2006). DHA modulates Na⁺, K⁺ ATPase, an integral membrane protein associated with nodes of Ranvier in neurons (Marszalek and Lodish, 2005). This enzyme generates and maintains Na⁺ and K⁺ gradients necessary for resting neural membrane potential. Supplementation of DHA results in increased Na⁺, K⁺-ATPase activity in sciatic nerve (Gerbi et al., 1998). In contrast, in human endothelial cells DHA significantly reduces Na⁺, K⁺-ATPase activity.

8.5.3 Modulation of Inflammation and Immunity by DHA

Immune system, which protects the host against pathogens, consists of innate and acquired functional divisions. These systems involve various blood-born factors and cells. Low intake of DHA promotes immune functions, whereas high intake inhibits antigen presentation and adhesion molecule expression (Harbige, 2003). DHA not only reduces the expression of pro-inflammatory cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor- α (Caughey et al., 1996; Wu and Meydani, 1998), but also modulates the expression of interferon- γ and

adhesion molecule reflecting faster maturation of the immune system. A variety of other molecular mechanisms may also explain how DHA interferes with immune cell function. These mechanisms include alterations in eicosanoid synthesis, orphan nuclear receptor activation (e.g., peroxisome proliferator-activated receptors, liver X receptors), and T lymphocyte signaling by changing the molecular composition of lipid rafts (Stulnig, 2003).

DHA competes with AA for incorporation into neural membrane glycerophospholipids. Increase in DHA incorporation in neural membranes not only decreases levels of AA in glycerophospholipids, but prevents the release of AA and oxygenation of AA by cyclooxygenases and lipoxygenases. These processes decrease the production of PGE₂ and LTB₄ (Calder, 2003, 2004). In non-neural cells, DHA induces changes in localization of IL-2R, STAT5a and STAT5b signaling in lipid rafts, and suppresses the expression of JAK1, JAK3, and tyrosine/phosphotyrosine in soluble membrane fractions (Li et al., 2006). It also decreases lymphocyte proliferation and natural killer cell activity, but increases macrophages. DHA incorporates T-cell membrane glycerophospholipids and produces changes in lipid homeostasis by shifting the metabolic pathways toward energy supply. Changes in lipid homeostasis result not only in reduction of sphingomyelin content in lipid rafts, but also in suppression of protein kinase C Θ signaling (Fan et al., 2004). This optimizes the function of immune cells. Through the modulation of inflammatory processes and immune cell activation, DHA induces positive effects on various states of immune deficiencies and diseases with hyper-inflammatory character (Fan et al., 2004; Grimm et al., 2002). Collectively, these studies suggest that DHA modulates immunosuppressive and autoimmune effects in neural and non-neural tissues in humans and animals (Li et al., 2005; Horrocks and Farooqui, 2004).

8.5.4 Modulation of Learning and Memory by DHA

Long-term potentiation (LTP) and long-term depression (LTD) of hippocampal synaptic transmission are two forms of activity-dependent synaptic plasticity that underlie learning and memory. Intracerebroventricular injections of DHA retard the induction of CA1 LTP, but have no effect on the dentate gyrus LTP. In rat brain slices during tetanic stimulation, endogenously released DHA triggers the expression of LTP (Fujita et al., 2001). Addition of glycerophospholipids containing DHA enhances LTP in the rat hippocampal CA1 region (Izaki et al., 1999). Collective evidence suggests that DHA is crucial for the induction and maintenance of long-term potentiation (LTP).

DHA also blocks the induction of long-term depression (LTD). This process requires the activation of NMDA receptors in hippocampal CA1 neurons (Young et al., 1998). The molecular mechanism of this process remains unknown. However, it is proposed that DHA acts on postsynaptic sites (Zucker, 1989). DHA also inhibits function of the K^+ channels (Poling et al.,

1995; Honore et al., 1994). Since the K⁺ channel blocker, tetraethylammonium ion, produces NMDA-independent long-lasting modulation in the Schaffer collateral-CA1 synapses of hippocampal slices (Aniksztejn and Ben Ari, 1991), it is likely that DHA acts through modulation of the K⁺ channel. Another possibility is that DHA blocks GABA-induced chloride channel activity and potentiates NMDA responses of dissociated rat substantia nigra neurons (Nabekura et al., 1998). Based on this observation, it is proposed that DHA may improve memory formation by blocking the inhibitory actions of GABA on LTP (Das, 2003). Collectively, these studies suggest that the complete blockade of NMDA receptors prevents both LTP and LTD induction, whereas a moderate concentration of the NMDA antagonist, D-APV, shifts the direction of synaptic plasticity to the induction of LTD (Young et al., 1998).

Incorporation of DHA in neural membranes from transgenic mice overexpressing the Tg2576 gene partly protects the mice from NMDA receptor subunit loss. Thus, DHA stabilizes the structure of NMDA receptors (Calon et al., 2005). Collectively, these studies suggest that DHA not only improves cognitive development, but also increases neuroplasticity of neural membranes. These processes contribute to synaptogenesis and may be involved in synaptic transmission.

8.5.5 Modulation of Apoptotic Cell Death by DHA

DHA has anti-apoptotic effects in neural cell cultures. Thus DHA protects rat retinal photoreceptor cells from apoptotic cell death. It prevents apoptotic cell death in serum-deprived Neuro 2A cells (Kim et al., 2000; Kishida et al., 1998; Rotstein et al., 1997, 1998). The molecular mechanism associated with antiapoptotic effects of DHA remains unknown. However, the translocation of Raf-1 kinase, reduction in caspase-3 activity, and inhibition of the phosphatidylinositol 3-kinase pathway by DHA may be involved in the prevention of apoptotic cell death induced by staurosporine in Neuro 2A cells (Kim et al., 2000; Akbar and Kim, 2002; Akbar et al., 2005). DHA mediates neuronal survival by facilitating membrane translocation/activation of Akt (a serine/ threonine kinase involved in cell survival) through its capacity to increase PtdSer. Docosapentaenoic acid (22:5 n-6) (Fig. 8.1), which replaces DHA during n-3 deficiency was less effective in accumulating PtdSer and translocating Akt, and thus less effective in preventing apoptotic cell death (Akbar et al., 2005). The extent of the anti-apoptotic effect of DHA correlates with a timedependent increase in the PtdSer content. Treatment of Neuro 2A cells with DHA in serine-free culture medium has no effect on caspase-3 and phosphatidylinositol 3-kinase activities. Thus, an increase in PtdSer content is necessary for staurosporine-induced apoptosis.

In contrast, in non-neural tumor cells and HL-60 cells, DHA induces apoptotic cell death (Siddiqui et al., 2001; Miura et al., 2004). The molecular mechanism

associated with DHA-mediated cell death remains elusive. However, DHA is known to increase ceramide levels and reduces the amount of phosphorylated retinoblastoma protein (pRb), a protein, which is involved in the cell cycle and growth (Siddiqui et al., 2001, 2003, 2004). Thus, DHA induces cell cycle arrest and apoptosis by activating protein phosphatases. Besides dephosphorylation of retinoblastoma protein, protein phosphatases also interact with Bcl-2, an apoptotic protein that regulates the release of cytochrome c from mitochondria, and eventually, activation of caspase-3 (Siddiqui et al., 2004; Bougnoux, 1999).

8.5.6 DHA and Generation of Docosanoids

Cyclooxygenases do not oxidize DHA. The action of an enzyme resembling 15-lipoxygenase on DHA produces docosatrienes, resolvins, and neuroprotectins (Fig. 8.3) (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Serhan, 2005a). As stated earlier docosatrienes, resolvins, and neuroprotectins antagonize the effects of eicosanoids, modulate leukocyte trafficking, and downregulate the expression of cytokines in glial cells and modulate interactions among neurons, astrocytes, oligodendrocytes, microglia, and cells of the microvasculature (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Bazan, 2006). The neuroprotectins comprise docosatrienes and resolvins of the D series (Serhan, 2005a). The infusion of neuroprotectin D1 (NPD1), following ischemic reperfusion injury or during oxidative stress in cell culture, downregulates oxidative stress and apoptotic DNA damage. NPD1 also upregulates the anti-apoptotic Bcl-2 proteins, Bcl-2 and bclxL, and decreases the expression of the proapoptotic proteins, Bax and Bad (Bazan, 2005a). Furthermore, this metabolite inhibits caspase-3 activity, and blocks IL-1-mediated expression of cyclooxygenase-2.

In non-neural cells, resolvins of D series (RvD1 to RvD6) are generated from DHA. Administration of RvDs and RvD1 protects kidney tissues from ischemia/reperfusion injury. RvDs and RvD1 act by reducing the number of infiltrating leukocytes and block TLR-mediated activation of microglial cell macrophages in kidney (Duffield et al., 2006). The generation of RvDs and RvD1 may be an internal neuroprotective mechanism for preventing tissue damage (Hong et al., 2003; Marcheselli et al., 2003; Serhan, 2005b; Mukherjee et al., 2004; Bazan, 2005a; Lukiw et al., 2005; Muckova et al., 2006).

8.5.7 DHA and Neurite Outgrowth

A deficiency of DHA in the hippocampus not only decreases neuronal soma size in hippocampus (Ahmad et al., 2002) and levels of PtdSer and PlsEtn (Hamilton et al., 2000; Farooqui and Horrocks, 2001), but also interferes with learning and memory processes (Moriguchi et al., 2000). Restoration of memory formation in DHA-deficient animals occurs when these animals were fed a DHA-adequate diet. Similarly, supplementation of DHA in cell culture media not only induces neurite outgrowth in PC12 cells (Ikemoto et al., 1997), hippocampal cells (Calderon and Kim, 2004), and rat cortical primary neuronal cultures (Cao et al., 2005), but also promotes branching. DHA inclusion in the culture medium increases the neurite length of DHA-deficient neurons to the level of DHA-adequate neurons (Calderon and Kim, 2004). The molecular events associated with DHA-mediated neuritogenesis and differentiation is not fully understood. However, the overexpression of long-chain acyl-CoA synthetase 6 specifically promotes DHA internalization, activation to DHA-CoA, and accumulation in differentiating PC12 cells (Marszalek et al., 2005). These observations suggest that DHA plays an important role in brain development (Calderon and Kim, 2004; Cao et al., 2005).

In addition to glycerophospholipid synthesis, neurite outgrowth requires the synthesis of protein (Ikemoto et al., 1999). Cultured neurons treated with DHA have strong growth-associated protein-43 (GAP-43) immunoreactivity and higher PtdSer and EtnGpl contents but a lower PtdCho content than control neurons (Cao et al., 2005). Fatty acid analyses of DHA-supplemented neurons show significantly increased DHA contents in PtdSer and EtnGpl, suggesting that these lipids may be the direct precursors of lipid mediators associated with signal transduction processes involved in the induction of neurite outgrowth in DHA-supplemented cell cultures. Supplementation of serum-free medium with hormones and DHA permits the full development of synapses of cultured mouse fetal hypothalamic cells as attested by the increase in number and the regular shape and diameter of synaptic vesicles.

DHA supplementation also affects the morphology of astrocytes from primary culture. The three populations of astrocytes are observed in DHA-treated astrocytic cultures (Champeil-Potokar et al., 2006). They differ from each other in their n-3: n-6 PUFA ratios in glycerophospholipids. DHA-supplemented cells have a physiological high n-3: n-6 ratio (1.58), unsupplemented cells have a low n-3: n-6 ratio (0.66). In contrast, AA-supplemented cells have a very low n-3: n-6 ratio (0.36), with excess n-6 PUFA. DHA-supplemented astrocytes have greater gap junction capacity than unsupplemented cells or AA-supplemented astrocytes. The enhanced gap junction coupling of DHA-enriched astrocytes may be associated with a more functional distribution of connexin 43 at cell interfaces and more of the main phosphorylated isoform of connexin 43. These findings suggest that the high n-3: n-6 PUFA ratio that occurs naturally in astrocyte membranes is required for optimal gap junction coupling and astrocyte neuronal communication (Champeil-Potokar et al., 2006).

8.5.8 DHA in Visual Function

DHA is a major constituent of photoreceptor membranes (Neuringer and Connor, 1986; Anderson et al., 1976). It accumulates in rod outer segment disks and plays an important role in disordering disk membranes, creates an adequate environment for conformational rhodopsin changes, and modifies the

activity of retinal enzymes. Biophysical and biochemical properties of DHA modulate photoreceptor membrane function by altering permeability, fluidity, thickness, and lipid phase properties (SanGiovanni and Chew, 2005). Tissue DHA status modulates retinal cell signaling associated with phototransduction. DHA promotes signaling cascades not only by upregulating membrane-bound retinal proteins but also by rhodopsin regeneration. Tissue DHA insufficiency causes changes in retinal function. Visual processing deficits have been ameliorated with DHA supplementation in some cases (SanGiovanni and Chew, 2005). In retina, DHA has been shown to modulate retinal cell gene expression, cellular differentiation, and cellular survival. As stated above, DHA activates a number of nuclear hormone receptors that operate as transcription factors for molecules that modulate reduction-oxidation-sensitive and pro-inflammatory genes; these include the peroxisome proliferator-activated receptor-alpha (PPAR- α) and the retinoid X receptor. Interactions of DHA with PPAR- α prevent endothelial cell dysfunction and vascular remodeling through inhibition of vascular smooth muscle cell proliferation, inducible nitric oxide synthase production, interleukin-1 induced cyclooxygenase (COX)-2 production, and thrombin-induced endothelin 1 production. DHA also modulates the production and activation of angiogenic growth factors, eicosanoids. Changes in DHA levels alter the electro-retinogram and visual acuity tests in human and nonhuman primates (Connor et al., 1990). Thus, DHA-induced changes in neural membrane fatty acid composition may lead to restoration of many membrane properties such as membrane fluidity, permeability, receptor affinities, ion fluxes, and activities of membrane-bound enzymes. Dietary DHA has antiarrhythmic properties, which have been attributed to its ability to modulate Na⁺, Ca²⁺, and several K⁺ channels resulting in heart rate viability (Ismail, 2005). Similarly, in retina and hippocampal neurons, Na⁺, Ca²⁺, and K⁺ channels respond to electrophysiological effects of DHA and promote stabilizing effect on electrical activities of neuronal membranes (Leaf et al., 2003).

8.5.9 DHA in Nociception (Pain)

Nociception is a complex process. It is characterized by peripheral and central mechanisms (Svensson and Yaksh, 2002; Broom et al., 2004). It is generally agreed that both mechanisms are involved in neuropathic and inflammatory pain. The induction of nociception is accompanied by an increase in the expression of COX-2 activity and generation of PGI₂ and PGE₂ (Svensson and Yaksh, 2002). Intrathecal or local injections of COX-2 inhibitors prevent the development of nociception. DHA and EPA directly interact with TRPV1, an ion channel associated with nociceptive neurons and brain (Matta et al., 2007). These fatty acids not only modulate TRPV1 ion channel enhancement responses to extracellular protons in a phosphorylation-dependent manner, but also displace binding of the ultrapotent TRPV1 ligand [³H]resiniferatoxin. In

contrast to their agonistic properties, DHA and EPA competitively block the responses of vanilloid agonists. These actions occur in mammalian cells in the physiological concentration range of 1–10 nmol. Significantly, DHA exhibits the greatest efficacy as an agonist, whereas EPA and LA are markedly more effective inhibitors. Similarly, EPA but not DHA profoundly inhibits capsaicinmediated pain behavior in mice. These effects are independent of alterations in membrane elasticity because the micelle-forming detergent Triton X-100 only minimally affects TRPV1 properties. Thus, DHA and EPA differentially regulate TRPV1 and this form of neural cell signaling may contribute to their biological effects. Collectively, these studies suggest that dietary supplementation with selective n-3 fatty acids would be most beneficial for the treatment of pain (Matta et al., 2007).

8.6 Alterations in DHA Levels in Aging and Neurological Disorders

Aging is a natural process that is defined as a progressive deterioration of biological functions after the organism has attained its maximal reproductive competence. The most important risk factors for neurodegenerative diseases in humans are old age and a positive family history. The onset of neurodegenerative diseases is often subtle, and usually occurs in mid to late life and their progression depends on not only genetic but also environmental factors. Mitochondrial dysfunction, levels of ATP, oxidative stress and inflammation, changes in redox status, and ion permeability in neurodegenerative diseases lead to progressive cognitive and motor disabilities with devastating consequences to their patients (Farooqui et al., 2007, 2008).

8.6.1 DHA Levels in Normal Aging Brain

DHA is retained in brain during early development, however, generation of free radicals and ROS during aging process causes a detrimental decline in DHA levels in neural membranes (Lauritzen et al., 2001; Horrocks and Yeo, 1999). Aging is associated not only with a decrease in the brain content of DHA, but also with a reduction in neuroplasticity. The glutamate receptor subunits, GluR2 and NR2B, play a significant role in forebrain synaptic plasticity (Farooqui et al., 2008). Aging decreases levels of GluR2 and NR2B subunits. The decrease in GluR2 and NR2B subunits can be reversed by DHA supplementation. It is also shown that decline in DHA levels with age is accompanied by loss of memory and learning. Reduction in DHA levels with age can be restored by DHA supplementation. The loss of DHA with aging may be due to increased activity of plasmalogen-selective PLA₂ is also coupled with the loss of PtdEtn, PlsEtn, and PtdSer during aging. Dietary supplementation of DHA not only

restores the levels of DHA, but also increases cerebral choline and acetylcholine levels that improve passive avoidance performance in stroke-prone spontaneously hypertensive rats and also in rat hippocampus during aging (Barceló-Coblijn et al., 2003). Collectively, these studies suggest that during aging in neural membranes, DHA supplementation not only promotes normal membrane fluidity, but also turns on certain genes that maintain normal cognition and brain function (Farkas et al., 2000; Kitajka et al., 2002; Barceló-Coblijn et al., 2003).

8.6.2 DHA Levels in Neurological Disorders

Introduction of vegetable oils (corn, sunflower seeds, safflower seeds, cottonseed, and soybeans) in early 1950s has resulted in an enormous increase in the consumption of n-6 fatty acids. Today, in American diets, the ratio of n-6 to n-3 fatty acids ranges from approximately 17–20:1 instead of the traditional range of 1-2:1. A high intake of n-6 fatty acids shifts the physiological state to one that is prothrombotic and proaggregatory as characterized by increase in blood viscosity, vasospasm, and vasoconstriction and reduction in bleeding time. Consumption of n-6 fatty acids in vegetable oils elevates eicosanoids and upregulates the expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α . In contrast, *n*-3 fatty acids have anti-inflammatory, antithrombotic, anti-arrhythmic, hypolipidemic, and vasodilatory properties (Simopoulos, 2004, 2002, 2006; Farooqui et al., 2007, 2008). These beneficial effects of n-3 fatty acids retard brain damage in a number of neurological diseases such as stroke, spinal cord injury, Alzheimer disease and chronic visceral diseases such as coronary heart disease, hypertension, type 2 diabetes, rheumatoid arthritis, ulcerative colitis, Crohn disease, and chronic obstructive pulmonary disease. Most of these studies on n-3 fatty acids have been performed with fish oils [eicosapentaenoic acid (EPA) and DHA]. However, α linolenic acid, found in green leafy vegetables, flaxseed, rapeseed, and walnuts, desaturates and elongates in the human body to EPA and DHA with low rates and may have beneficial effects on normal human health and in chronic visceral and neurodegenerative diseases.

Levels of DHA in hippocampus of Alzheimer disease (AD) patients are significantly decreased compared to age-matched controls (Söderberg et al., 1991) (Fig. 8.8). This may be due to significant decrease in plasmalogen levels in AD patients (Söderberg et al., 1990, 1991; Wells et al., 1995; Guan et al., 1999; Han et al., 2001; Pettegrew et al., 2001). Loss of DHA and plasmalogens is reflected in the loss of synapses and cognitive impairment in AD. Chronic pre-administration of DHA to a rat model of AD prevents β -amyloid-induced impairment of an avoidance ability-related memory function (Hashimoto et al., 2002). Thus, DHA is beneficial in preventing the learning deficiencies in this AD model. DHA also affects amyloid precursor protein processing by inhibiting



Fig. 8.8 Beneficial effects of DHA on neurological disorders

 α - and β -secretase activities (de Wilde et al., 2003). Supplements of DHA produce a neuroprotective effect on β -amyloid deposition without significant toxic effects.

DHA protects the brain tissue against ischemic and excitotoxic damage in rats (Okada et al., 1996; Terano et al., 1999; Gamoh et al., 1999; Bas et al., 2007). DHA may act as an antioxidant (Hossain et al., 1998). DHA induces antioxidant defenses by enhancing cerebral activities of catalase, glutathione peroxidase, and levels of glutathione (Hossain et al., 1999). Dietary supplementation of fish oil enhances resistance to ROS and reduces lipid peroxidation. To counteract the effects of ROS, neural cells induce a number of genes encoding phase II detoxifying enzymes and antioxidant proteins. A cis-acting transcriptional regulatory element, called as antioxidant response element (ARE), mediates the transcriptional activation of genes such as heme oxygenase-1, gamma-glutamylcysteine synthetase, thioredoxin reductase, glutathione-S-transferase, and NADPH: quinone oxidoreductase. Nuclear factor-erythroid 2-related factor 2 (Nrf2), a member of the Cap nno Collar family of basic region-leucine zipper (bZIP) transcription factors, plays a crucial role in ARE-mediated antioxidant gene expression. Kelch-like ECH-associated protein-1 (Keap1) normally sequesters Nrf2 in the cytoplasm in association with the actin cytoskeleton, but upon oxidation of cysteine residues Nrf2 dissociates from Keap1, translocates to the nucleus where it interacts with ARE sequences leading to transcriptional activation of antioxidant and phase II detoxifying genes. Oxidized n-3 fatty acids interact directly with the negative regulator of Nrf2, Keap1, initiating Keap1 dissociation with Cullin3,

thereby inducing Nrf2-directed gene expression. Lipidomics analyses of oxidized EPA have indicated the presence of novel cyclopentenone-containing molecules termed J3-isoprostanes in vitro and in vivo and have been shown to mediate Nrf2-directed gene expression. It seems likely that formation of J-ring compounds generated from oxidation of EPA and DHA in vivo can reach concentrations high enough to induce Nrf2-based cellular defense systems (Gao et al., 2007). These results support the view that DHA may be an effective dietary supplement in the management of various diseases in which oxidant/antioxidant defense mechanisms are disturbed (Erdogan et al., 2004). It is proposed that DHA also exerts a beneficial effect, not only by antagonizing the metabolism of arachidonic acid and its downstream metabolites, but also by modulating oxidative stress. In addition, DHA downregulates NF- κ B via a PPAR- γ -dependent pathway. Collective evidence suggests that PPAR- γ activation by EPA and DHA may be another underlying mechanism for the beneficial effects of fish oil.

Treatment of Zellweger syndrome patients with purified DHA partially improves visual function, increases levels of plasmalogens, and reduces levels of saturated very long-chain fatty acids (Martínez et al., 2000). The level of DHA is also low in patients with multiple sclerosis (Fig. 8.8). Fish oil supplements with vitamins improve the clinical outcome in patients (Nordvik et al., 2000). DHA supplementation reduces stress-induced aggression in students, so DHA may have psychotropic effects (Hamazaki et al., 1996).

Many other neurological disorders are characterized by DHA deficiency, including infantile Refsum disease, neonatal adrenoleukodystrophy, X-linked adrenoleukodystrophy, and adrenomyeloneuropathy (Martínez et al., 2000). Retinitis pigmentosa and Usher's syndrome have low levels of DHA in the retina (Anderson et al., 1999). Low levels of DHA are also associated with depression and attention-deficit hyperactivity disorders (Burgess et al., 2000). DHA is decreased in cultured skin fibroblasts from schizophrenic patients compared to bipolar patients and normal subjects (Mahadik et al., 1996). DHA must have a special place in neuronal function to be involved in so many neurological disorders. Supplementation of diet with DHA in the above-mentioned human pathological conditions indicates that DHA has many positive effects on human health (Hodge et al., 2006), but large double-blind human trials are required recommendation of DHA consumption at a larger scale.

8.6.3 Dietary DHA and Cancer

It is well known that AA and its metabolites are closely associated with cancer cell proliferation and cancer biology. Epidemiological studies strongly link fish oil with low incidences of several types of cancer. The inhibitory effects of DHA on cancer development and progression are based on studies with cultured cells and animal models. The exact molecular mechanism associated with the effect

of DHA on breast cancer is not fully understood. However, it is proposed that DHA decreases cell proliferation and induces apoptotic cell death in human breast cancer cells, possibly by decreasing signal transduction through the Akt/ NF- κ B cell survival pathway (Schley et al., 2005). DHA also downregulates the expression of Her-2/neu oncogene in human breast cancer cells. It is shown that n-3 fatty acids act at the transcriptional level, which, in turn, interacts synergistically with anti-HER2 trastuzumab-based immunotherapy during cancer treatment. Furthermore, the involvement of suppression of tumor eicosanoid biosynthesis, reduction of nitric oxide synthase activity, increased lipid peroxidation, and neutral sphingomyelinase in inhibition of breast cancer growth in nude mice by dietary fish oil and inhibition of breast cancer cell growth in culture by treatment with DHA has also been reported (Rose et al., 1995, 2005). These results provide the rationale for in vivo preclinical investigations in primates. Fat-1 mice are a useful model for elucidating the role of n-6/n-3 fatty acid ratio in tumorigenesis because they can convert n-6 to n-3 fatty acids and have a balanced ratio of n-6 to n-3 fatty acids in their tissues and organs, independent of diet (Kang et al., 2004). Studies on implantation of mouse melanoma B16 cells into transgenic and WT littermates indicate a dramatic decrease in melanoma formation and growth in fat-1 transgenic mice (Xia et al., 2006). The levels of n-3 fatty acids and their lipid mediator prostaglandin E₃ (PGE₃) are



Fig. 8.9 Beneficial effects of DHA on non-neural disorders

markedly elevated, but the n-6/n-3 ratio is much lower, in the tumor and surrounding tissues of fat-1 mice than that of WT animals. The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) gene is significantly upregulated in the fat-1 mice. In vitro experiments indicate that addition of n-3fatty acid eicosapentaenoic acid or PGE₃ prevents the growth of B16 cell line and upregulates the expression of PTEN. This can be partially attenuated by the inhibition of PGE₃ synthesis, suggesting that PGE₃ may act as an antitumor lipid mediator (Xia et al., 2006). These studies demonstrate that an anti-cancer (antimelanoma) effect of n-3 fatty acids may be in part through the activation of PTEN pathway mediated by PGE₃ (Xia et al., 2006). In addition, dietary n-3fatty acids improve responsiveness of cancer cells to chemotherapy with doxorubicin, mitomycin, and cyclophosphamide due to their ability to enhance membrane permeability to drugs (Stillwell and Jenski, 2002). DHA and EPA also have beneficial effects in heart disease, rheumatoid arthritis, autoimmune diseases, psoriasis, diabetes, cystic fibrosis, obesity, and sepsis (Fig. 8.9).

8.7 The Adverse Effects of DHA

Despite the above benefits, there have been several concerns about the safety of DHA. Antithrombotic effects of fish oils may promote the risk for bleeding and increase the risk of hemorrhagic stroke (Rabbani et al., 2001; Clarke et al., 2005; Mozaffarian and Rimm, 2006; Bays, 2007; Harris, 2007). In rats, high intake of n-3 fatty acids is associated with severe motor impairment and a poorer functional outcome (Clarke et al., 2005). A significant reduction in absolute amount of serum HDL and a significant increase in relative liver and spleen weights are seen in both male and female rats with the high dose of fish oil concentrate. High doses of fish oil concentrate decrease serum iron and vitamin E levels (Rabbani et al., 2001). A reduction in osmotic fragility of red blood cells and an increase in red blood cells deformity occur in rats treated with high doses of fish oil concentrate. These rats show a significant overall increase in white blood cells count. Collectively, these studies indicate that in rats, subchronic consumption of high levels of fish oil concentrate can be beneficial, but may also have side effects such as increased red cell deformity, increased relative liver and spleen weights, and reduced serum HDL, iron, and vitamin E concentrations (Rabbani et al., 2001; Bays, 2007; Harris, 2007). The susceptibility of DHA preparations to undergo lipid peroxidation contributes to patient intolerance and potential toxicity. Finally, consumption of large amounts of fish may cause exposure to environmental toxins such as mercury, polychlorinated biphenyls, dioxins, and other contaminants. Thus, physicians and health professionals should be aware of the available guidelines provided by FDA and EPA to assess the relative safety of fish oil preparation (Mozaffarian and Rimm, 2006; Bays, 2007; Harris, 2007). Although, in vivo data on this topic are inconclusive, due in part to limitations in the methodologies, recent studies on

the measurement of neuroprostanes, a reliable measure of in vivo lipid peroxidation and oxidative stress, do not support the above views on adverse effects of DHA (Mori, 2004). This is temping to speculate that benefits of fish oil intake exceed its potential health risks.

Fish oil supplementation may not be beneficial for everyone (Englyst et al., 2007). The molecular mechanism associated with susceptibility of some human subjects to fish oil and not others is not understood. However, it is suggested that resistance to activated protein C (APC), a serine protease with potent antiinflammatory effects, may play an important role. Since APC resistance is modulated with the nature of fatty acids in glycerophospholipids, analysis of APC resistance along with protein concentrations and platelet fatty acids are important parameters for coagulant activity. In clotting assay, APC ratio indicates increased anticoagulant activity. Fish oil supplementation in healthy men decreases the APC ratio in plasma. The decrease in APC ratio equates to an increase in APC resistance. Fish oil reduces the APC ratio by (a) increasing lowdensity lipoprotein (LDL) cholesterol and apolipoprotein B and (b) increasing platelet microparticles. In vitro, purified LDL reduces the APC ratio and increases microparticle formation. These changes affect the anticoagulant APC may contribute toward a prothrombotic state potentially explaining the observation that fish oil supplementation may not always be of benefit (Englyst et al., 2007).

8.8 Conclusion

DHA is a 22-carbon essential fatty acid with six double bonds. It is mostly located at the sn-2 position of glycerol moiety of plasmalogens and phosphatidylserine. Acyl chain length and the number of double bonds present in fatty acids have a major influence on the physical properties of glycerophospholipids that contain them. In the retina, DHA-enriched glycerophospholipids are highly concentrated in the immediate environment of rhodopsin and play an important role in visual transduction. Although details of the molecular mechanisms remain unknown, DHA incorporation into neural membrane glycerophospholipids not only alters their physicochemical properties, but also markedly affects the signal transduction processes associated with optimal brain function. Thus, dietary intake of DHA is important for normal mental function. In the brain, DHA and its metabolites modulate neurotransmitter release, gene expression, immunity and inflammation, learning and memory, apoptosis, and activities of membrane-bound enzymes, ion channels, and receptors. Inadequate amounts of DHA are linked to a wide variety of abnormalities ranging from visual acuity to learning and memory. DHA deficiency is associated with AD and many other neurological disorders such as schizophrenia, depression, hyperactivity, and stroke. The involvement of DHA in so many neurological disorders suggests that this fatty acid provides neural membranes with specific physicochemical properties and also modulates specific genes involved in neurological disorders. Long-term DHA supplementation may not only restore signal transduction processes associated with behavioral deficits and learning activity but also produce several neuroendocrinological and immunological effects on brain tissue.

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Chapter 9 Effects of Statins and *n*–3 Fatty Acids on Heart and Brain Tissues: The Clash of the Titans

9.1 Introduction

Statins are HMG-CoA (3-hydroxy-3-methylglutaryl co-enzyme A) reductase inhibitors that significantly reduce risk for cardiovascular and cerebrovascular diseases (Endres, 2005; Vaughan, 2003). Beneficial effects of statins in cardiovascular and cerebrovascular systems are due to their anti-excitotoxic, antioxidant, and anti-inflammatory properties. In general, statins are well-tolerated drugs, but in some individuals they may cause a variety of skeletal muscle-associated abnormalities ranging from muscle pain to rhabdomyolysis. Little is known about mechanisms associated with statin-mediated muscle abnormalities. However, it is proposed that muscle abnormalities may be due to statin-mediated mitochondrial abnormalities along with the inhibition of ubiquinone, isoprenoids, and dolichols synthesis (Kaufmann et al., 2006). These processes result into uncoupling of oxidative phosphorylation and consequent release of cytochrome c initiating apoptotic cell death. Fish oil n-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid, have similar anti-excitotoxic, antioxidant, and anti-inflammatory effects in cardiovascular and cerebrovascular tissues (Horrocks and Farooqui, 2004; Farooqui and Horrocks, 2007; Farooqui et al., 2007a). These effects of *n*-3 fatty acids may be due to the generation of recently discovered lipid mediators called resolvins, protectins, and neuroprotectins. These lipid mediators not only retard inflammation and oxidative stress but also prevent apoptotic cell death. Like statins, ingredients of fish oil inhibit generation of β -amyloid and stabilize the integrity of membranes, reduce platelet aggregation, and improve endothelial cell function. Collective evidence suggests that antioxidant, anti-inflammatory, and anti-apoptotic properties of statins and fish oil may contribute to the clinical efficacy of treating cardiovascular and cerebrovascular disorders with statins and fish oil. Based on present knowledge, it can be proposed that there is an overlap between biochemical events associated with cardiovascular and cerebrovascular injuries (Farooqui et al., 2007a).

9.2 Properties, Metabolic Sites and Mechanism of Action of Statins

Statins, the most popular drugs of this decade, competitively inhibit HMG-CoA reductase and retard cholesterol synthesis by blocking the formation of mevalonate (Fig. 9.1). Statins are classified into three categories: naturally occurring statins, semisynthetic statins, and synthetic statins. The naturally occurring statins include lovastatin and pravastatin. Semisynthetic statins are represented by simvastatin, and the synthetic statins include atorvastatin, fluvastatin, cerivastatin, rosuvastatin, and pitavastatin. Structural differences among statins (Figs. 9.2 and 9.3) determine their lipophilicity, half-lives, and potency in mammalian tissues. Some statins such as mevastatin, lovastatin, and simvastatin are lipophilic and have a closed-ring structure (lactone) while others have an open ring structure (pravastatin and fluvastatin). Lovastatin reduces cholesterol contents in both cytofacial and exofacial membrane leaflets while



Fig. 9.1 Biosynthesis of cholesterol, ubiquinone, and dolichol in brain. 3-Hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase (1); farnesylpyrophosphate (FPP) synthase (2); squalene synthase (3); farnesyltransferase (4); geranylgeranylpyrophosphate (GGPP) synthase (5); Geranylgeranyltransferase I (6); isopentenylpyrophosphate (IPP); geranylpyrophosphate (GPP); farnesylpyrophosphate (FPP); farnesyl protein transferase (FPTase); geranylgeranylpyrophosphate (GGPP); geranylgeranyl protein transferase (GGPTase); and endothelial nitric oxide synthase (eNOS). Notice that HMG-CoA reductase is inhibited by statins and fish oil



Fig.9.2 Chemical structures of statins. Mevastatin (compactin) (**a**); lovastatin (Mevacor) (**b**); cerivastatin (Baycol) (**c**); fluvastatin (lesocol RXL) (**d**); and atorvastatin (Lipitor) (**e**)

simvastatin reduces cholesterol in cytofacial membrane leaflet. Because of their lipophilicity simvastatin, lovastatin, and cerivastatin pass the blood-brain barrier. In contrast, due to their hydrophilicity pravastatin, fluvastatin, and atorvastatin do not pass the blood-brain barrier to any significant extent (Vuletic et al., 2006). Although hydrophilic statins, such as rosuvastatin and pravastatin, do not penetrate the cell membrane as efficiently as the lipophilic statins, they have similar affects in humans as the lipophilic statins. Lipophilic statins are more susceptible to metabolism by the cytochrome P450 system, except for pitavastatin, which undergoes limited metabolism via this pathway. Pravastatin and rosuvastatin are relatively hydrophilic and not significantly metabolized by cytochrome P450 enzymes. All statins enter liver. Lipophilic statins are efficient through uptake system while hydrophilic statins are taken up by active carrier-mediated processes. In recent years, drug companies have developed many statins. These statins have different chemical and commercial names (Table 9.1), but do not differ significantly in their IC50 values for HMG-CoA reductase (Table 9.2). In addition to cholesterol-lowering properties, statins have many different effects. They modulate enzymic activities, gene expression, and have a variety of "pleiotropic effects" in visceral and brain tissues (Johnson-Anuna et al., 2005, 2007; Kirsch et al., 2003).



Fig. 9.3 Chemical structures of more statins. Pitavastatin (a); Ezetimibe (b); rosuvastatin (Crestor) (c); pravastatin (Pravachol) (d); and simvastatin (Zocor) (e)

| Generic name | Trademark | Pleiotropic effects | Reference |
|------------------------------|------------------|--|--|
| Atorvastatin | Lipitor | Antioxidant/anti- inflammatory | Amarenco (2005); Endres (2005) |
| Lovastatin | Mevacor, Altocor | Antioxidant/anti- inflammatory, antithrombotic | Amarenco (2005); Endres (2005) |
| Cerivastatin Lipobay, Baycol | | Antioxidant/anti- inflammatory, antithrombotic | Rajanikant et al. (2007); Vaughan (2003) |
| Fluvastatin | LescolRXL | Antioxidant/anti- inflammatory | Endres (2005); Vaughan (2003) |
| Mevastatin | Compactin | Antioxidant/anti- inflammatory, antithrombotic | Amarenco (2005); Vaughan (2003) |
| Pitavastatin | Livalo, Pitava | Antioxidant/anti- inflammatory, antithrombotic | Amarenco (2005); Vaughan (2003) |
| Pravastatin | Pravachol | Antioxidant/anti- inflammatory, antithrombotic | Rajanikant et al. (2007); Vaughan (2003) |

Table 9.1 Statins, their commercial names and pleiotropic effects

| Generic name | Trademark | Pleiotropic effects | Reference |
|----------------------------|--------------|--|----------------------------------|
| Rosuvastatin | Crestor | Antioxidant/anti- inflammatory, antithrombotic | Endres (2005); Vaughan (2003) |
| Simvastatin | Zocor, Lipex | Antioxidant/anti- inflammatory, antithrombotic | Endres (2005); Vaughan (2003) |
| Ezetimibe + Simvastatin | Vytorin | Antioxidant/anti- inflammatory, antithrombotic | Endres (2005); Vaughan (2003) |

Table 9.1 (continued)

| Fable 9.2 | IC50 | values o | of | statins | for | HMC | G-CoA | reductase |
|------------------|------|----------|----|---------|-----|-----|-------|-----------|
| | | | | | | | | |

| Statin | IC50 (nM) |
|-----------------|---------------|
| Mevastatin | 23 |
| Simvastatin | 11 |
| Fluvastatin | 28 |
| Cerivastatin | 10 |
| Atorvastatin | 8 |
| Rosuvastatin | 5 |
| G . 1.6 G 1.(20 | 001) 0 1 1 11 |

Summarized from Stuve et al. (2003b); Schmitz and Langmann (2006); Vaughan (2003); and Rajanikant et al. (2007).

Mevalonate, the key intermediate of cholesterol pathway, is a precursor for not only cholesterol but also for ubiquinone, dolichols, and other isoprenoids (Laufs and Liao, 2000). Isoprenoids include geranylpyrophosphate, farnesylpyrophosphate, geranylgeranylpyrophosphate, and squalene. Farnesylpyrophosphate and geranylgeranylpyrophosphate serve as important lipid attachments for post-translational modification of proteins including heterotrimeric G-proteins and small GTP-binding proteins. Inhibition of isoprenoid synthesis by statins suppresses Rho GTPase activity and relocates Rho GTPase to the cytoplasm, rendering it inactive. This suggests that isoprenylation is a critical step for activity and trafficking of many proteins inducing their translocation and covalent attachment. Thus, a statin-mediated decrease in isoprenoid intermediates may have harmful metabolic consequences for neural and non-neural cells (Laufs and Liao, 2000; Holstein and Hohl, 2004; Endres, 2005).

Co-enzyme Q10 (CoQ10, a ubiquinone) is an important lipophilic molecule, which transfers electrons from mitochondrial respiratory chain complexes I and II to complex III (Beal, 2004). Co-enzyme Q is the only lipid-soluble antioxidant synthesized endogenously. It is synthesized in the inner mitochondrial membrane. CoQ10 consists of a ubiquinone head group attached to a tail of 10 five-carbon isoprenoid units that anchors the molecule to the membrane. CoQ10 functions as an antioxidant and protects mitochondrial inner-membrane proteins and DNA against oxidative damage during oxidative stress (Fig. 9.4) (Albano et al., 2002). CoQ10 also functions as membrane stabilizer.



Fig. 9.4 Role of co-enzyme Q in cardiovascular and cerebrovascular systems

Long-term treatment with stating decreases CoO10 levels resulting in energy metabolism impairment in heart, skeletal muscle, and liver (Wilkins and Bliznakov, 1998). This may be associated with cardiomyopathy. Supplementation of the diet with CoQ10 can reverse many symptoms of myopathy (Langsjoen et al., 2005). In its reduced form, CoOH2 (ubiquinol) inhibits protein and DNA oxidation and its effect on lipid peroxidation has been thoroughly investigated. Ubiquinol not only blocks the peroxidation of cell membrane lipids, but also retards the peroxidation of lipoprotein lipids present in the circulation (Littarru and Tiano, 2007). In apolipoprotein E-deficient mice fed with a high-fat diet, CoQ10 has a direct anti-atherogenic effect. In this model, supplementation with CoQ10 at pharmacological doses reduces the absolute concentration of lipid hydroperoxides in atherosclerotic lesions minimizing the size of atherosclerotic lesions in the whole aorta. Furthermore, CoQ10 not only has a direct effect on endothelial function, but also modulates the expression of genes involved in human cell signaling, metabolism, and transport (Littarru and Tiano, 2007).

The most common side effect of statin is muscle pain and weakness, a condition called rhabdomyolysis (Fig. 9.5), most likely due to the depletion of CoQ10, a nutrient that supports muscle function. During cholesterol synthesis, statins block synthesis of farnesylpyrophosphate, an intermediate in the synthesis of ubiquinone or CoQ10 (Fig. 9.1). This fact, plus the role of CoQ10 in mitochondrial energy production, has prompted the hypothesis that statin-induced CoQ10 deficiency is involved in the pathogenesis of statin myopathy. This hypothesis is supported by studies that indicate the relationship between statin therapy and lower plasma ubiquinone level (Chu et al., 2006). Collective evidence suggests that there is insufficient evidence to prove the etiologic role of



Fig. 9.5 Side effects of statins in cardiovascular and cerebrovascular systems

CoQ10 deficiency in statin-associated myopathy and that large double-blind well-designed clinical trials are required to address this issue.

9.3 Composition of Fish Oil and Its Importance in Human Nutrition

Brain contains n-3 polyunsaturated fatty acids (docosahexaenoic acid, DHA and eicosahexaenoic acid, EPA) and n-6 (arachidonic acid, AA) polyunsaturated fatty acids, which are mainly located at the sn-2 position of glycerol moiety of neural membrane glycerophospholipids. Brain tissue lacks the ability to synthesize n-3 and n-6 through de novo synthesis. Although astrocytes can synthesize some DHA from α -linolenic acid (18:3n-3, ALA) (Williard et al., 2002), it is not enough to meet the need of brain tissue. Therefore, n-3 and n-6fatty acids are obtained either directly from the diet or synthesized from their main dietary n-3 precursors (ALA) and linoleic acid (LA) in the liver (Igarashi et al., 2007b, a). Fish oil is the richest source of n-3 fatty acids. n-6 fatty acids are enriched in vegetable oils from corn, sunflower seeds, cottonseed, and soybean. Diet enriched in n-6 fatty acid shifts the physiologic state not only to one that is prothrombic and proaggregatory with increased blood viscosity, but also to provasospasmic with decreases in bleeding time. In contrast, diet enriched in n-3 fatty acids is hypolipidimic, antithrombotic, and anti-inflammatory. Both n-6 and n-3 fatty acids are essential and have beneficial effects on human health, but it is the ratio between n-6 to n-3 fatty acid in diet that is crucial. Our ancestors lived and survived on a diet, which had n-6 and n-3 fatty acid ratio of 1:1. Changes in eating habits (natural versus processed food) and agriculture development within the past 100–200 years have changed the n-6 to n-3 fatty acid ratio in favor of omega-6 fatty acids. Thus, the present day Western diet has n-6 to n-3 ratio of 15:1 (Simopoulos, 2002, 2004, 2006; Cordain et al., 2005). This diet promotes the pathogenesis of many chronic diseases such as heart disease, cancer, autoimmune diseases, and neurodegenerative diseases. In contrast, diet enriched in n-3 fatty acids exerts cardioprotective, immunosuppressive, and neuroprotective effects (Simopoulos, 2006), suggesting that the ratio between n-6 and n-3 fatty acid is very important in human diet.

These days fish oil is available in the form of a purified liquid with different flavors (Table 9.3). Several human trials are underway with FDA-approved fish oil preparation "Omacor" (Davidson et al., 2007). For vegetarians *n*–3 fat preparation "O-Mega-Zen^{3TM}" is commercially available. "O-Mega-Zen^{3TM}" is derived from golden marine algae with little fishy odor or aftertaste. These fish oil preparations increase intracellular degradation of apolipoprotein B-100-containing lipoproteins, lower plasma triacylglycerol level, depress LDL, increase bleeding time, downregulate gene expression for platelet-derived growth factor, lower blood pressure, decrease the number of endothelial adhesion molecules, improve lipoprotein size, and decrease the risk of cardiovascular and cerebrovascular diseases.

In heart and brain tissue, the incorporation of n-3 and n-6 fatty acids not only influences physicochemical properties membranes (fluidity, permeability), but also modulates expression of many genes (De Caterina and Massaro, 2005; Deckelbaum et al., 2006). The metabolic utilization of n-3 fatty acids differs from that of n-6 fatty acid metabolism. Oxygenation of n-6 fatty acids generates pro-inflammatory mediators such as prostaglandins, leukotrienes, and

| Commercial name | Manufacturer |
|---------------------------------|--|
| Purified fish oil | Carlson Laboratories Inc., Arlington Heights, IL |
| Omegaven TM | Kabi Bad Homburg, Germany |
| Lipoplus TM (LMF541) | B. Braun Melsungen, AG |
| OmacorTM | Reliant Pharmaceutical Inc., Corner, NJ |
| Cardiozen TM | Equazen Nutraceuticals, London, UK |
| DHActive TMCL | Nutrinova, Montpellier, France |
| O-Mega-Zen ^{3™} | Nu Tru Inc., Lincolnwood, IL |
| Vegan Omega-3 | Deva Nutrition, Chelsea, AL |
| Krill oil | Jedwards International Inc., Quincy, MA |

 Table 9.3 Commercial names and manufacturers of fish oil preparations

thromboxanes (eicosanoids). In contrast, oxygenation of EPA by cyclooxygenases and 5-lipoxygenase generates 3-series prostaglandins, thromboxanes, and 5-series leukotrienes. EPA-derived eicosanoids (thromboxanes A_3 , B_3 and leukotriene B_5) are much less active than AA-derived eicosanoids. EPA-derived resolving E1 reduces inflammation by suppressing the activation of nuclear factor- κB (NF- κB) and consequently the expression and synthesis of cytokines and chemokines. At nanomolar levels, RvE1 dramatically reduced dermal inflammation, peritonitis, dendritic cell migration, and interleukin IL-12 production. Its action is mediated by the ChemR23 receptor. Treatment of dendritic cells with small-interference RNA specific for ChemR23 sharply reduces RvE1 regulation of IL-12 (Arita et al., 2005a). These results demonstrate novel counter-regulatory responses in inflammation initiated via RvE1 receptor activation that provide the first evidence for potent endogenous agonists of antiinflammation derived from EPA (Arita et al., 2005a, b). Another possible mechanism of RvE1 may be that this metabolite prevents the binding of LTB4 to its receptor and therefore blocks the propagation of a pro-inflammatory signal.

DHA is not a substrate for cyclooxygenase. Actions of a 15-lipoxygenaselike enzyme on DHA produce docosanoids (17S-resolvins, 10-,17S-docosatrienes, protectins and neuroprotectins) (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Serhan and Savill, 2005; Farooqui and Horrocks, 2006; Phillis et al., 2006; Farooqui et al., 2007b). Excessive production of eicosanoids results in inflammation and oxidative stress that ultimately produce cellular injury, whereas generation of docosanoids is cardioprotective and neuroprotective and retards cellular injury and cellular death (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Serhan and Savill, 2005; Phillis et al., 2006; Farooqui et al., 2007b). Collectively, these studies suggest that n-3 fatty acids oppose the effect of n-6 fatty acid-derived metabolites by promoting the synthesis of less active thromboxanes A₃, B₃, and leukotriene B₅ as well as docosanoids.

9.4 Biochemical Effects of Statins on Cardiovascular System

Lowering of cholesterol levels is not the primary mechanism of beneficial effects of statins (Bottorff, 2006; Chen et al., 2003; Cucchiara and Kasner, 2001). Statins produce beneficial effects due to their "pleiotropic" (non-lipid-lowering actions) effects such as stabilization of atherosclerotic plaques. The "pleiotropic" effects of statins include improvement in endothelial function, enhancement of the stability of atherosclerotic plaques, increase in blood flow, reduction in oxidative stress and inflammation, and inhibition of the thrombogenic response (Fig. 9.6). In addition, growing evidence indicates that statins lower plasma levels of high-sensitivity C-reactive protein (hs-CRP), an important indicator of inflammation and predictor of cardiovascular events, in a manner



Fig. 9.6 Pleiotropic effects of statins in cardiovascular and cerebrovascular systems

largely independent of LDL-cholesterol lowering. The "pleiotropic" effects of statins in visceral tissues also include the activation of enzymes such as protein kinase Akt, endothelial nitric oxide synthases, inhibition of apoptosis in endothelial cells (Fig. 9.7), and upregulation of angiogenesis (Mason, 2006; Li et al., 2006; Endres, 2005).



Fig. 9.7 Modulation of enzymic activities by statins

Statins activate the protein kinase Akt/PKB in endothelial cells and this process is related to nitric oxide synthase. Thus, in vitro simulatin-mediated phosphorylation of the endogenous Akt substrate, endothelial nitric oxide synthase (eNOS), not only prevents apoptosis but also stimulates vascular structure formation in an Akt-dependent manner. It is proposed that similar to vascular endothelial growth factor (VEGF) treatment, both simvastatin treatment and stimulated Akt signaling in the endothelium promote angiogenesis in ischemic limbs of normocholesterolemic rabbits (Kureishi et al., 2000). In contrast, the Rho/Rho-kinase (ROCK) pathway downregulates the expression of eNOS suggesting the involvement of two distinct mechanisms for modulating eNOS activity in endothelial cells (Rikitake and Liao, 2005). The mechanism of this process probably involves, in part, ROCK-mediated changes in the actin cytoskeleton, which leads to a decrease in eNOS mRNA stability. The regulation of eNOS by PtdIns 3 K/Akt and Rho GTPases pathways, therefore, may be an important mechanism underlying the cardiovascular protective effect of statins. By increasing NO generation, statins may interfere with atherosclerotic lesion development, stabilize plaque, inhibit platelet aggregation, improve blood flow, and protect against ischemia. Therefore, the ability of statins to improve endothelial function through the release of NO may partially account for their beneficial effects at reducing the incidence of cardiovascular events. It should be noted that higher doses of statins inhibit endothelial cell migration and angiogenesis. Thus, stating have biphasic potential to either promote or inhibit angiogenesis. Low statin doses induce a pro-angiogenic effect through Akt activation and increase nitric oxide production, whereas high statin doses may decrease protein prenylation and inhibit cell growth. Furthermore, low doses of statins reduce metalloproteinase expression (Furman et al., 2004). This enzyme modifies elements of the atherosclerotic plaques and has implications for plaque stability.

The anti-inflammatory effects of the statins are due to their ability to reduce the levels of isoprenyl intermediates in the cholesterol biosynthetic pathway. These effects of statins are not retarded by exogenous cholesterol supplementation, but the addition of the isoprenyl precursors, mevalonic acid, and geranylgeranylpyrophosphate prevents the statin-mediated downregulation of oxidative stress and inflammation. Similar to statin's effect, treatment with GGT1-286 (an inhibitor of protein isoprenylation) blocks the inflammatory response. These observations support the view that the anti-inflammatory effects of statins are distinct from their cholesterol-lowering effects (Cordle and Landreth, 2005). These anti-inflammatory and antioxidant effects of statins contribute to the clinical efficacy of statins in treating cardiovascular as well as cerebrovascular diseases in humans (Stoll et al., 2004). Physicochemical properties and molecular differences of statins affect their metabolism, solubility, and intramembrane localization. This in turn can influence the efficacy and safety of these drugs. In addition, these properties may have a differential impact on the pleiotropic effects of statins, including their ability to improve endothelial function and to affect proliferation and apoptosis in vascular tissues (Mason, 2006). Collectively, these studies suggest that statins not only inhibit an early step in the cholesterol biosynthetic pathway, but also retard the synthesis of isoprenoids such as farnesylpyrophosphate and geranylgeranylpyrophosphate, which are important post-translational lipid attachments for intracellular signaling molecules such as the Rho GTPases (Fig. 9.1). Indeed, decrease in Rho GTPase responses as a consequence of statin treatment increases the production and bioavailability of endothelium-derived NO. The mechanism involves, in part, Rho/Rho-kinase (ROCK)-mediated changes in the actin cytoskeleton, which leads to decrease in the stability of eNOS mRNA. The regulation of eNOS by Rho GTPases, therefore, may be an important mechanism underlying the cardiovascular protective effect of statins (Rikitake and Liao J.K, 2005).

In addition to their effects on endothelial cells and platelets, statins may have important anti-inflammatory effects in the heart tissue. Inflammation is closely linked to the production of reactive oxygen species (ROS), which are derived from many sources including mitochondria, xanthine oxidase, uncoupled nitric oxide synthases, and NADPH oxidases. NADPH oxidases are a major source of super-oxide in cardiovascular system. Superoxide-mediated oxidative stress promotes endothelial dysfunction. The molecular basis of anti-inflammatory and antioxidant effects of statins may relate to their ability to block the production and/or activity of ROS (Stoll et al., 2004). Statins inhibit ROS generation not only through interference with NADPH oxidase expression and superoxide production but also through the upregulation of nitric oxide synthase, the enzyme that generates NO (Fig. 9.7). As stated above, the release of NO from the endothelium regulates blood flow, inflammation, and platelet aggregation; and consequently its disruption during endothelial dysfunction can decrease plaque stability encouraging the formation of atherosclerotic lesions and thrombi (Laufs, 2003).

Lipoprotein-associated phospholipase A₂ (Lp-PLA₂), a member of the phospholipase superfamily, circulates bound primarily to low-density lipoprotein. It is associated with cardiovascular disease risk in epidemiologic studies. This enzyme also hydrolyzes platelet-activating factor (PAF). An increase in its activity is an index for cardiovascular and cerebrovascular diseases (Caslake and Packard, 2003; Saougos et al., 2007). Its reaction products (lysophosphatidylcholine and oxidized nonesterified fatty acids) mediate the pro-inflammatory role of Lp-PLA₂. These bioactive lipid mediators are generated in lesion-prone vasculature and to a lesser extent in the circulation (e.g., in electronegative LDL). They elicit several inflammatory responses. Statins inhibit Lp-PLA₂ activity (Nambi and Ballantyne, 2006; Saougos et al., 2007). Thus, statins decrease Lp-PLA₂ activity by 26% in cardiac patients. The change in Lp-PLA₂ may be related to changes in low-density lipoprotein cholesterol. These studies indicate greater differences in patients with coronary heart disease compared with controls in $Lp-PLA_2$ in the fed state than in the fasting state. Among various statins, atorvastatin is more effective than fluvastatin, lovastatin, pravastatin, or simvastatin in decreasing not only low-density lipoprotein cholesterol, but also C-reactive protein (CRP) and Lp-PLA₂ activity (Schaefer et al., 2005).

The expression of sPLA₂-IIA is negatively regulated by RhoA/Rho-associated kinase and MEK/ERK signaling pathways and statins, due to their downregulatory effect on these pathways, can potentiate the IFN- γ -mediated sPLA₂-II expression at transcriptional and post-transcriptional levels (Menschikowski et al., 2005). sPLA₂ is pro-atherogenic in the circulation and in the arterial wall (Hurt-Camejo et al., 2001). In blood, this enzyme modifies the circulating lipoproteins and so induces the formation of small dense LDL particles that are associated with increased risk for cardiovascular diseases. Statins also increase arachidonic acid synthesis in hypercholesterolemic patients through increased $\Delta 5$ desaturation (Rise et al., 2001). Furthermore, simvastatin represses the high glucose-mediated Rho GTPase/p21 signaling in glomerular mesangial cells. Thus, these studies provide another molecular basis for the use of statins, independently of their cholesterol-lowering effect, in early stages of diabetic nephropathy (Danesh et al., 2002).

It must be noted that sudden withdrawal of statin treatment may acutely impair vascular function and increase morbidity and mortality in patients with cardiovascular and cerebrovascular diseases (Endres, 2006). This is because statin withdrawal increases oxidative stress, which elicits endothelial dysfunction. Studies on mice have indicated that statin withdrawal-mediated animal death is common in normal mice, but does not occur in gp91^{phox} knockout mice (gp91^{phox-/-}) (Vecchione and Brandes, 2002). The molecular mechanism involved in this process remains unknown. However, it is known that in human umbilical vein endothelial cells, statins retard the association of NADPH oxidase-activating Rac-1. This process causes an increase in GTPase activity in whole-cell lysates. Withdrawal of statins from human umbilical vein endothelial cells induces the translocation of Rac-1 from the cytosol to the membrane and transiently increases NADPH-induced lucigenin chemiluminescence in membrane preparations. Rac-1 inactivation by Clostridium difficile toxin B blocks the statin-mediated oxygen radical production in human umbilical vein endothelial cells. These observations indicate that the withdrawal of statins induces endothelial dysfunction. The underlying mechanism associated with this may involve the activation of a gp91^{phox} containing NADPH oxidase by Rac-1 and the subsequent scavenging of endothelium-derived NO by superoxide anions generated from this enzyme (Vecchione and Brandes, 2002; Jung et al., 2004). These observations indicate that oxidative stress may be the causal event in statin-mediated morbidity.

9.5 Biochemical Effects of Statins on Brain

Statins regulate activities of many enzymes (Fig. 9.7) including nitric oxide synthase, NADPH oxidase, paraoxonase, protein kinases, Lp-PLA₂, cycloox-ygenase-2, and HMG-CoA reductase. Statins also modulate cytokine and chemokine gene expression in brain tissue (Fig. 9.8) (Stepien et al., 2005;



Fig. 9.8 Modulation of immune function, gene expression, and excitotoxicity by statins

Schmitz and Langmann, 2006). Like cardiovascular "pleiotropic," statins have anti-excitotoxic, anti-inflammatory, and antioxidant effects in brain tissue. These effects result in neuroprotection through the improvement in platelet and endothelial cell function, increase in blood flow, and reduction in oxidative stress and inflammation.

Neurochemical effects of statins on enzymic activities can be divided into two categories: cholesterol-independent effects and cholesterol-dependent effects. The effects depend upon not only the chemical nature of statin but also pharmacokinetic behavior, tissue distribution, metabolism, and bioavailability. For example interactions of statins with other drugs such as cyclosporin, erythromycin, and itraconazole may inhibit cytochrome P450 enzymes and increase bioavailability of statins leading to their harmful effects such as myotoxicity (Williams and Feely, 2002).

9.5.1 Cholesterol-Independent Effects of Statins

In some experimental model of ischemia, statins act through cholesterol-independent effect and enhance functional outcome and neural plasticity. Atorvastatin induces phosphorylation of Akt and Erk in cultured primary cortical neurons. The molecular mechanism through which statins modulate neuroplasticity and improvement of neurologic outcome in stroke patients is not fully understood. However, this may be due to statin-induced increase in the expression of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) (Chen et al., 2005). Collectively, these studies indicate that statins promote synaptogenesis, neurogenesis, and angiogenesis in a rat model of stroke (Fig. 9.8) (Chen et al., 2003, 2005).

Inhibition of α - and β -secretase pathways by statins directly effects the processing of amyloid precursor protein (Sjögren et al., 2003, 2006). Statins robustly block the A β -mediated expression of interleukin-1 β and inducible nitric oxide synthase and the production of nitric oxide in microglia and monocytes (Cordle and Landreth, 2005). Statin treatment also prevents the rac1-dependent activation of NADPH oxidase and superoxide production. As stated above, these anti-inflammatory actions of the statins are due to their ability to inhibit the levels of isoprenyl intermediates in the cholesterol biosynthetic pathway (Cordle and Landreth, 2005; Cordle et al., 2005). The addition of the isoprenyl precursors, mevalonic acid, and geranylgeranylpyrophosphate (GGpp) attenuates the statin-mediated downregulation of inflammatory markers. Inhibition of protein isoprenylation through the blockage of Rho-family function by *Clostridium difficile* toxin A retards the inflammatory response suggesting that the anti-inflammatory actions of statins are due to the inhibition of isoprenylation (Koudinov and Koudinova, 2003).

Treatment of rat hippocampal neurons with pravastatin induces neuritogenesis. This neuritogenesis is accompanied by increase in levels of phosphatidylcholine (Pooler et al., 2006). Detailed investigations have indicated that pravastatin treatment increases neurite length and branching, but does not affect neural cell levels of cholesterol (Pooler et al., 2006). Co-incubation of mevalonate, but not cholesterol, blocks the stimulatory effect of pravastatin on neurite outgrowth. Addition of isoprenoids also prevents the pravastatin-induced increase in neurite growth. This observation indicates the involvement of isoprenylation signaling through the Rho family of small GTPases (Pooler et al., 2006). Specific inhibitors of geranylgeranylation, but not farnesylation, increase the stimulatory effect of pravastatin on neuritogenesis. Thus, many pleiotropic effects of statins are mediated through the inhibition of isoprenoids, which serve as lipid attachments for intracellular signaling molecules. In particular, inhibition of the small GTPbinding proteins, Rho, Ras, and Rac, whose proper membrane localization and function are dependent on isoprenylation, may play an important role in mediating the pleiotropic effects of statins (Liao and Laufs, 2005). ROCK1 is the immediate downstream target of Rho A. Although ROCKS have been implicated in several cellular functions such as cell shape, motility, secretion, proliferation, regulation of vascular tone, inflammation, oxidative stress, and gene expression it is not known how ROCKS are regulated, what some of their downstream targets are, and whether ROCK1 and ROCK2 are involved in different cellular functions (Liao and Laufs, 2005). It is becoming increasingly evident that the RhoA/ROCK pathway is closely associated with pathophysiology of cardiovascular disease and that inhibition of ROCK pathway by ROCK inhibitors or statin may be beneficial for heart disease.

In neurons and glial cells, cholesterol maintains microtubule stability through the modulation of the tau phosphorylation state (Fan et al., 2001). Compactin, a HMG-CoA reductase inhibitor, and TU-2078, a squalene epoxidase inhibitor, inhibit dendrite outgrowth, but not that of axons, and attenuate axonal branching (Fan et al., 2002). Compactin-induced alterations can be prevented by concurrent treatment of cultured neurons with β -migrating very-low-density lipoproteins or cholesterol. Thus, experimental manipulation of cholesterol levels in neural membrane may alter tau and MAP2 phosphorylation inducing a selective inhibition of dendrite outgrowth due to the decreased stability of microtubules (Fan et al., 2002; Ohm and Meske, 2006).

9.5.2 Cholesterol-Dependent Effects of Statins

In mouse brain neuronal cultures, statins protect neurons from NMDAmediated excitotoxic damage through a cholesterol-dependent mechanism (Fig. 9.9). The rank order of neuroprotective potency is rosuvastatin =simvastatin > atorvastatin = mevastatin > pravastatin. This rank of potency is similar to the known rank order of potency for inhibition of the HMG-CoA reductase. This effect is cholesterol dependent because alterations in cholesterol by β -cyclodextrin modulate excitotoxicity (Zacco et al., 2003). Simvastatin protects neurons from oxygen and glucose deprivation (OGD)/reoxygenation-mediated neuronal death by blocking the generation and cytotoxicity of 4-hydroxynonenal (4-HNE), the final product of arachidonic acid peroxidation (Lim et al., 2006). Based on cell culture studies, it is proposed that 4-HNE mediates the cytotoxicity by synergistically increasing the N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, and simvastatin largely retards the potentiation of NMDA-mediated neurotoxicity by 4-HNE. Simvastatin also reverses the inhibition of NF- κ B activity induced by OGD/reoxygenation or 4-HNE (Lim et al., 2006). Simvastatin-mediated neuroprotection is significantly attenuated by various NF- κ B inhibitors, implying that simvastatin inhibits the cytotoxicity of 4-HNE at least in part by maintaining the activity of NF- κ B. Furthermore, simvastatin is directly known to ameliorate the cytotoxicity of 4-HNE (Zacco et al., 2003). In kainic acid-mediated neurotoxicity, lovastatin protects neurons by preventing the accumulation of 24-hydroxycholesterol and inhibiting 24-hydroxylase (Fig. 9.9) (He et al., 2006).

Collective evidence suggests that in addition to effects on cerebrovascular function, statins promote resistance to NMDA-mediated excitotoxic death as a result of changes in cellular glycerophospholipid and cholesterol homeostasis. Statins also modulate gene expression; these gene expression patterns vary considerably from one statin to another, and may be statin specific. Thus, lovastatin, pravastatin, and simvastatin alter the expression of genes associated with cell growth and signaling, trafficking, and survival proteins. The overlap in effects of the three statins on gene expression is quite high. However,



Fig. 9.9 Anti-excitotoxic, antioxidant, and anti-inflammatory effects of statins and fish oil on signal transduction process in brain tissue. Cytosolic phospholipase A₂ (cPLA₂); secretory phospholipase A₂ (sPLA₂); arachidonic acid (AA); platelet-activating factor (PAF); 4-hydro-xynonenal (4-HNE); reactive oxygen species (ROS); inhibitory form of nuclear factor kappa B (I κ B/NF- κ B); nuclear factor κ B-response element (NF- κ B-RE); inhibitory subunit of NF- κ B (I κ B); tumor necrosis factor- α (TNF- α); interleukin-1 β (IL-1 β); interleukin –6 (IL-6); cyclooxygenase-2 (COX-2); inducible nitric oxide synthase (iNOS); positive sign (+) indicates stimulation; and negative sign (–) indicates inhibition

simvastatin alters expression of more genes than the other statins (Johnson-Anuna et al., 2005, 2007). Bcl-2 is a pro-survival member of the Bcl-2 protein family and one of the most studied regulators of cell survival. The neuroprotective effect of simvastatin is due to the stimulation of Bcl-2 gene expression resulting from chronic drug treatment (Johnson-Anuna et al., 2007).

Lovastatin attenuates experimental autoimmune encephalomyelitis (EAE) with significant alterations in the expression of immunity-related genes in treated animals compared to untreated EAE controls (Paintlia et al., 2004). Statins modulate the expression of genes, which encode for leukocyte-specific markers and receptors, histocompatibility complex, cytokines/receptors, chemokines/receptors, adhesion molecules, components of the complement cascade, cellular activation, transcription factors, and signal transduction-related molecules. Interestingly, $T_{\rm H}^2$ phenotype cytokines such as interleukin-4,

interleukin-10, and transforming growth factor- β 1 and transcription factors such as peroxisome proliferator-activated receptor- γ (PPAR- γ) are upregulated in lumbar spinal cord by lovastatin. These findings suggest that PPARs-induced signal transduction processes may mediate the anti-inflammatory and immunomodulatory effects of lovastatin (Paintlia et al., 2004).

Statins also cause immunomodulatory effects in brain tissue. The molecular mechanism of statin-mediated immunomodulatory changes in brain is not known. However, this process may involve the inhibition of post-translational protein prenylation of small GTP-binding proteins (Weber et al., 2006). Statins also downregulate chemokine expression and chemokine receptor expression in human endothelial cells and macrophages via inhibition of the geranylgeranylpyrophosphate pathway (Veillard et al., 2006). Statins may also act by blocking the induction of MHC-II expression by INF- γ and thus as repressors of MHC-II-mediated T-cell activation (Kwak et al., 2000). Thus, statins have antiinflammatory properties beyond their lipid-lowering effects. Collectively, these studies suggest that stating may have both beneficial as well as harmful effects in brain tissue. The risks of muscle adverse events related to use of statins increase significantly with the addition of interacting drugs to a patient's therapy. The mechanism for most statin drug interactions involves the cytochrome P450 system, which provides an indication of which drugs may interact (Bottorff, 2006). However, it is difficult to predict the probability of a drug interaction in a given patient because of individual differences in sensitivity to increased statin drug levels. Drug metabolism studies show simvastatin and lovastatin to be especially sensitive to the inhibiting effects of other drugs on the cytochrome P450 3A4 (CYP3A4) isozyme (Bottorff, 2006).

9.6 Biochemical Effects of Fish Oil on Heart

In non-neural tissues, the dietary uptake of n-3 fatty acids reduces pro-atherogenic cytokines, improves endothelial function, reduces vascular occlusion, and mitigates the course of coronary atherosclerosis. The heart rate is reduced, and the heart rate variability is increased. An anti-arrhythmic effect is also observed at the supraventricular and the ventricular levels (von Schacky, 2006). Replacement of n-6 with n-3 fatty acids in membrane glycerophospholipids decreases the transcriptional activation of many genes including adhesion molecules, chemoattractants, and inflammatory cytokines (De Caterina and Massaro, 2005). These genes mediate responses to inflammatory and pro-inflammatory stimuli through the downregulation of the NF- κ B system of transcription factors (Fig. 9.9). Such regulation of gene expression by n-3 fatty acids may be related to the presence of six double bonds in the fatty acid chain in n-3compared with n-6 fatty acids. By similar mechanisms, n-3 fatty acids downregulate cyclooxygenase-2 gene and upregulate metalloproteinase gene in plaque angiogenesis and plaque rupture. The quenching of gene expression of pro-inflammatory pro-atherogenic genes by n-3 fatty acids has consequences on the extent of leukocyte adhesion to vascular endothelium, early atherogenesis, and later stages of plaque development and plaque rupture, ultimately yielding a plausible comprehensive explanation for the vasculoprotective effects of n-3 fatty acids (De Caterina and Massaro, 2005).

DHA and EPA differ from each other not only in expression of specific genes but also in many biochemical and physicochemical effects on neural and nonneural cells (Verlengia et al., 2004). For example EPA is metabolized by cyclooxygenase and 5-lipoxygenase generating 3-series prostaglandins, thromboxanes, and 5-series leukotrienes. These eicosanoids are less active than AA-derived eicosanoids. In contrast, DHA is not metabolized by cyclooxygenases. EPA is hypotriglyceridemic and hypocholesterolemic. DHA has a lesser effect on plasma triacylglycerols than EPA (Hashimoto et al., 1999). DHA is less effective than EPA in inhibiting vascular smooth muscle proliferation. DHA is a more potent inhibitor of lymphocyte adhesion to endothelial cells than EPA (Hashimoto et al., 1999).

DHA inhibits voltage-stimulated Na⁺ channels, whilst EPA has no effect on membrane excitability or Na⁺ channels in hippocampal neurons (Xiao and Li, 1999) Similarly, DHA modulates certain voltage-gated K⁺ channels in Chinese hamster ovary cells, whereas EPA has no effect on K⁺ channels. EPA modulates DHA synthesis in SH-SY5Y neuroblastoma cell cultures (Fig. 9.10). EPA has antidepressant and antipsychotic activity whilst DHA does not. Quantitation of the mRNA levels of genes encoding for several key enzymes of both the endoplasmic reticulum and peroxisomal steps of fatty acid metabolism indicates that EPA downregulates the enzymes involved in DHA synthesis



Fig. 9.10 Effects of fish oil (n-3 fatty acids) on brain and vascular system

and decreases DHA synthesis from its precursor, α -linolenic acid (Poumès-Ballihaut et al., 2001; Langelier et al., 2005). Collectively, these studies suggest that EPA and DHA differ on their effects on plasma lipid profiles, gene expression, and neural membrane structure.

n-3 fatty acids have similar effects on cholesterol synthesis in non-neural cells. Thus in Reuber H35 hepatoma cells, EPA and DHA downregulates HMG-CoA reductase (Bastianetto and Quirion, 2004; Duncan et al., 2005; Choi et al., 1989), a rate-limiting intrinsic membrane protein (96 kDa), whose proteolysis releases an enzymically active soluble fragment (52–56 kDa) that regulates the synthesis of cholesterol. The downregulation of HMG-CoA reductase by EPA and DHA is not due to decreased protein synthesis, since similar levels of protein are found in the presence and absence of this fatty acid. In contrast, myristic acid upregulates HMG-CoA reductase protein; therefore, protein synthesis is required for the increase of HMG-CoA reductase activity (Bastianetto and Quirion, 2004).

9.7 Biochemical Effects of Fish Oil on Brain

Actions of a 15-lipoxygenase-like enzyme on DHA produce 17S-resolvins, 10, 17S-docosatrienes, and protectins (Hong et al., 2003; Marcheselli et al., 2003) (Serhan et al., 2004; Serhan and Savill, 2005). As stated in Chapters 1, 2, and 8, these second messengers are collectively called docosanoids. They are potent endogenous anti-inflammatory and pro-resolving chemical lipid mediators (Serhan, 2006). They antagonize the effects of eicosanoids, the lipid mediators of n-6 fatty acids, modulate leukocyte trafficking, and downregulate the expression of cytokines in glial cells (Hong et al., 2003) (Marcheselli et al., 2003; Mukherjee et al., 2004; Serhan et al., 2004). Aspirin impinges on these systems, inducing formation of the epimeric 17R-series RvDs - denoted as "aspirintriggered RvDs" - which possess bioactivity in vivo equivalent to that evoked by their 17S-series counterparts (i.e., RvDs). Like eicosanoids, docosanoids are stereoselective agonists that act through specific receptors and modulate the duration and magnitude of inflammation. Docosanoid receptors include resolvin D receptors (resoDR1), resolvin E receptors (resoER1), and neuroprotectin D receptors (NPDR). Characterization of these receptors in brain tissue is in progress (Hong et al., 2003; Marcheselli et al., 2003; Serhan et al., 2004; Mukherjee et al., 2004). The generation of n-3 fatty acid metabolites may be an internal anti-inflammatory protective mechanism for preventing heart and brain damage in cardiovascular and cerebrovascular diseases. The discovery of docosanoids opens new avenues and approaches to therapeutic interventions via accelerated resolution of inflammation (Hong et al., 2003; Marcheselli et al., 2003; Serhan, 2005); Mukherjee et al., 2004; Bazan, 2005b; Ariel et al., 2005).

DHA retards soluble β -amyloid oligomer-mediated neuronal apoptosis and significantly increases neuronal survival by preventing cytoskeleton perturbations,

caspase activation, and promoting extra signal-related kinase pathways (Park et al., 2004). DHA attenuates endothelial cyclooxygenase-2 induction through NADPH oxidase and protein kinase C ϵ inhibition (Massaro et al., 2006). DHA promotes antioxidant response of human fibroblasts by upregulating γ -glutamyl-cysteinyl ligase and glutathione reductase activities (Arab et al., 2006). Docosanoids retard inflammation and oxidative stress through several mechanisms including upregulation of γ -glutamyl-cysteinyl ligase and glutathione reductase activities, inhibition of p65 subunit transcription factor NF- κ B, preventing cyto-kine secretion, blocking the synthesis of eicosanoids, blocking toll-like receptor-mediated activation of macrophages, and reducing leukocyte trafficking (Faroo-qui and Horrocks, 2006; Farooqui et al., 2006; Farooqui and Horrocks, 2007; Duffield et al., 2006; Massaro et al., 2006; Li et al., 2005a).

The dietary depletion of DHA stimulates caspases and downregulates NMDA receptors in Tg2576 mouse brain. Dietary supplementation of DHA partially protects the mice from NMDA receptor subunit loss (Calon et al., 2005). DHA also promotes neuronal survival not only by facilitating neural membrane translocation/activation of Akt but also through its ability to increase PtdSer levels in neural membranes (Akbar et al., 2005). In addition, DHA stimulates extracellular signal-regulated kinase in photoreceptors (German et al., 2006) and enhances cerebral activities of catalase and glutathione peroxidase and increases levels of glutathione in the cerebral cortex (Hossain et al., 1999). DHA also modulates physiological processes such as long-term potentiation and memory processes, and inhibits arachidonic acid and its metabolite-mediated oxidative stress in brain tissue (Fujita et al., 2001; McGahon et al., 1999; Hashimoto et al., 2006). DHA consumption reduces reactive oxygen species in the hippocampus of A β -infused and aged rats (Hossain et al., 1998, 1999).

Collective evidence suggests that n-3 fatty acids have many beneficial effects on brain tissue through their action on neural membrane structure and serving as precursors for potent lipid mediators. They are involved not only in cognitive development, memory-related learning, plasticity of nerve membranes, synaptogenesis, and synaptic transmission but also in neuroprotection and neural cell survival. Recent trial in 1000 free living subjects, the intake of n-3 fatty acids is independently associated with lower levels of pro-inflammatory markers (IL-6, IL-1ra, TNF- α , C-reactive protein) and higher levels of anti-inflammatory markers (soluble IL-6r, IL-10, TGF- β) (Ferrucci et al., 2006).

9.8 Therapeutic Value of Statins and DHA in Cardiovascular and Cerebrovascular Systems Disorders

9.8.1 Statins in Cardiovascular System

Statins are the most commonly prescribed drugs for the prevention of cardiovascular disease. By blocking cholesterol synthesis in the liver, statins activate hepatocyte low-density lipoprotein (LDL) receptors and produce reductions in circulating LDL cholesterol with improvements in cardiovascular risk by retarding atherosclerosis in all major arteries (Shepherd, 2006). In addition to their vascular effects, such as stabilization of atherosclerotic plaques and decreased carotid intimal-medial thickness, statins have additional properties such as endothelial protection via actions on the nitric oxide synthase system as well as antioxidant, anti-inflammatory, anti-platelet aggregation, and antithrombotic effects.

Type 2 diabetes is a major risk factor (two- to four fold) for coronary heart disease and stroke. Endothelial cells dysfunction is an initial trigger of the progression of atherosclerosis in patients with diabetes mellitus and hyperglycemia (Nakagami et al., 2005). NADPH oxidase, a major source of superoxide in cardiovascular cells, can be activated in hyperglycemia through the protein kinase C pathway. From the viewpoint of these molecular mechanisms, statins inhibit the high glucose-induced NADPH oxidase activation through inhibition of Rac activity and finally prevent the increase in ROS production in diabetes (Nakagami et al., 2005). Paraoxonase, a phosphotriesterase that acts as an antioxidant in cardiovascular system, is also stimulated by statins. This enzyme is associated with serum high-density lipoprotein (HDL)-containing apolipoprotein AI (apoAI) and prevents low-density lipoprotein (LDL) peroxidation (Muacevic-Katanec et al. 2007).

In cardiovascular system, statins both upregulate endothelial nitric oxide synthase (eNOS) and inhibit inducible nitric oxide synthase (iNOS). They improve arterial tone by inducing local nitric oxide-mediated vascular relaxation that limits acetylcholine-induced vasoconstriction (Andersson et al., 1990). These effects are potentially neuroprotective. Statins influence platelet function by enhancing availability of nitric oxide, reducing the generation of thromboxane, and decreasing membrane cholesterol content (Vaughan et al., 2001). In addition, statins protect the myocardium against ischemia-reperfusion injury via a mechanism unrelated to cholesterol-lowering effect. Statins inhibit isoprenylation and thereby prevent activation of proteins such as RhoA. It is proposed that the cardioprotection and the increase of the RhoA cytosol-tomembrane ratio mediated by statins in vivo are inhibited by geranylgeranylpyrophosphate. The modulation of cytosol/membrane-bound RhoA is an important factor for the protective effect of statins against myocardial ischemia-reperfusion injury (Bulhak et al., 2007).

9.8.2 Stains in Cerebrovascular System

Statins affect brain function by inhibiting rac-dependent activation of NADPH oxidase, retarding cytokine upregulation, and downregulating isoprenylation of microglial and macroglial cell signaling proteins. Statins retard glutamatemediated neurotoxicity in cortical cultures (Zacco et al., 2003; Bosel et al., 2005). Whether this anti-excitotoxic effects can be achieved in vivo with tolerable doses of statins remains to be seen. The effects of statins on brain may also vary with respect to their ability to cross the blood–brain barrier (Sparks et al., 2002). Statins modulate cerebral blood flow by upregulating endothelial nitric oxide synthase and decreasing infarct volume following focal ischemic injury in rats (Laufs and Liao, 2000; Sironi et al., 2003). Collective evidence suggests that statins have potential therapeutic implications in acute neural trauma (stroke, spinal cord trauma, and head injury) and neurodegenerative diseases (Alzheimer disease, Parkinson disease, multiple sclerosis, and primary brain tumors) (Cucchiara and Kasner, 2001; Rajanikant et al., 2007; Stuve et al., 2003b) (Table 9.4).

Thus, statins reduce the incidence of ischemic stroke through stabilizing precerebral atherosclerotic plaques and decreasing infarct size and also by antithrombotic mechanisms mediated by statins involving inhibition of metalloproteinase activity (Vaughan et al., 2001; Stepien et al., 2005). Statins also attenuate the inflammatory cytokine responses that accompany cerebral ischemia. They possess antioxidant properties that likely ameliorate ischemic oxidative stress in the brain. Statin administration reduces the levels of molecular markers of inflammation such as NF- κ B, ICAM-1, and iNOS (Pahan et al., 1997; Sironi et al., 2006). Statins may also act by inhibiting the synthesis of isoprenoid intermediates in the cholesterol biosynthetic pathway. Isoprenoid

| Neurological | a | Mechanism of | D 0 |
|-----------------------------|-----------------------------|--|---|
| disorders | Statin | action | References |
| Ischemia | Atorvastatin | Anti-inflammatory, antioxidant | Tuñón et al. (2007) |
| Spinal cord trauma | Atorvastatin | Anti-inflammatory, | Pannu et al. (2005) |
| | Simvastatin | antioxidant Anti-inflammatory, | Holmberg et al. (2006) |
| | | antioxidant | |
| Head injury | Atorvastatin | Anti-inflammatory, antioxidant | Qu et al. (2005); Wang et al. (2007) |
| Alzheimer disease | Atorvastatin Simvastatin | Anti-inflammatory, antioxidant Anti-inflammatory, antioxidant | Sparks et al. (2005) Petanceska et al. (2002); Eckert et al. (2005) |
| Multiple sclerosis | Atorvastatin | Anti-inflammatory | Neuhaus et al. (2005) |
| EAE | Lovastatin | Anti-inflammatory | Paintlia et al. (2006); Stuve et al. (2003a) |
| Neurofibromatosis type 1 | Lovastatin | Anti-inflammatory, antioxidant | Li et al. (2005b) |
| Migraine | Statin | Anti-inflammatory, antioxidant | Liberopoulos and Mikhailidis (2006) |
| Parkinson disease | Statin | Anti-inflammatory, antioxidant | Lieberman et al. (2005) |

 Table 9.4
 Therapeutic effects of statins in animal models of acute neural trauma and neurodegenerative diseases

intermediate are important lipid attachments for intracellular signaling molecules. They may have direct noncholesterol-dependent effects on inflammatory and endothelial cells. In addition to reducing stroke, statins exhibit a number of important neuroprotective properties (antioxidant and anti-inflammatory properties) that may attenuate the effects of ischemia on the brain vasculature and parenchyma.

Alzheimer disease (AD) is the most common and irreversible neurodegenerative disorder. It is characterized by a progressive memory deficit and cognitive impairment accompanied by the appearance of two pathological hallmarks in specific brain areas: neurofibrillary tangles and amyloid plaques (Yamada and Toshitaka, 2002). β -Amyloid peptide (A β), the major component of the senile plaques, is the end product of the abnormal post-translational processing of its precursor, amyloid precursor protein (APP). The compartmentation of the APP molecule within the cell membrane is regulated by the cholesterol content of the lipid bilayers. The APP molecule can be present either inside or outside of the membrane rafts, which are enriched in cholesterol and sphingolipids (Urano et al., 2005).

Statins activate α -secretase and reduce A β generation and amyloid accumulation in a transgenic mouse model of AD (Pedrini et al., 2005; Zimmermann et al., 2005). Statins inhibit the isoprenoid pathway, thereby modulating the activities of the Rho family of small GTPases, Rho A, B, and C. Statins also block activities of Rac and cdc42. Rho proteins, in turn, exert many of their effects via Rho-associated protein kinases (ROCKs) (Pedrini et al., 2005). In cell cultures, statin-mediated reduction in A β production correlates with an inhibition of β -secretase dimerization into its more active form at several concentrations of statin (Parsons et al., 2006). These effects can be reversed by the administration of mevalonate indicating the involvement of pathways dependent on 3-hydroxy-3-methylglutaryl-CoA. At a low statin concentration (1 μ M), reduction in A β production and inhibition of β -secretase dimerization are mediated by inhibition of isoprenoid synthesis, but at high (>10 μ M) concentrations of statins, an inhibition of β -secretase palmitovlation occurs. Statins also modulate the phosphorylation of tau in humans and this effect may depend on the CNS availability of statins. This may be another mechanism by which statins reduce the risk of AD (Riekse et al., 2006). Collective evidence suggests that statins are novel and powerful drugs to study cerebrovascular biology, including protein isoprenylation, small G-protein function, antioxidant and anti-inflammatory activities, and endothelial cell function. Based on results in several studies, it is proposed that the long-term use of low doses of statins, starting as early as possible during AD development, may slow down or even prevent the progression of AD. This has resulted in several patents (Wolozin, 2002).

Statins through their antioxidant and anti-inflammatory effects may not only retard the oxidative effects of reactive oxygen species, but also inhibit the release of pro-inflammatory cytokines (Sparks et al., 2005). Administration of simvastatin enhances memory and protects against the development of behavioral deficits in an established mouse model of AD (Li et al., 2006). Statins inhibit an A β -mediated inflammatory response through their ability to prevent the isoprenylation of members of the Rho family of small G-proteins, resulting in the functional inactivation of these G-proteins (Cordle et al., 2005; Cole et al., 2005b). Treatment of microglia with statins results in perturbation of the cytoskeleton and morphological changes due to alteration in Rho family function. Statins also retard A β -stimulated phagocytosis through inhibition of Rac action. Paradoxically, the statin-mediated inactivation of G-protein function is associated with increased GTP loading of Rac and RhoA. Statin treatment also disrupts the interaction of Rac with its negative regulator, the Rho guanine nucleotide dissociation inhibitor (RhoGDI), an interaction that is dependent on protein isoprenylation (Cordle et al., 2005; Cordle and Landreth, 2005).

In a rat model of spinal cord injury (SCI), atorvastatin treatment results in attenuation of the SCI-induced expression of iNOS, TNF- α , and IL-1 β (Pannu et al., 2005; Holmberg et al., 2006; Pannu et al., 2007). Atorvastatin also protects against SCI-induced tissue necrosis, neuronal and oligodendrocyte apoptosis, demyelination, and reactive gliosis. Furthermore, rats treated with atorvastatin score much higher on the locomotor rating scale after SCI than untreated rats. Collectively, these studies indicate that atorvastatin may have beneficial effect on SCI-related neurochemical changes and pathology.

In an animal model of traumatic brain injury (TBI), atorvastatin administration increases neuronal survival in the CA3 region and the lesion boundary zone, and prevents the loss of neuronal processes of damaged neurons in the hippocampal CA3 region, but not in the lesion boundary zone on day 15 after TBI. The protective effect of atorvastatin on the injured neurons perhaps is mediated by increasing the density of vessels in the lesion boundary zone and the hippocampus after TBI. Collective evidence suggests that statins may promote significant restoration of spatial memory but not reduction of sensorimotor functional deficits in TBI (Qu et al., 2005).

Statins exert pleiotropic immunomodulatory effects. These effects may be of therapeutic benefit in autoimmune disorders (Paintlia et al., 2004, 2006; Stuve et al., 2003a). The combination of lovastatin and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside lessens inflammation-associated neurodegeneration in the central nervous system of experimental autoimmune encephalomyelitis (EAE). The primary mechanism by which statins alter immune function appears to be mediated through the inhibition of post-translational protein prenylation of small GTP-binding proteins and is largely independent of lipid lowering (Weber et al., 2006). Collectively, these studies suggest that in EAE, a mouse model for multiple sclerosis (MS), statins prevent or reverse paralysis. Treatment of scrapie-infected mice with simvastatin significantly prolongs their survival times (193 versus 183 days). These results indicate that low-dosage simvastatin treatment affects the progression of experimental scrapie and supports the concept that statin treatment may influence prion pathogenesis (Kempster et al., 2007).

9.8.3 n–3 Fatty Acids in Cardiovascular System

In cardiovascular system, the dietary n-3 fatty acids downregulate pro-atherogenic cytokines, improve endothelial function, reduce vascular occlusion, and mitigate the course of coronary atherosclerosis. They reduce heart rate and stabilize heart rhythm (von Schacky, 2006). Replacement of *n*-6 with *n*-3 fatty acids in heart muscle glycerophospholipids downregulates genes for adhesion molecules, chemoattractants, and inflammatory cytokines (De Caterina and Massaro, 2005). These genes mediate responses to inflammatory and proinflammatory stimuli through the downregulation of the NF- κ B system of transcription factors (Fig. 9.9). In addition, n-3 fatty acids downregulate cyclooxygenase-2 gene and upregulate metalloproteinase gene in plaque angiogenesis and plaque rupture (De Caterina and Massaro, 2005). FDA-approved n-3 preparation (OmacorTM) decreases cardiovascular deaths and mainly fatal arrhythmias after myocardial infarction (Ducobu, 2005; Pater et al., 2003). At present, attempts are being made to develop novel DHA-derived lipid mediator-based compounds that can selectively downregulate neuroinflammatory responses in heart tissues.

n-3 fatty acids affect not only heart but other visceral organs. OmacorTM has been used for the treatment of IgA nephropathy (Donadio and Grande, 2004). n-3 PUFA prevents renal disease progression by interfering with a number of effector pathways triggered by mesangial immune-complex deposition.

9.8.4 n-3 Fatty Acids in Cerebrovascular System

It is becoming increasingly evident that oxidative stress and inflammation plays a major role in pathogenesis of neurological disorders, such as AD, PD, stroke, traumatic brain and spinal cord injuries (Farooqui and Horrocks, 2007). A substantial body of biochemical and clinical data supports the use of n-3 fatty acids as anti-inflammatory agents (Mori and Beilin, 2004) (Table 9.5). They reduce oxidative stress and neuroinflammation in several ways: (a) they decrease the formation of AA by blocking the activity of Δ 5-desaturase; (b) they inhibit the synthesis of eicosanoids (Calder, 2005); and (c) they promote and facilitate the generation of resolvins and neuroprotectins (Serhan, 2005, 2006); Bazan, 2005c, a). All these processes decrease oxidative stress and inflammation in brain tissue.

n–3 fatty acids play an important role in normal neurological and cognitive function (Horrocks and Farooqui, 2004). Levels of DHA are markedly decreased in neural membranes obtained from brains of healthy elderly people and also from patients with neurological disorders (Bechoua et al., 2003; Horrocks and Farooqui, 2004). This DHA deficiency may be responsible for the loss of synapses in AD. It correlates not only with cognitive decline and accumulation of A β but also with generation of elevated levels of F₄-

| Neurological | | |
|-------------------------------------|---|---|
| disorder | Mechanism of action | Reference |
| Ischemia | Anti-inflammatory, antioxidant, anti- excitotoxic | Högyes et al. (2003); Bas et al. (2007) |
| Alzheimer disease | Anti-inflammatory, antioxidant, anti- excitotoxic | Hashimoto et al. (2005); Olivo and Hilakivi- Clarke (2005); Cole et al. (2005a) |
| Parkinson disease | Anti-inflammatory, antioxidant, anti- excitotoxic | Samadi et al. (2006); de Lau et al. (2005) |
| Epilepsy | antioxidant, anti- excitotoxic | Yuen et al. (2005) |
| Spinal cord injury | Anti-inflammatory, antioxidant, anti- excitotoxic | King et al. (2006) |
| Multiple sclerosis | Anti-inflammatory, antioxidant | Nordvik et al. (2000) |
| AIDS dementia | Anti-inflammatory, antioxidant, anti- excitotoxic | Das (2005); Pocernich et al. (2005) |
| Glaucoma | Anti-inflammatory, antioxidant | Ren et al. (2006); Hodge et al. (2006) |
| Schizophrenia | Anti-inflammatory, antioxidant, anti- excitotoxic | Horrobin (2003); McNamara et al. (2006) |
| Depression and mood disorders | Anti-inflammatory, antioxidant | Zanarini and Frankenburg (2003); McNamara et al. (2006); Freeman (2006); Leonard and Myint (2006) |

Table 9.5 Therapeutic effects of fish oil (n-3 fatty acids) in animal models of acute neural trauma and neurodegenerative diseases

isoprostanes in aged as well as AD brain tissue. DHA-enriched diets can prevent these effects (Calon et al., 2004; Olivo and Hilakivi-Clarke, 2005). Chronic pre-administration of DHA blocks β -amyloid-mediated impairment of an avoidance ability-related memory function in a rat model of AD (Hashimoto et al., 2005) and protects mice from synaptic loss and dendritic pathology in another model of AD (Calon et al., 2004). DHA modulates amyloid precursor protein processing by inhibiting α - and β -secretase activities (de Wilde et al., 2003; Walsh and Selkoe, 2004; Olivo and Hilakivi-Clarke, 2005), and inhibits β -amyloid secretion from aging brain cells through the synthesis of neuroprotectin D1, which retards apoptotic cell death induced by β -amyloid. Thus, DHA is beneficial in preventing the learning deficiencies in animal AD models (Lukiw et al., 2005).

DHA protects the brain against ischemic and excitotoxic damage in rat brain and hippocampal slice cultures (Strokin et al., 2006). It inhibits oxidative stressmediated apoptotic changes in hippocampus of ischemic rats. Thus, dietary supplementation of n-3 fatty acids may be beneficial against ischemic cerebral vascular disease (Bas et al., 2007).

DHA administration reduces L-DOPA-induced dyskinesias in monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Samadi et al., 2006). This suggests that DHA can reduce the severity or delay the development of L-DOPA-induced dyskinesias in a nonhuman primate model of Parkinson disease. A DHA-enriched diet may represent a new approach to improve the quality of life of Parkinson disease (PD) patients.

EPA and DHA have also been used for the treatment of Huntington disease (HD), which is caused by a mutation in exon 1 of the huntingtin gene that encodes a stretch of polyglutamine (polyQ) residues close to the N-terminus of the huntingtin protein. Randomized, placebo-controlled, double-blind studies show that highly unsaturated fatty acids are beneficial to HD patients (Puri, 2005; Das and Vaddadi, 2004), suggesting that unsaturated fatty acids may either prevent or arrest polyQ aggregation, inhibit histone deacetylation, or activate the ubiquitin-proteasome system (Das and Vaddadi, 2004).

Spinal cord injury (SCI) is accompanied by glutamate release, free fatty acid generation, and production of pro-inflammatory eicosanoids. These metabolites induce excitotoxicity and oxidative stress and inflammation. α -Linolenic acid (LNA) and DHA have been used for the treatment of rats with spinal cord injury. Thus, the administration of LNA or DHA 30 min after spinal cord hemisection injury in adult rats results in increased neuronal and glial survival, and a significantly improved neurological outcome (King et al., 2006). In severe compression model of SCI, intravenous DHA administration results in better neurological outcome than saline-injected rats. The acute DHA treatment increases neuronal and glial cell survival. The analysis of spinal cord tissue after DHA administration indicates that the DHA significantly decreases lipid peroxidation, protein oxidation and RNA/DNA oxidation, and the induction of COX-2. Parallel studies in a facial nerve injury model in mice also show proregenerative effects of chronic dietary administration of DHA after nerve lesion. These observations suggest that treatment with n-3 fatty acids may represent a promising therapeutic approach in the management of spinal cord injury (King et al., 2006; Michael-Titus, 2007).

9.9 Effects of Combination of Statin and Fish Oil in Cardiovascular and Neurological Disorders

Administration of a combination of statins and EPA indicates that serum total cholesterol and triglyceride levels are significantly decreased 3 months after the statins and EPA administration, and serum high-density lipoprotein cholesterol concentrations are significantly increased. Plasma EPA concentrations and the ratio to arachidonic acid in plasma are also significantly increased 3 months after the treatment. These results suggest that the combination therapy of statin

and EPA combination may be effective for patients with hyperlipidemia (Nakamura et al., 1999). Also, atorvastatin and n-3 fatty acids have independent and additive effects in correcting dyslipidemia in viscerally obese men. Improvement in abnormalities in remnant lipoproteins may occur with use of atorvastatin only. Thus combination treatment with statin and fish oil may, however, offer an optimal therapeutic approach for globally correcting dyslipidemia in obesity (Chan et al., 2002).

Collective evidence from cardiovascular studies indicates that n-3 fatty acids efficiently potentiate the cholesterol-lowering effect of statins, counteract the fasting insulin-elevating effect of statins, and, unlike statins, do not decrease serum levels of β -carotene and CoQ10 (ubiquinol-10) (Jula et al., 2005; Tavazzi et al., 2004). These are small trials. Thus, large-scale, randomized, double-blind trials are needed to test the efficacy of combination of statin and n-3 fatty acid in cardiovascular and cerebrovascular disorders.

9.10 Adverse Effects of Statins and *n*–3 Fatty Acids

Although statins are commonly used drugs for the treatment of hypercholesterolemia and dyslipidemia associated with coronary heart disease and atherosclerosis, beneficial effects of statin therapy are accompanied by their toxicity. In cardiovascular system the myotoxic side effects range from mild myopathy (an inflammation of the muscles) to serious rhabdomyolysis (muscle toxicity with marked CK elevation) (Wilkins and Bliznakov, 1998; Kaufmann et al., 2006). Statin-mediated rhabdomyolysis produces skeletal muscle injury leading to fatal irreversible renal damage through a series of biochemical reactions (Fig. 9.5) (Tiwari et al., 2006). Statin-mediated myopathy may lead to myoglobulinuria secondary to rhabdomyolysis. It is a rare but potentially fatal complication of statin therapy. Cerivastatin (baycol), a statin approved for use in humans, has been voluntarily withdrawn from the market by Bayer because of many deaths caused by rhabdomyolysis.

Cytochrome P450 3A4 is the main isoenzyme involved in statin metabolism. Reduced activity of this enzyme due to either reduced expression or inhibition by other drugs (cyclosporin or itraconazole) may increase plasma concentrations of statin not only leading occasionally to myotoxicity but also in asymptomatic elevations in serum levels of aminotransaminases (Williams and Feely, 2002). In addition, statins are substrates for P-glycoprotein, a drug transporter present in the small intestine that may influence their oral bioavailability. Statins may also alter the concentrations of other drugs such as warfarin or digoxin. This effect may interfere with clinical monitoring. Thus, the knowledge of the pharmacokinetic properties of the statins is necessary for avoiding the majority of drug interactions and complication (Williams and Feely, 2002). The risk of myopathy with statin therapy can be exacerbated by compromised hepatic and renal function, hypothyroidism, and diabetes. The use of statins at high dose may cause increased incidence of nausea, vomiting, stomach pain, brown or dark-colored urine, yellowish eyes, cataract, neoplasia, sleep disturbance, skin problems, and some psychiatric disorders (Wilkins and Bliznakov, 1998; Langsjoen et al., 2005; Buajordet et al., 1997). Long-term use of statins may be involved in drug-induced lupus erythematosus and other autoimmune disorders.

Adverse effects of statins in the brain tissue include aggression, nervousness, depression, anxiety, sleeping disorders, memory loss, confusion, and impotence (Wilkins and Bliznakov, 1998; Langsjoen et al., 2005; Buajordet et al., 1997). In some patients, long-term use of statins causes statin-induced peripheral polyneuropathy. The mechanisms associated with statin-induced neuropathy remain unknown. However, it is proposed that inhibition of HMG-CoA reductase may alter neural membrane function because cholesterol is an important component. Furthermore, statins also inhibit the synthesis of ubiquinone disturbing neuronal energy utilization and thereby promoting neuropathy (Gaist et al. 2002). These findings emphasize the need to be vigilant in looking for these reactions as they can have a significant personal impact on a patient (Tatley and Savage, 2007). More adverse effects may be caused by antiproliferative and proapoptotic properties of the statins. Depending on their chemical structure, metabolism, and survival in cardiovascular and cerebrovascular systems, some statins may have lower adverse effects than others, which may be an important determinant of safety during long-term therapy. Grapefruit juice consumption should be avoided during statin therapy.

As stated above, the best source of n-3 fatty acids is fish oil. High doses of fish oil increase the bleeding time. Individual who bruise easily or taking bloodthinning medications should be careful in taking fish oil (Carroll and Roth, 2002; Harris, 2007). The most common adverse effects of fish oil include fishy smell and aftertaste in the mouth, nausea, and gastrointestinal discomfort with belching and bloating, diarrhea, and flatulence (Tosaki et al., 2007; Burns et al., 2004). Occasional nosebleeds are also observed in some individuals taking high doses of fish oil. The susceptibility of n-3 fatty acid preparations to undergo oxidation may contribute to potential toxicity. Availability of high-quality purified lemon and orange flavor fish oil has eliminated the problems of fishy smell and aftertaste. These commercially available preparations are stabilized with adequate amounts of vitamin E and are packaged in brown bottles to protect fish oil ingredients from light and oxygen. Emulsified fish oil preparations are much better absorbed than the straight oils in gelatin capsules.

9.11 Conclusions

Statins, the 3-hydroxy-3-methyl co-enzyme A (HMG-CoA) reductase inhibitors, specifically inhibit the rate-limiting step in cholesterol biosynthesis and thus reducing plasma cholesterol levels. Numerous studies have consistently demonstrated that cholesterol lowering with statin therapy reduces morbidity and mortality from coronary heart disease. Recent evidence indicates that benefits of statin therapy may also extend into stroke prevention. In addition to their cholesterol-lowering properties, statins produce a number of pleiotropic, neuroprotective, and vasculoprotective effects including improvement of endothelial function, increased nitric oxide (NO) bioavailability, antioxidant properties, stabilization of atherosclerotic plaques, regulation of progenitor cells, downregulating the production of pro-inflammatory cytokines such as TNF- α , interleukin-1, interleukin-2, and interleukin-6, and immunomodulatory actions. These processes result in reducing oxidative stress and vascular inflammation. Like statins, n-3 fatty acids stabilize plaques, dilate arteries through upregulation of nitric oxide synthase, inhibit the release of cytokines, and lower blood pressure. n-3 fatty acids also produce anti-arrhythmic, blood triacylglycerol lowering, antithrombotic, and endothelial relaxation effects (Holub and Holub, 2004). Statins and n-3 fatty acids are also known to improve vascular and platelet function.

Collective evidence suggests that both statins and n-3 fatty acids have antiexcitotoxic, antioxidant, and anti-inflammatory effects in brain tissue. Both inhibit HMG-CoA reductase, stimulate nitric oxide, and act as diuretics, antihypertensives, and anti-atherosclerotic molecules. Unlike higher doses of statins, n-3 fatty acids do not cause myopathy and neuropathy. n-3 fatty acids lower plasma concentrations of fibringen and have no adverse or side effects even after long-term use in patients with cardiovascular and cerebrovascular diseases. It is becoming increasingly evident that sudden withdrawal of statin treatment may acutely impair vascular function and increase morbidity and mortality in patients with vascular disease (Endres, 2006). The withdrawal of n-3 fatty acids slows metabolism, but has no effect on morbidity. In addition, n-3 fatty acids reduce the inflammatory response, have positive effects on eyesight, and increase tolerance to organ transplantation by improving the function of the graft and preventing impaired cellular immunity (Holub and Holub, 2004). Statins and n-3fatty acids also prevent neurodegeneration and improve learning and memory. They reduce the risk of stroke and dementia. Only n-3 fatty acids reduce the risk of macular degeneration and metabolic syndrome (Johnson and Schaefer, 2006; Carpentier et al., 2006; Zararsiz et al., 2006).

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Chapter 10 Apoptosis and Necrosis in Brain: Contribution of Glycerophospholipid, Sphingolipid, and Cholesterol-Derived Lipid Mediators

10.1 Introduction

Neural membranes, the dynamic physical barriers, are not only involved in the transport of nutrients, gasses, and specific ions, but also associated with sensing, receiving, processing, and transmitting information from plasma membrane to the nucleus and other subcellular organelles through lipid mediators derived from glycerophospholipid, sphingolipid, and cholesterol (Fig. 10.1). As stated in Chapters 1 and 2 neural membranes are composed of glycerophospholipids, sphingolipids, cholesterol, and proteins, which are asymmetrically distributed between the two bilayers. Asymmetry distribution of aminoglycerophospholipid is determined by sites of glycerophospholipid synthesis, glycerophospholipid transporters, and maintained by an aminoglycerophospholipid translocase that transports phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) from inner to outer leaflet (Farooqui et al., 2000a, 2004). Maintenance of proper lipid asymmetry is necessary for neural cell functions. Neuronal functions and viability are maintained through a network of signaling pathways that involves the participation of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators. These signaling pathways can be perturbed in response to cellular stimulus. Under normal conditions, the cross talk among glycerophospholipids, sphingolipids, and cholesterol-derived lipid mediators is necessary for neural cell proliferation, neuritogenesis, and neural cell survival (Farooqui et al., 2007a). High levels of lipid mediators under pathological conditions not only disturb the signaling networks and result in loss of communication among various lipid mediators inducing oxidative stress, and inflammation, but also induce morphological changes such as membrane blebbing and nuclear shrinkage. These changes promote neural cell injury and death. Thus, neural cell injury and death are not the result of one well-defined signaling pathway, but also include consequences of extensive cross talk between several neurochemical and molecular events involving glycerophospholipid, sphingolipid, and cholesterolderived lipid mediators at different cellular and subcellular levels (Farooqui et al., 2000a; Kihara and Igarashi, 2004).

Apoptosis and necrosis are the two basic mechanisms of cell death that occur in brain and spinal cord (Table 10.1). They are triggered by a variety of stimuli



Fig.10.1 Roles of biomembrane in brain

| Characteristics | Apoptosis | Necrosis |
|----------------------------|---------------------------------------|------------------------------------|
| Stimuli | Physiological | Pathological |
| Nature | Controlled process | Uncontrolled process |
| Energy requirement | Energy required (active) | Energy not required (passive) |
| Morphology | Nuclear fragmentation | Nuclear desolution |
| Membrane integrity | No loss of membrane integrity | Loss of membrane integrity |
| Levels of Ca ²⁺ | Low until late in apoptosis | Early increase in Ca ²⁺ |
| Inflammation | Absent | Present |
| Phagocytosis | Present | Absent |
| Cell involved | Individual or small group of cells | Large group of cells |
| Lysosomal enzyme release | Absent | Present |
| Effect of cycloheximide | Protection | No effect |

Table 10.1 Comparison of general features of apoptosis with necrosis

including developmental signals, disruption of cell cycle, withdrawal of neurotrophic factors, release of excitatory amino acids, treatment with $A\beta$ peptide, inflammatory reactions, and oxidative stress. During apoptotic and necrotic cell death, neural cells undergo events that are controlled by intricate interplay among glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators, intracellular enzymes, changes in integrity of subcellular organelles, and levels of ATP (Richter et al., 1996; Miller and Kaplan, 2001; Sastry and Subba Rao, 2000; Farooqui et al., 2004). In addition to neuronal cell death, apoptosis also contributes to synaptic dysfunction and breakdown of neural circuitry (Mattson et al., 2000).

10.2 Apoptosis and Necrosis Death in Brain

Morphologically, apoptosis is characterized by nuclear chromatin condensation, DNA fragmentation, cell shrinkage, and bleb and apoptotic body formation. Plasma membrane and other subcellular organelles such as mitochondria and endoplasmic reticulum remain active during apoptosis. An important feature of neuronal apoptosis in brain is breakdown of neurites. These structures are also damaged during necrosis, but morphological features of apoptotically damaged neuritis are different from necrotically damaged neuritis (Mattson et al., 1998; Mattson and Duan, 1999; Mattson et al., 2000). In brain, approximately 50% of the neurons are apoptosed during neurogenesis before the maturation of nervous system. Apoptosis also occurs in acute metabolic trauma (stroke), mechanical trauma (head injury and spinal cord injury), and neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS) (Mattson et al., 2000; Kitamura et al., 1999). During the execution phase of apoptosis, the asymmetric distribution of glycerophospholipid in lipid bilayer is lost due to the externalization of PtdSer from inner leaflet to the outer leaflet where it functions as a tag on the dying cell for recognition (eat-me signal) and removal by phagocytosis (Fadok et al., 1992). Apoptotic bodies are engulfed by macrophages with formation of tight-fitting phagosomes, similar to the "zipper"-like mechanism of phagocytosis. In contrast, necrosis usually occurs when cells are traumatized by extreme physical stress or physical challenges, where they are beyond repair. This process is characterized not only by massive ion influx and mitochondrial swelling, but also by nonspecific DNA breakage in the nuclei (Table 10.2). In apoptosis, dead cell corpses are engulfed by macrophages to avoid neural inflammation while in necrosis dead cells are either lysed and their contents cause neuroinflammation or internalized by a macropinocytotic mechanism (Krysko et al., 2006; Farooqui et al., 2007b). In brain tissue, an efficient elimination of apoptotic cells is crucial for neural cell homeostasis. Secreted "find-me," exposed "eat-me," and lacking "don't-eat-me" signals constitute the central mechanisms of apoptotic cell removal. These processes prevent the liberation of intracellular contents into the surrounding brain region (Lauber et al., 2004; Peter et al., 2008). The externalization of PtdSer on a

| Table 10.2 Tests for the detection of apoptosis | | |
|---|---|--|
| Test | Kit name and manufacturer | |
| Caspase activation assay | Caspase-Glo 3/7 assay, Promega | |
| Tunel assay | ApopTag Fluorescein direct in situ assay, Chemicon International | |
| Annexin V assay | Annexin V-biotin assay, Calbiochem | |
| Cytochrome c release assay | Cytochrome c release assay, Calbiochem | |
| Mitochondrial membrane potential assay | JC-1 Mitochondrial membrane potential assay, Cell Technology | |
| ATP quantification assay | ApoGlow rapid apoptosis assay, Cambrex | |

 Table 10.2
 Tests for the detection of apoptosis

cell surface during apoptosis can be visualized by fluorescent conjugate of annexin V (Zhang et al., 1997). These processes have been extensively characterized in non-neural tissues, but recognition of these processes and removal of apoptotic cells in the brain have only recently begun to be unraveled (De Simone et al., 2004). In brain tissue, interactions between microglial cells and PtdSerexpressing apoptotic neurons do not promote the inflammatory cascade, but rather facilitate the elimination of damaged neurons through anti-inflammatory and neuroprotective functions. Based on detailed investigation, it is proposed that the anti-inflammatory microglial phenotype mediates the activation of the specific PtdSer receptor (PtdSerR), which is expressed by both resting and activated microglial cells. This process may be relevant to the final outcome of pathogenesis neurodegenerative diseases, in which apoptosis and oxidative stress play a major role (De Simone et al., 2004). In contrast, during necrosis some cells are lysed, and others are internalized by a macropinocytotic mechanism involving formation of multiple ruffles directed toward necrotic debris (Krysko et al., 2006).

10.2.1 Mechanisms Associated with the Activation of Caspases

Several pathways of apoptotic cell death have been described in neural and non-neural cells. They include mitochondrial apoptotic pathway (intrinsic or stress-mediated), receptor-mediated pathway, and p53-mediated pathway (Nicotera and Lipton, 1999; Nicotera et al., 1999; Gorman et al., 1998; Asker et al., 1999; Morrison et al., 2003). The mitochondrial pathway is a wellcharacterized pathway that is activated by a wide variety of toxic agents that promote the depolarization of mitochondrial membrane, opening of the permeability transition pore, release of cytochrome c, and other apoptogenic proteins from mitochondria. Cytochrome c then facilitates the activation of caspase cascade (Sastry and Subba Rao, 2000; Fiskum et al., 1999). This pathway is initiated by the withdrawal of growth factors and modulated by Bcl-2 family of proteins. Among Bcl-2 protein family, Bax, Bcl-Xs, Bak, Bid, and Bad are proapoptotic, whereas, Bcl-2 and Bcl-X1 are anti-apoptotic proteins. The proapoptotic Bcl-2 proteins promote the opening of permeability transition pore and facilitate the release of cytochrome c, whereas, anti-apoptotic Bcl-2 proteins conserve membrane potential and prevent the release of cytochrome c (Merry and Korsmeyer, 1997).

Receptor-mediated pathway proceeds through the recruitment of death receptor family members including Fas/CD95, TRAIL R1, TRAIL R2, and TNF-R1 receptors. These receptors share a homologous intracellular domain called death domain, whose integrity is needed to induce apoptotic signal. Death domain triggers activation of caspase cascade (Fig. 10.2), which results in the cleavage of a variety of cellular proteins leading to the orderly demise of the cell through apoptosis (Graham and Chen, 2001; Farooqui et al., 2004).



Fig. 10.2 Involvement of Fas and NMDA receptors in apoptotic and necrotic cell death. Fas ligand (FasL); *N*-methyl-D-aspartate receptor (NMDA-R); phosphatidylcholine (PtdCho); phospholipase A_2 (PLA₂); arginine (Arg); nitric oxide synthase (NOS); nitric oxide (NO); superoxide (O₂⁻); peroxynitrite (ONOO⁻); hydroxyl radical ('OH)reactive oxygen species (ROS); arachidonic acid (AA); lysophosphatidylcholine (lyso-PtdCho); cytochrome c (Cytc); apoptosome complex with apoptosis activating factor-1 (Apaf-1); and poly(ADP)ribose polymerase (PARP)

The third less characterized apoptotic pathway is associated with p53mediated DNA damage. Tumor suppressor p53 halts the cell cycle and/or triggers apoptotic cell death in response to a variety of stimuli such as oxidative stress, oncogene activation, and ribonucleotide depletion (Fig. 10.3) (Asker et al., 1999; Morrison et al., 2003). p53-inducible genes encoding the BH3-only proteins of the Bcl-2 family, Noxa and Puma, have been recently identified. Gene knockout studies reveal that both Noxa and Puma are involved in apoptosis induction in oncogene-expressing cells. Although most apoptotic cell death pathways are aided by caspases, still cell death can occur even when the caspase cascade is blocked, revealing the existence of nonapoptotic alternative pathway(s) of apoptotic cell death (Broker et al., 2005). Caspase-independent cell death pathways are important safeguard mechanisms associated with neuronal protection against unwanted and potential harmful effects of microglial cells such as release of cytokines, when caspase-mediated routes fail, but can also be triggered in response to cytotoxic agents or other death stimuli.

Caspases are a family of at least 14 aspartate-specific cysteine proteases that are essential in the initiation and execution of apoptosis and the proteolytic maturation of inflammatory cytokines such as IL-1 β and IL-18 (Creagh et al.,



Fig. 10.3 Stress signal-mediated accumulation of the p53 protein and induction of p53mediated apoptosis, which invlove transactivation of specific target gene, transrepression of other genes, and protein–protein interactions. Insulin-like growth factor (IGP); redox-related p53-induced gene (PIG); Bcl-2 family member (Bax); microtubule-associated protein 4 (MAP-4); insulin-like growth factor 1 receptor (IGF1-R); and presenilin 1 (PS1)

2003; Cohen, 1997). Caspases are normally expressed as inactive proenzymes (zymogens) that become activated during apoptosis and proceed to dismantle the cell from within. Caspases differ in the molecular mass and sequence of their N-terminal prodomain and can be classified into two subgroups. Caspases with long prodomains include procaspases-1, -2, -4, -5, -8, -9, -10, and -14, whereas caspases with short prodomains include procaspases-3, -6,-7,-11, and -13 (Zhivotovsky B et al., 1999). All members of the caspase family share a number of amino acid residues crucial for substrate binding and catalysis. Amino acid residues Cys-285 and His-237 participate in catalysis and Arg-179, Gln-283, Arg-341, and Ser-347 are associated with carboxylate binding pocket of all caspases except caspase-8. Once activated, these enzymes proceed to cleave not only other downstream caspases but a variety of enzymes, cytokines, cytoskeletal, nuclear, and cell cycle regulatory proteins (Tables 10.3, 10.4, and 10.5) (Cohen, 1997). Most substrates of apoptotic caspases are involved in cellular dismantling, but some substrates are linked to the role of caspases in cellular responses such as cell differentiation, proliferation, and NF- κ B activation. Caspase activities in neural and non-neural tissues is regulated not only by the

| Substrate | Caspase | Function of substrate | Reference |
|----------------------------|------------------------------|-----------------------------------|----------------------------|
| Protein kinase C δ | Caspase-3 | Activated | Emoto et al. (1995) |
| PKN | Caspase-3 | Activated | Takahashi et al. (1998) |
| c-jun N-terminal kinase | Caspase-1, -3, -8, and -9 | Activated | Zaitseva et al. (2008) |
| cPLA ₂ | Caspase-3 | Activated | Atsumi et al. (1998, 2000) |
| iPLA ₂ | Caspase-3 | Activated | Atsumi et al. (1998, 2000) |
| NADPH oxidase | Caspase-3 | Activated | Arroya et al. (2002) |
| PARP | Caspase-3 and -7 | DNA repair | Janicke et al. (1998) |
| DNA-PK _{cs} | Caspase-3 | DNA double strand break repair | Janicke et al. (1998) |
| U1-70 kDa | Caspase-3 | Splicing of RNA | Song et al. (1996) |

 Table 10.3 Enzymes that are substrates for caspases

Table 10.4 Cytoskeletal proteins that are substrate for caspases

| Substrate | Caspase | Function of substrate | Reference |
|--------------------|-----------|--------------------------|-----------------------|
| Tau protein | Caspase-3 | Effector of apoptosis | Fasulo et al. (2000) |
| Huntingtin | Caspase-3 | Stimulation of caspase-1 | Nasir et al. (1996) |
| Presenilin-2 | Caspase-3 | _ | Kim et al. (1997) |
| Actin | | Cytoskeletal alterations | Nunez et al. (1998) |
| Lamin | Caspase-6 | Cytoskeletal alterations | Nunez et al. (1998) |
| Fodrin | Caspase-3 | Cytoskeletal alterations | Janicke et al. (1998) |
| α -Spectrin | Caspase-3 | Cytoskeletal alterations | Nunez et al. (1998) |
| β -APP | Caspase-3 | Cytoskeletal alterations | Xie et al. (2004) |
| Vimentin | Caspase-3 | Cytoskeletal alterations | Nunez et al. (1998) |
| Gelsolin | Caspase-3 | Cytoskeletal alterations | Nunez et al. (1998) |
| Bcl-2 family | Caspase-3 | Modulation of apoptosis | Nunez et al. (1998) |

Table 10.5 Other protein substrates for caspases

| Substrate | Caspase | Function of substrate | Reference |
|---|---------------|------------------------------|------------------------|
| Heteroribonuclear proteins C1 and C2 | Caspase-3, -7 | Pre-mRNA processing | Cohen (1997) |
| SREBP1 | Caspase-3, -7 | Sterol regulatory binding | Cohen (1997) |
| SREBP2 | Caspase-3, -7 | Sterol regulatory binding | Cohen (1997) |
| Rb | Caspase-3 | Cell cycle regulation | Cohen (1997) |
| D4-GDP inhibitor | Caspase-3 | Rho GTPase regulation | Cohen (1997) |
| MCM3 | Caspase-3, -9 | DNA replication | Nunez et al. (1998) |
| Rad51 | Caspase-3, -7 | DNA replication | Nunez et al. (1998) |

occurrence of procaspases (zymogens) level but also by members of Bcl-2 family and certain cellular inhibitor of apoptosis proteins (cIAPs) that can bind to and inhibit caspase activity.

10.2.2 Biochemical Changes Associated with Apoptosis

Biochemical changes during apoptosis include minor alterations in calcium ions, stimulation of isoforms of PLA₂, SMases, and production of low levels of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), which include nitric oxide (NO) and NO adducts (*S*-nitrosothiols and peroxynitrite) (Nicotera and Lipton, 1999) (Fig. 10.4). Peroxynitrite is a potent oxidant that not only inhibits mitochondrial respiration by inactivating complexes I–III, but plays an important role in mediating, at least in part, DNA strand breaking and inducing the activation of poly(ADP-ribose) polymerase (PARP), which in turn hydrolyzes NAD into nicotinamide and ADP-ribose. This futile process depletes intracellular ATP and promotes apoptotic cell death



Fig. 10.4 Involvement of glycerophospholipids, sphingolipids, and cholesterol in apoptotic cell death. Plasma membrane (PM); non-*N*-methyl-D-aspartate receptor (non-NMDA-R); tumor necrosis factor- α (TNF); tumor necrosis factor- α receptor (TNF-R); sphingomyelin (SM); sphingomyelinase (SMase); phospholipase A₂ (PLA₂); nitric oxide synthase (NOS); nitric oxide (NO); superoxide (O₂⁻); and reactive oxygen species (ROS)

(Virag et al., 1998). PARP cleavage prevents induction of necrosis and ensures the appropriate execution of caspase-mediated apoptosis. Thus, proteolytic cleavage of PARP by caspases is a hallmark of apoptosis. Increase in transglutaminase activity is another marker of apoptosis (Piacentini et al., 1991). This enzyme promotes the formation of cross-linked protein envelopes, which are insoluble in detergents and chaotropic agents.

Immunohistochemical analysis using an anti-transglutaminase antibody indicates that this enzyme accumulates within typical apoptotic bodies suggesting that transglutaminase mediates the formation of high molecular mass protein polymers, which maintain the integrity of cells and bodies and prevent leakage of their contents into the extracellular space. In addition, generation of ceramide, synthesis of 24-hydroxycholesterol, and translocation of one or more B-cell leukemia-2 protein (Bcl-2 protein) family members from cytosol to mitochondrial membrane (Putcha et al., 1999; Fiskum et al., 1999) have also been reported to occur in apoptotic cell death. The translocation of Bcl-2 proteins increases the capacity of mitochondria to accumulate Ca²⁺ and inhibits permeability transition while providing resistance to Ca²⁺-induced respiratory damage (Fiskum et al., 1999). In addition, apoptotic cells release membrane vesicles. These vesicles contain biologically active oxidized glycerophospholipids. The involvement of oxidized glycerophospholipids in the pathogenesis of chronic visceral inflammatory diseases as well as neurodegenerative diseases is increasingly recognized (Kadl et al., 2004; Kronke et al., 2003). Oxidized glycerophospholipids induce several pro-inflammatory genes, such as monocyte chemoattractant protein 1 or interleukin-8. Oxidized glycerophospholipids exert antiendotoxin effects by inhibiting lipopolysaccharide-induced signaling. It is proposed that they represent a possible feedback loop during gram-negative infection. Oxidized glycerophospholipids modulate the resolution of acute inflammation by inducing heme oxygenase-1 gene. Furthermore, oxidized glycerophospholipids serve as recognition signals on apoptotic cells facilitating phagocytosis. Collective evidence suggests that generation of oxidized glycerophospholipids in apoptotic cells (a) propagates chronic inflammation, and (b) contributes to the resolution of acute inflammation (Kadl et al., 2004).

10.2.3 Biochemical Changes Associated with Necrosis

In contrast, necrosis is accompanied by massive Na⁺ and Ca²⁺ influxes, rapid ATP depletion, high levels of ROS, onset of rapid and prolonged MPT, activation of calpains, and other Ca²⁺-dependent enzymes. Like caspases, calpains also exist as proenzyme heterodimers (mol. mass 29–80 kDa) in resting cells, but are activated by sustained elevation of cytosolic Ca²⁺ levels and autolytic processing into activated heterodimer (mol. mass 18–78 kDa) (Wang, 2000). The activity of calpains is regulated by endogenous protein inhibitor called calpastatin. Unlike caspases, calpains preferentially cleave at Val, Leu, or Ile



Fig. 10.5 Involvement of NMDA and TNF- α receptors in necrotic cell death. Plasma membrane (PM); *N*-methyl-D-aspartate receptor (NMDA-R); tumor necrosis factor- α (TNF); tumor necrosis factor- α receptor (TNF-R); sphingomyelin (SM); sphingomyelinase (SMase); ceramide 1-phosphate (ceramide-1-*P*)phospholipase A₂ (PLA₂); nitric oxide synthase (NOS); nitric oxide (NO); superoxide (O₂⁻); 4-hydroxunonenal (4-HNE); and reactive oxygen species (ROS)

residues in their protein substrates. Their substrates include cytoskeletal proteins, growth factor receptors, signal transduction-related proteins, and transcription factors (Wang, 2000). Collective evidence suggests that high levels of Ca^{2+} ions, increased activity of Ca^{2+} -dependent enzymes, and elevated levels of ROS irreversibly damage proteins and DNA leading to necrosis (Fig. 10.5).

10.3 Apoptosis and Necrosis-Mediated Alterations in Glycerophospholipid, Sphingolipid, and Cholesterol Metabolism

Considerable changes occur in neural membrane constituents during apoptotic and necrotic cell death. These changes include enhancement of glycerophospholipid, sphingolipid, and cholesterol metabolism and alterations in levels of glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators. These processes along with changes in activities of phospholipases, sphingomyelinases, ceramidases, kinases, and caspases may be responsible for abnormal signal transduction processes that bring about neural cell demise through apoptosis (Nicotera and Lipton, 1999; Farooqui et al., 2004). During apoptosis, these changes occur in an orderly fashion due to sufficient levels of ATP that maintains normal ion homeostasis. The dead cells are removed from the tissue through apoptotic body formation and phagocytosis.

In contrast, in necrotic cell death rapid permeabilization of plasma membrane, rapid decrease in ATP, sudden loss of ion homeostasis, glutathione depletion, and activation of lysosomal enzymes result in a passive cell death through lysis (Nicotera and Lipton, 1999; Farooqui et al., 2004). The release of cellular contents is accompanied by neuroinflammation and oxidative stress (Farooqui et al., 2007b). Apoptosis and necrosis are caused by common stimuli (cytokines, ischemia, heat, irradiation, pathogens) and signaling pathways (death receptors, phospholipases A₂, sphingomyelinases, kinase cascades, and mitochondrial depolarization). These findings support the view that apoptosis and necrosis are interrelated processes and there is considerable overlap between biochemical events associated with apoptotic and necrotic cell death. The transformation of apoptosis into necrosis and vice versa is an arguable issue. Recent studies indicate that in cell cultures, by modulating levels of stimulus, it is possible to transform apoptosis into necrosis. For example in rat C6 glioma cells, arachidonic acid, a product of PLA₂-catalyzed reaction, promotes cell demise by changing apoptotic cell death to necrotic cell death through lipid peroxidation initiated by lipid hydroperoxides generated by 12-lipoxygenase under the reduced glutathione depletion (Higuchi and Yoshimoto, 2002; Higuchi et al., 2007). This suggestion is supported by observations that anti-apoptotic mechanisms (e.g., Bcl-2/Bcl-x proteins, heat shock proteins) are equally effective in protecting neural cells from apoptosis and necrosis. Furthermore, cortical neurons rapidly die via necrotic cell death due to poor glucose uptake in the low-density (LD) culture under serum-free condition without any supplements (Fujita and Ueda, 2003). This suggestion is based on scanning and transmission electron microscopic analysis, which indicates membrane disruption, mitochondrial swelling, and loss of cytoplasmic electron density. In contrast, high-glucose treatment delays neuronal death by suppressing necrosis, but inducing apoptosis through increase in Bax levels, cytochrome c release, caspase-3 activation, and DNA ladder formation. Thus, high-glucose treatment transforms neuronal cell death mode from necrosis to apoptosis, which can be blocked by neurotrophic factors. The cell death mode transformation from necrosis to apoptosis is also modulated by intracellular ATP levels (Fujita et al., 2001; Ueda and Fujita, 2004). As stated above apoptosis is an active process and appropriate levels of ATP are essential for this type of cell death, while the ATP depletion results in necrosis suggesting that ATP plays a key role in determining the mode of cell death. Preliminary investigations indicate that the topical use of glucose to ischemic retina or brain protects these tissues from injury through the cell death mode switch (Ueda and Fujita, 2003). The degree of Ca^{2+} overload and levels of ROS may also influence the decision point between apoptosis and necrosis. ROS not only

modulate caspase activity, but also regulate opening of MPT pore (Denecker et al., 2001). Collective evidence suggests that in low and high-density neural cell cultures levels of glucose, Ca^{2+} , and ROS can be used to study transformation of necrosis into apoptosis and vice versa.

10.3.1 Apoptosis and Necrosis-Mediated Changes in Glycerophospholipid Metabolism

Remarkable changes occur in neural membrane constituents during apoptotic and necrotic cell death. As stated above, apoptosis is accompanied by externalization of PtdSer. In addition, externalization of PtdEtn has also been reported during apoptosis (Brand and Yavin, 2001). Transbilayer movement of PtdEtn has been documented using Ro09-0198, a peptide that specifically recognizes PtdEtn. The exposure of PtdEtn correlates well with PtdSer exposure on the outer leaflet. This supports the view that a complete disruption of loss of glycerophospholipid asymmetry occurs during apoptosis (Emoto et al., 1997; Brand and Yavin, 2001; Wang et al., 2004). The loss of lipid asymmetry produces looser lipid packing in membrane, which may be prone to PLA_2 attack in the outer leaflet. This view is supported by the observation that inhibitors of PLA₂ activity block apoptosis (Farooqui et al., 2004, 2006). Treatment of CHO-K1 and GOTO cells with exogenous PtdSer results in time- and dose-dependent apoptosis (Uchida et al., 1998). This cytotoxic effect is specific for PtdSer. Other glycerophospholipids, PtdCho, PtdEtn, PtdIns, and PtdH, show no effect on cell viability. PtdSer-mediated apoptotic cell death is not caused by the detergent action of lyso-PtdSer. A high concentration of lyso-PtdSer is needed to produce cell death by lyso-PtdSer. Treatment of cell cultures with lyso-PtdSer results in extensive membrane fragmentation and swelling, whereas PtdSer-treated cells become round and show a dramatic reduction in cellular volume while maintaining the membrane integrity of cellular membranes. The mechanism of PtdSer-mediated apoptosis is not known. However, it is becoming increasingly evident that during apoptosis before externalization PtdSer undergoes peroxidation via cytochrome c, which is released from mitochondria (Bavir et al., 2006). Since PtdSer contents of apoptotic cells are three times higher than control cells, it is suggested that accumulation of cellular PtdSer may be responsible for abnormal signaling pathways that induce apoptotic cell death (Uchida et al., 1998). It is also shown that in HN 2-5 cells, PtdSer externalization during apoptosis not only detaches cells from culture plates but also promotes rapid phagocytosis by microglial cells (Adayev et al., 1998).

In an oligodendroglia-like cell line (OLN 93), during H_2O_2/Fe^{2+} -induced oxidative stress externalization of PtdEtn is a very early event that peaks at 30 min, with 40% PtdEtn externalized to the outer leaflet (Brand and Yavin, 2001). Several hours following oxidative stress, in OLN 93 a reversal or relocation of PtdEtn occurs in the lipid bilayer. In contrast to PtdSer, which acts as an

eat-me signal through phagocytosis, externalization and relocation of PtdEtn act as a typical signaling molecule, which follows biphasic kinetics. It is proposed that externalization and relocation of PtdEtn act as a way of controlling or triggering specific enzymes and signaling cascades that facilitate apoptosis (Brand and Yavin, 2001).

Cardiolipin (Ptd₂Gro) is an essential constituent of inner and outer leaflets of the mitochondrial internal membrane. It is crucial not only for the ATP synthesis but for the functionality of several mitochondrial proteins (Garcia Fernansez et al., 2002). Ptd₂Gro participates in the initiation and execution phases of apoptosis (McMillin and Dowhan, 2002; Gonzalvez and Gottlieb, 2007). Some investigators propose that Ptd₂Gro binds to cytochrome c and with the involvement of mitochondrial cytochrome oxidase and the adenine nucleotide translocase facilitates apoptosis, while others suggest that in mitochondrial membrane cytochrome c acts as a cardiolipin oxygenase and generates ROS resulting in selective oxidation of cardiolipin (McMillin and Dowhan, 2002; Jiang et al., 2003; Bavir et al., 2006; Gonzalvez and Gottlieb, 2007). The oxidized Ptd₂Gro is associated with the release of proapoptotic factors from mitochondria into the cytosol. Furthermore, Bid (a proapoptotic protein of Bcl-2 family protein) binds to Ptd₂Gro and transports it into mitochondrial membrane to maintain the integrity of mitochondrial membrane (Esposti, 2002; Gonzalvez and Gottlieb, 2007). Collective evidence indicates that Ptd₂Gro has multiple roles in apoptosis and that relationship among Bcl-2, Ptd₂Gro, and cytochrome c is important in apoptotic cell death progression. Regulation of Ptd₂Gro oxidation and/or deacylation represents a possible therapeutic target for preventing apoptotic cell death (Choi et al., 2007).

In the cytosol, the released cytochrome c not only interacts with Apaf-1 in forming apoptosomes and mediating caspase-9 activation but also oxidizes another anionic phospholipid, PtdSer, and catalyzes its oxidation. Peroxidized PtdSer promotes its externalization essential for the recognition and clearance of apoptotic cells by microglia. Thus, oxidation of plasma membrane PtdSer constitutes an important redox-dependent function of cytochrome c. Oxidized glycerophospholipids are essential for the transduction of two distinctive apoptotic signals: (a) one is participation of oxidized cardiolipin in the formation of the mitochondrial permeability transition pore that facilitates release of cytochrome c into the cytosol and (b) the other is the contribution of oxidized PtdSer to the externalization and recognition of PtdSer on the cell surface by specialized receptors of phagocytes (Kagan et al., 2004). This function of cytochrome c contributes to phagocytosis. Collective evidence suggests that cytochrome c acts as an anionic phospholipid-specific oxygenase, which is activated and required for the execution of essential stages of apoptosis (Bavir et al., 2006; Tyrina et al., 2004; Kagan et al., 2004). Recent studies have also indicated that generation of ROS in mitochondria plays a key role in apoptotic cell death (Seleznev et al., 2006). The main target of ROS is mitochondrial cardiolipins because they are not only enriched in polyunsaturated fatty acids, but also located in the inner mitochondrial membrane near the ROS-producing sites. Under normal conditions mitochondria have the ability to repair peroxidative damage in part through the deacylation-reacylation cycle that involves PLA₂ isoforms and acyl-coenzyme A-dependent acyltransferase (Farooqui et al., 2000b, 2004). It is reported that in staurosporine-treated INS-1 cells, iPLA₂ translocated to mitochondria before the induction of apoptotic cell death, and most iPLA₂-associated mitochondria are intact in apoptosis-resistant cells. Expression of iPLA₂ in INS-1 cells not only inhibits the loss of mitochondrial membrane potential and attenuates the release of cytochrome c, Smac/DIABLO, but also reduces mitochondrial ROS generation. Furthermore, Staurosparin downregulates endogenous iPLA₂ transcription in both INS-1 and iPLA₂-expressing INS-1 cells without affecting the expression of group IV Ca^{2+} -dependent PLA₂. Collective evidence suggests that iPLA₂ is associated with the protection of mitochondrial function from oxidative damage during apoptosis and downregulation of endogenous iPLA₂ by STS may result in the loss of mitochondrial membrane repair functions and this process may facilitate apoptotic cell death (Seleznev et al., 2006).

Apoptotic cells also secrete lyso-PtdCho, a chemotactic seek-me signal that mediates the attraction between monocytes and apoptotic cells in a caspase and calcium-independent phospholipase A_2 (iPLA₂) dependent manner (Lauber et al., 2003; Peter et al., 2008). Association of lyso-PtdCho with seek-me signal has been confirmed by a detailed analysis. It is clearly shown that none of the lyso-PtdCho metabolites or other lysophospholipids represent the essential apoptotic attraction signal able to trigger a phagocyte chemotactic response. Furthermore, using RNA interference and expression studies, it is shown that the G-protein-coupled receptor G2A is closely associated with monocytes chemotaxis. Caspases and iPLA₂ inhibitors not only retard the increased release of lyso-PtdCho but also block the enhanced attraction between monocytes and apoptotic cells. Collective evidence suggests that caspase-mediated stimulation of iPLA₂ and generation of lyso-PtdCho play an important role in attracting monocytes (Atsumi et al., 1998, Atsumi 2000); Peter et al., 2008).

cPLA₂ and iPLA₂ are inhibited by PLA₂-inhibitory proteins called annexins. These proteins occur in multiple forms designated as annexin 1 to annexin 5. In addition to PLA₂ activity and arachidonic acid release, annexin 1 (ANXA1) modulates several components of the inflammatory reaction. Thus, ANXA1 not only specifically targets cytosolic PLA₂, but also inhibits the expression and/or activity of other enzymes like inducible nitric oxide synthase (iNOS) in macrophages and inducible COX-2 in activated microglia. The inhibition of iNOS expression may be caused by the stimulation of IL-10 release mediated by ANXA1 in macrophages. ANXA1 has inhibitory effects on both neutrophil and monocyte migration in inflammation. Several mechanisms may participate in the ANXA1-mediated cell migration. They include the activation of formyl peptide receptor (FPR) and the lipoxin A4 receptor (ALXR), the shedding of L-selectin, and the binding to $\alpha 4\beta$ 1-integrin and carboxylated N-glycans (Parente and Solito, 2004). Furthermore, ANXA1 promotes inflammatory cell apoptosis mediated by transient rise in intracellular calcium and caspase-3

activation. This annexin also has been identified as one of the "eat-me" signals on apoptotic cells to be recognized and ingested by phagocytes. Thus, PLA₂ inhibitory protein, ANXA1, may contribute to the anti-inflammatory signaling that allows safe post-apoptotic clearance of dead cells.

Apoptosis promotes a significant increase (10–20%) in the proportion of saturated fatty acids in the acyl chains of PtdEtn, PtdSer, and PtdIns, but not in PtdCho, in HN2-5 cells (Singh et al., 1996). Treatment of NGF-differentiated PC12 cells with stearic and palmitic acids for 24 h induces apoptotic cell death (Ulloth et al., 2003) that is accompanied by caspase-8 and caspase-3 activation, but pan-caspase inhibitor z-VAD-fmk has no effect on cell death. RT-PCR and RNA blot experiments indicate an upregulation of the Fas receptor and ligand mRNA in saturated fatty acid-mediated PC12 cell death. Collectively, these studies suggest that changes in fatty acid acyl group composition of neural membrane glycerophospholipids may be associated with cell shrinkage, deformation, and porosity of membranes. These changes in membrane structures may allow the diffusion of deoxyribonucleases into cell nuclei inducing the fragmentation of chromosomal DNA that occurs in apoptosis (Singh et al., 1996; Ulloth et al., 2003).

Among glycerophospholipid-derived lipid mediators, 15-deoxy- $\delta(12,14)$ prostaglandin J_2 (15d-PGJ₂) has emerged as a potent antineoplastic agent among cyclopentenone prostaglandin derivatives (Kim et al., 2003; Nakata et al., 2006). It interacts with peroxisome proliferator-activated receptor- γ (PPAR- γ). Death receptor 5 (DR5) is a specific receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). It is one of the most promising candidates for new cancer therapeutics (Nakata et al., 2004). 15d-PGJ₂ mediates DR5 expression at both mRNA and protein levels, resulting in the synergistic sensitization of TRAIL-induced apoptosis in Jurkat human leukemia cells or PC3 human prostate cancer cells. 15d-PGJ₂ significantly elevates DR5 mRNA stability but has no effect on DR5 promoter activity. Synthetic PPAR- γ agonists, such as pioglitazone or rosiglitazone, do not mimic the DR5inducing effects of 15d-PGJ₂. A potent PPAR- γ inhibitor GW9662 has no effect on 15d-PGJ₂-mediated DR5 induction suggesting the involvement of PPAR- γ independent mechanisms (Nakata et al., 2006). Cotreatment with 15d-PGJ₂ and TRAIL stimulates caspase cascade and Bid. DR5/Fc chimera protein, zVAD-fmk pan-caspase inhibitor, and caspase-8 inhibitor efficiently prevent the activation of these apoptotic signal mediators and the induction of apoptotic cell death enhanced by cotreatment with 15d-PGJ₂ and TRAIL. Similarly, a double-stranded small interfering RNA targeting DR5 gene, which retards DR5 upregulation by 15d-PGJ₂, significantly attenuates apoptosis induced by cotreatment with 15d-PGJ₂ and TRAIL. These studies suggest that 15d-PGJ₂ is a potent sensitizer of TRAIL-mediated cancer therapeutics through DR5 upregulation (Nakata et al., 2006).

In addition, 15-A2t-IsoP, the novel electrophilic lipid peroxidation products, induces apoptosis in neuronal cultures at submicromolar concentrations (Musiek et al., 2006). 15-A2t-IsoP-mediated neuronal apoptosis involves

decrease in glutathione levels and enhancement in generation of ROS, followed by 12-lipoxygenase activation and phosphorylation of extracellular signalregulated kinase 1/2, and the redox-sensitive adaptor protein p66shc-induced caspase-3 cleavage. 15-A2t-IsoP application also dramatically potentiates oxidative glutamate neurotoxicity at concentrations as low as 100 nM, indicating the functional importance of these arachidonate-derived lipid mediators with neurodegeneration (Musiek et al., 2006; Farooqui and Horrocks, 2007).

During necrosis, stimulus-mediated activation of PLA_2 markedly increases arachidonic acid cascade resulting in high levels ROS, and at the same time inhibition of respiratory chain arrests ATP production producing a rapid decrease in ATP and inducing mitochondrial explosion-mediated cell death. Collective evidence suggests that mitochondrial respiratory chain function is essential for controlling the decision of the cell to enter apoptotic or necrosis process (Proskuryakov et al., 2003). Furthermore, pronounced increase is also shown to occur in *N*-acylethanolamine in post-decapitative cerebral ischemic brain and glutamate-induced neuronal cytotoxicity (Natarajan et al., 1986; Hensen et al., 1995), indicating that rapid hydrolysis of glycerophospholipid through the activation of lipases and phospholipases is an important event associated with necrosis. In addition to lipases and phospholipases, a number of other enzymes are also elevated in necrotic cell death (Table 10.6).

10.3.2 Apoptosis and Necrosis-Mediated Changes in Sphingolipid Metabolism

Sphingomyelin (SM) is the major membrane sphingolipid and is the precursor for the bioactive products. Ceramide is formed from SM by the action of sphingomyelinases (SMases). These enzymes are activated by a variety of receptor molecules and stimuli including CD95, the tumor necrosis factor receptor (TNF-R), CD40, CD28, LFA-1, CD5, during development, irradiation, heat shock, UV light, or bacterial and viral infections (Gulbins and Grassme, 2002). Ceramide mediates its biological effects by the activation of

| Table 10.0 Calcium-dependent enzymes associated with necrosis | | |
|---|-----------|--|
| | Effect on | |
| Enzyme | activity | Reference |
| Cytosolic phospholipase A ₂ | Increased | Atsumi et al. (1998); Farooqui et al. (2004) |
| Ca ²⁺ -independent phospholipase A ₂ | Increased | Farooqui et al. (2004); Atsumi et al. (1998) |
| Phospholipase C | Increased | Farooqui et al. (2004) |
| Nitric oxide synthase | Increased | Farooqui et al. (2004) |
| Calpain | Increased | Ray et al. (2003); Farooqui et al. (2004) |
| Endonuclease | Increased | Wang et al. (1999) |

Table 10.6 Calcium-dependent enzymes associated with necrosis

several intracellular signaling molecules including cathepsin D, phospholipase A_2 , or the kinase suppressor of Ras. Moreover, by regulating the permeability of the mitochondrial outer membrane (Van Blitterswijk et al., 2003), ceramide facilitates the release of cytochrome c. In the effector phase of apoptotic cell death, hydrolysis of plasma membrane sphingomyelin to ceramide is a consequence of lipid scrambling and may modulate apoptotic body formation. Collective evidence suggests that ceramide modulates many biochemical and cellular processes that lead to apoptosis depending not only on its concentration, but also on activation or differentiation status of the cell.

Ceramide is hydrolyzed by ceramidases to yield sphingosine, and sphingosine can be phosphorylated by sphingosine kinase to yield sphingosine 1-phosphate (Vaena de Avalos et al., 2004; Edsall et al., 2001). Cellular balance between ceramide and sphingosine 1-phosphate is crucial for a cell's decision to either undergo apoptosis or proliferate. Two events modulate apoptotic cell death in neural and non-neural tissues. The first event is the generation of ceramide, which is a proapoptotic process, and second event is the production of sphingosine 1-phosphate. This lipid mediator mediates cellular proliferation and protects neural cell from apoptotic cell death (Farooqui et al., 2007a; Singh and Hall, 2007). Under physiological conditions, there is a balance between ceramide formation and sphingosine 1-phosphate synthesis. Processes that tip the balance in favor of ceramide generation, i.e., stimulation of sphingomyelinase and serine palmitoyltransferase or by inhibition of ceramidase or sphingosine kinase, may promote apoptotic cell death. It is proposed that inhibition of these reactions may effectively block apoptotic cell death (Maceyka et al., 2002; Huwiler and Zangemeister-Wittke, 2007). The molecular mechanism associated with ceramide-mediated apoptosis remains unknown. However, several mechanisms have been proposed. They include (a) ceramide-mediated mitochondrial channel formation during the induction phase of apoptosis, release of cytochrome c, proapoptotic proteins, and activation of caspase-3 (Siskind, 2005); (b) interactions of ceramide with the p75 low-affinity NGF receptor (Barrett, 2000) (Fig. 10.6); and (c) stimulation of cPLA₂ and PlsEtn-PLA₂ and liberation of arachidonic acid by ceramide (MacEwan, 1996; Pettus et al., 2004: Subramanian et al., 2005: Latorre et al., 2002) and induction of arachidonic acid cascade generating ROS, a process closely associated with the onset of apoptosis (Farooqui et al., 2000a,b; Farooqui and Horrocks, 2007).

Very little is known about ceramide-mediated necrotic cell death in brain tissue, but in non-neural, A20 B lymphoma and Jurkat T cells, C6-ceramide (C6) produces necrosis in a time- and dose-dependent manner (Villena et al., 2007). Rapid formation of ROS within 30 min of C6-ceramide results in loss of mitochondrial membrane potential. The ceramide-mediated necrotic cell death can be blocked by *N*-acetylcysteine or ROS scavengers like Tiron. Furthermore, adenovirus-mediated expression of catalase in A20 cells also prevents necrotic cell death. C6-ceramide-mediated necrotic cell death observed is accompanied by a pronounced decrease in ATP levels, and Tiron significantly delays ATP depletion in both A20 and Jurkat cells. Collective evidence suggests that



Fig. 10.6 Hypothetical diagram showing the link between p75 receptor and apoptotic cell death. Plasma membrane (PM); nerve growth factor (N); sphingomyelinase (SMase); and poly(ADP)ribose polymerase (PARP)

ceramide-mediated necrotic death is linked to the loss of mitochondrial membrane potential, production of ROS, and intracellular ATP depletion (Villena et al., 2007). Similarly, treatment of hepatoma HepG2 cells with C2-ceramide causes necrotic cell death by DNA smearing in DNA electrophoresis (Gentil et al., 2003). It is reported that C2-ceramide not only inhibits the respiratory chain, but also markedly increases ROS production. These processes arrest ATP production inducing necrotic cell death.

10.3.3 Apoptosis and Necrosis-Mediated Changes in Cholesterol Metabolism

Oxysterols arise from the enzymic or non-enzymic oxidation of cholesterol. They occur in normal brain tissue and brain tissue from patients with neurological disorders (Lizard et al., 2000; Kolsch et al., 2001; Teunissen et al., 2007). Oxysterols produce cytotoxic effects in vivo in brain tissue and in vitro in neural and non-neural cell lines. The cytotoxicity and neurodegenerative effect of oxysterol are blocked by lovastatin, a cholesterol synthesis inhibitor (Ong et al., 2003; He et al., 2006). In hippocampal slices, glutathione protects neurons from cholesterol oxidation products-mediated toxicity. Based on detailed investigations (Ong et al., 2003; He et al., 2006), it is proposed that cholesterol oxidation products may be a key factor in aggravating oxidative damage to neurons following neuronal injury induced by excitotoxins (Ong et al., 2003). The exact molecular mechanism through which oxysterols induce cytotoxicity has not been fully elucidated. However, 7β -hydroxycholesterol (7β -OH-Chol), 24-hydroxycholesterol (24-OH-Chol), and other cholesterol oxides-mediated cell death in cell culture is accompanied by oxidative stress, cytochrome c release, and caspase-3 activation, suggesting that hydroxycholesterol-induced apoptosis may occur via the mitochondrial pathway (O'Callaghan et al., 2001; Ryan et al., 2005, 2006; Kölsch et al., 1999; Lemaire-ewing et al., 2005). In addition, phosphatidylserine externalization, loss of mitochondrial potential, increased permeability to propidium iodide, and occurrence of cells with swollen, fragmented, and/or condensed nuclei are also observed. It is also proposed that in neural and non-neural cell cultures 7-ketocholesterol triggers the stimulation of NADPH oxidase, generation of superoxide anions, and loss of mitochondrial transmembrane potential ($\Delta \Psi m$). These processes are closely associated with the apoptotic cell death (Lizard et al., 2000). 7-Oxycholesterol not only modulates Ca^{2+} signals, but also inhibits the phosphorylation of endothelial nitric oxide synthase and cPLA₂ (Millanvoye-Van Brussel et al., 2004). In non-neural tissues, it is shown that among oxysterols oxidized at C7 $(7\alpha, 7\beta$ -hydroxycholesterol, and 7-ketocholesterol) 7β -hydroxycholesterol and 7-ketocholesterol are potent inducers of cell death and probably play central roles in atherosclerosis and 7-ketocholesterol might be a causative agent of vascular damage by inducing apoptosis and enhancing superoxide anion production (Miguet-Alfonsi et al., 2002).

Treatment of SH-SY5Y with 7α -hydroperoxycholesterol results in neuronal cell death through necrosis (Rao et al., 1999; Kolsch et al., 2000). This suggestion is not only based on the absence of DNA fragmentation and caspase-3 activity but also on rapid release of and marked increase in the generation of ROS.

10.4 Interactions Among Glycerophospholipid, Sphingolipid, and Cholesterol Metabolism in Apoptosis and Necrosis

In brain catabolism of glycerophospholipid by PLA₂ (arachidonic acid release and generation of eicosanoids) and hydrolysis of sphingomyelin by acid and neutral SMases (generation of ceramide) are closely associated with apoptotic cell death (Wissing et al., 1997; Atsumi et al., 1998, 2000); Hannun and Obeid, 1995). Modulation of arachidonic acid- and ceramide-derived lipid mediators involves kinases, phospholipases, cyclooxygenases, and various transcription factors and cytokines including AP1, NF- κ B, and TNF- α (Wissing et al., 1997; Ohanian and Ohanian, 2001). PLA₂ and acid SMase require a cytoplasmic death domain (p55). Upon binding with TNF- α , the death domain (p55) of TNF- α receptor interacts with an adaptor protein TRADD that in turn recruits three additional proteins, TRAF2, FADD, and RIP (Wallach, 1997).

Recruitment of FADD is essential for the stimulation of PLA₂ and acid SMase activity and generation of arachidonic acid and ceramide (MacEwan, 1996; Schwandner et al., 1998). Ceramide-induced cell death requires the release of AA and the stimulation of cPLA₂ activity (Havakawa et al., 1993; MacEwan, 1996; Jayadev et al., 1997). Another potential site of interaction between ceramide and glycerophospholipid metabolism is phosphatidylinositol 3-kinase (PtdIns 3-K) pathway. Receptor-mediated stimulation of PtdIns 3-K, PDK, and Akt/PKB retards apoptosis through Bcl-2 phosphorylation. C2-ceramide blocks this pathway either by downregulating PtdIns 3 K or by inhibiting Akt/ PKB activation (Ohanian and Ohanian, 2001). Ceramide stimulates its own synthesis by directly inhibiting mitochondrial complex III resulting in increased generation of ROS, which upregulates sphingolipid synthesis via serine palmitovltransferase (Gudz et al., 1997). Furthermore, ceramide stimulates mitochondrial oxyradical release by inhibiting Bcl-2 via activation of phosphatase-2A (Ruvolo et al., 1999). Collectively, multiple studies support the view that during apoptotic cell death a controlled and coordinated interplay (cross talk) occurs between glycerophospholipid and sphingomyelin-derived lipid mediators.

As stated above, glycerophospholipid and sphingolipid lipid mediatorsmediated necrotic cell death is characterized by mitochondrial dysfunction, rapid depletion of ATP, sustained calcium ion overload, and generation of high levels of reactive oxygen species (ROS) (Weber, 1999; Farooqui and Horrocks, 2007). A sustained increase in intracellular calcium ion is responsible for the stimulation of calcium-dependent enzymes (isoforms of PLA₂, calpains, and nitric oxide synthase) and lysosomal proteases and cathapsins. The stimulation of these enzymes not only initiates cellular lysis but also releases arachidonic acid from neural membrane glycerophospholipids and sets in motion an uncontrolled "arachidonic acid cascade." The later includes the synthesis and accumulation of eicosanoids, isoprostanes, and 4-hydroxynonenal (4-HNE) and generation of ROS (Farooqui et al., 2004) (Fig. 10.5). The 4-HNE impairs the activities of key metabolic enzymes including Na⁺, K⁺-ATPase, glucose 6phosphate dehydrogenase, and several kinases (Farooqui and Horrocks, 2006) and ROS activate NF- κ B, which not only induce the release of cytokine, but also increase the expression of inducible nitric oxide synthase (iNOS) and generation of peroxynitrite. Collectively, these studies suggest that necrotic cell death does not result through well-organized signaling cascade, but it is the consequence of extensive cross talk between several biochemical and molecular events at different cellular and subcellular levels. Serine/threonine kinase (RIP1), which contains a death domain and isoforms of PLA₂, may act as a central initiator. Calcium and reactive oxygen species (ROS) are key players during the propagation and execution phases of necrotic cell death, directly or indirectly provoking damage to proteins, lipids, and DNA, which culminates in disruption of organelle and cell integrity. During necrosis, dying cells also initiate pro-inflammatory signaling cascades by actively releasing inflammatory cytokines and by spilling their contents upon rupturing.

10.5 Apoptotic and Necrotic Cell Death in Neurological Disorders

Apoptosis is a physiologically important process in brain development. Approximately, 50% of the neurons die through apoptosis during brain maturation. However, premature apoptosis and/or aberrations in apoptosis control contribute to the pathogenesis of a variety of neurological disorders including acute head injury, spinal cord trauma, ischemia (stroke), and chronic neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS) (Ekshyyan and Aw, 2004). Although the exact cause of apoptosis is not known, a number of factors such as alteration in expression of p53 and Bcl-2, free radicals, insufficient levels of nerve growth factors, accumulation of self-aggregating proteins (amyloid β peptide, tau, α -synuclein, and huntingtin), and excessive levels of glutamate have been implicated.

In stroke, severe blockade in cerebral blood flow not only decreases oxygen and glucose delivery to the brain but also results in the build-up of potentially toxic products. Rapid depletion of ATP results in impaired ion homeostasis and commencement of excitotoxicity due to the release of high levels of glutamate; and high levels of generation of ROS are closely associated with neuronal injury and cell death (Siesjö, 1988; Farooqui and Horrocks, 1994; Farooqui and Horrocks, 2007). Neurons undergoing severe ischemia die rapidly (minutes to hours) by necrotic cell death at the core of ischemic injury, whereas penumbral region neurons display delayed vulnerability and die through apoptotic cell death (Farooqui et al., 2004).

In contrast, in head injury and spinal cord trauma, mechanical impact and shear forces traumatize brain and spinal cord tissue (McIntosh et al., 1998; Klussmann and Martin-Villalba, 2005). The mechanical impact produces a rapid deformation of brain and spinal cord tissues, leading to rupture of neural cell membranes, release of intracellular contents, and disruption of blood flow and breakdown of the blood-brain barrier. This process is accompanied by inflammation and glial cell reactions (scar formation) involving both activated microglia and astroglia and demyelination involving oligodendroglia (Beattie et al., 2000). At the same time like ischemic injury, glutamate is released from intracellular stores (Demediuk et al., 1988; Panter et al., 1990; Sundström and Mo, 2002). Accumulation of glutamate overstimulates glutamate receptors and produces excitotoxicity and overexpression of cytokines (Hayes et al., 2002; Ahn et al., 2004). Excitotoxicity, inflammation, and oxidative stress are major processes that are closely associated with the pathogenesis of stroke, head injury, and spinal cord trauma. Similar to stroke, in head injury and spinal cord trauma neurons die rapidly (hours to days) at the injury core by necrotic cell death, whereas in the surrounding area neurons undergo apoptotic cell death (several days to months) (McIntosh et al., 1998; Farooqui et al., 2004). In contrast, in experimental model of AD, PD, and HD, both extracellular amyloid, perkin, and huntingtin deposits and intracellular signal transduction

abnormalities caused by amyloid β -protein, huntingtin, and perkin fragments activate caspase-induced apoptosis. In AD, proteolytic cleavage of tau by caspases promotes the formation of neurofibrillary tangle formation, which correlates with dementia (Kitamura et al., 1999; Dickson, 2004; Rissman et al., 2004; Bamberger and Landreth, 2002). Similarly, in ALS cell culture model data and postmortem studies indicate that caspase activation mediates the death of motor neurons via apoptosis (Janik et al., 2001). In cell culture model of HD, caspase-mediated fragmentation of huntingtin results in accumulation of protein aggregates contributing to cell dysfunction and apoptotic neuronal death (Maglione et al., 2006a). Moreover, abnormalities in p53 expression, which control the cell cycle and repair of DNA, also contribute to apoptotic cell death in neurodegenerative diseases.

Apoptosis is an attractive mechanism for neuronal cell death in neurodegenerative diseases for several reasons (Dickson, 2004). In neurodegenerative diseases, cell demise involves site-specific premature and slow death of individual neuronal populations in a specific manner that allows phagocytosis of dead cell without causing neuroinflammation (Graeber and Moran, 2002; Farooqui et al., 2007b). For example in AD, neuronal degeneration occurs in the nucleus basalis; whereas in PD, neurons in the substantia nigra die. The most severely affected neurons in HD are striatal medium spiny neurons. Although selective neuronal populations in specific areas are vulnerable in different neurodegenerative diseases that have separate etiology with distinct morphological and pathophysiological characteristics, these chronic conditions share the same terminal neurochemical common processes such as activation of caspases, phospholipases A₂, excitotoxicity, oxidative stress, and inflammation (Farooqui and Horrocks, 1994; Roth, 2001; Farooqui and Horrocks, 2006; Farooqui et al., 2006; Dickson, 2004; Rissman et al., 2004; Farooqui et al., 2008). It remains controversial whether these processes are the cause or consequence of disease process (Roth, 2001; Andersen, 2004; Juranek and Bezek, 2005). Similarly, very little information is available on the rate of apoptotic or necrotic cell death and clinical expression of neurodegenerative diseases with age (Farooqui et al., 2004). Although the molecular mechanism of neurodegeneration in AD, PD, and HD remains illusive, however, it is becoming increasingly evident that in neurodegenerative diseases neurons die by abnormalities in levels of lipid mediators leading to apoptosis and necrosis. The most important risk factors for neurodegenerative diseases are old age and a positive family history. The onset of neurodegenerative diseases is often subtle and usually occurs in mid to late life and their progression depends not only on genetic but also on environmental factors (Graeber and Moran, 2002).

The loss of synapse by apoptosis takes place independent of cell body, and this process may play some role in synaptic remodeling not only during brain development but also in neurodegeneration (Mattson et al., 1998). During brain development, synapses are transiently formed and lost due to limited supply of target-derived growth factors. Alterations in trophic factor signaling in axon terminals may cause the alterations in mitochondrial function and stimulation

of caspases resulting in elimination of synapse. It is interesting to note that degenerative morphological changes in synapses are known to precede neuronal cell body damage in AD, PD, and ischemic injury (Anglade et al., 1996; Dekosky et al., 1996; Horner et al., 1996; Mattson et al., 1998). Apoptosismediated changes including minor alterations in Ca²⁺ and ROS levels, which propagate from postsynaptic dendritic regions to the cell body (Mattson, 1996), are modulated by redox status and ATP levels. Synaptic apoptosis may also be modulated by astrocytes that are located very close to synapse and play an important role not only in removing excess glutamate from synapse but also in providing growth factors for neuronal survival. It is becoming increasingly evident that impairment in astrocytic functions critically influences neuronal survival. It is proposed that astrocyte apoptosis may contribute to pathogenesis of acute neural trauma and chronic neurodegenerative diseases (Takuma et al., 2004; John et al., 2005). Astrocytic apoptosis also involves molecular mechanisms such as Ca^{2+} overload, oxidative stress, NF- κ B activation, mitochondrial dysfunction, endoplasmic reticulum stress, and protease activation. Collectively, these studies indicate that heat shock protein, mitogen-activated protein/extracellular signal-regulated kinase, phosphatidylinositol-3 kinase, and cyclic GMP phosphodiesterase may be important targets for anti-apoptotic drugs (Takuma et al., 2004). Furthermore, factors that enhance, maintain, and modulate activities of astrocytes in brain may be efficacious in treating neurodegenerative diseases (John et al., 2005).

10.6 Association of Mitochondrial Dysfunction with Apoptotic and Necrotic Cell Death in Neurological Disorders

It is well known that mitochondria not only generate ATP but also regulate intracellular calcium homeostasis. They are dynamically transported along lengthy neuronal processes, for appropriate distribution to those cellular regions that have high metabolic demand and need elevated intracellular calcium, such as synapses. Mitochondrial dysfunction causes impaired calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition, secondary excitotoxicity, depletion in ATP production, oxidative damage, and the induction of apoptosis, all of which are closely associated with the pathogenesis of acute neural trauma and neurodegenerative diseases (Fiskum et al., 1999; Tatton and Olanow, 1999; Zamzami et al., 1997). Excessive Ca²⁺ load inside the neural cell may cause an overstimulation of mitochondrial buffering capacity, uncoupling electron transport from energy generation, and consequent formation and release of ROS. Specific respiratory chain defect (complex I deficiency) in PD, complexes I, II, and III defect in AD, complex II/III deficiency in HD, and complexes I-III deficiency in Friedreich ataxia (FA) have been identified (Table 10.7). Thus, a potential pivotal role for mitochondrial dysfunction in apoptotic cell death in neurodegenerative diseases

| Disease | Caspase activity | Altered mitochondrial complex | Delayed cell death | Reference |
|-----------------------|------------------|-------------------------------------|-----------------------|---|
| Ischemia | Increased | Complexes I, II, III, and V | Apoptotic | Almeida et al. (1995) |
| Spinal cord injury | Increased | Complex I | Apoptotic | Sullivan et al. (2005); Wu et al. (2007) |
| Head injury | Increased | _ | Apoptotic | Robertson (2004); Lifshitz et al. (2004) |
| AD | Increased | Complexes II, III, and V | Apoptotic | Roth (2001) |
| PD | Increased | Complex I | Apoptotic | Schapira (1998); Vishwanath et al. (2001) |
| HD | Increased | Complex II | Apoptotic | Schapira (1998); Maglione et al. (2006b) |
| ALS | Increased | Complexes I and III | Apoptotic | Ilieva et al. (2007); Troost et al. (1995) |
| FA | Increased | Complexes II, III, and IV | Apoptotic | Schapira (1999) |

 Table 10.7
 Caspase activity, mitochondrial dysfunction, and nature of cell death in neurological disorders

Alzheimer disease (AD); Parkinson disease (PD); Huntington disease (HD); amyotropic lateral sclerosis (ALS); and Friedreich ataxia (FA).

has gained increasing acceptance (Schapira, 1998; Beal, 1998). Although, much evidence supports the view that the electron transport chain dysfunction in above conditions has both genetic and intracellular environmental causes, but alternative mechanisms have been proposed (Soane et al., 2007). These include direct, reversible inhibition of cytochrome oxidase by nitric oxide, release of mitochondrial cytochrome c, oxidative inhibition of mitochondrial matrix dehydrogenases and adenine nucleotide transport, the availability of NAD for dehydrogenase reactions, respiratory uncoupling by activities such as that of the permeability transition pore, and altered mitochondrial structure and intracellular trafficking. Thus, mitochondrial dysfunction can contribute to cell death in a number of ways: by disrupting their own electron transport and energy metabolism, by activating the mitochondrial permeability transition, and by releasing cytochrome c and other apoptosis-inducing factors (Calabrese et al., 2001). Collective evidence suggests that mitochondria perform important roles in regulating both apoptotic and necrotic cell death (Gogvadze and Orrenius, 2006; Orrenius et al., 2007). It is becoming increasingly evident that permeabilization of the outer mitochondrial membrane and subsequent release of intermembrane space proteins are closely associated with both types of cell death. Alterations in mitochondrial permeability transition is associated mainly with necrosis, whereas Bcl-2 family of protein-mediated release of caspaseactivating proteins (cytochrome c) is involved in apoptotic cell death. Multiple mechanisms of mitochondrial permeabilization under different conditions may explain diversities in the response of mitochondria to numerous apoptotic and necrotic stimuli in different types of neural and non-neural cells (Gogvadze and Orrenius, 2006; Orrenius et al., 2007). In brain mitochondria mechanisms involved in the release of these proteins not only depend on type of neural cell, but also on the nature of stimuli. A relationship between apoptotic and necrotic signaling cascades, disruption of mitochondrial energy metabolism, balance of cross talk between apoptotic and anti-apoptotic pathways, and duration of stimulus dictates the feasibility of mode of cell death (Soane et al., 2007).

10.7 Prevention of Apoptotic Cell Death by Inhibitors of Enzymes Associated with Exicitoxicity, Inflammation, and Oxidative Stress

Involvement of common stimuli, signaling pathways, and lipid second messengers that mediate apoptotic and necrotic cell death suggests considerable overlap between biochemical events associated with these processes. Since ATP is involved in transformation of apoptosis into necrosis and necrosis into apoptosis, and both types of cell deaths can be prevented by Bcl-2/Bcl-x proteins and heat shock proteins (Higuchi and Yoshimoto, 2002; Ueda and Fujita, 2004), many investigators believe that apoptosis and necrosis cannot be regarded as two separate entities. Thus, the same insult can promote neural cell death via apoptosis or necrosis depending on the intensity, duration, cell type, and the nature of insult (Mattson and Duan, 1999). In mild excitotoxicity (low concentration of glutamate), the neuronal death occurs over a 16–24 h period, while severe excitotoxicity (high concentration of glutamate) demises neurons within 2-8 h. Neurons are more susceptible to glutamate-mediated toxicity, inflammation, and oxidative stress than astroglial cells (Adibhatla et al., 2003; Ajmone-Cat et al., 2003). During neurodegeneration, the nature of stimulus and alterations in external milieu are sensed by death receptors, which convey the death message through cytoplasm to the nucleus via lipid mediator network. Collective evidence suggests that levels of lipid mediators and cross talk among various receptors not only control the intensity of cell death, but also modulate the commencement of apoptosis and necrosis.

It is well known that cell surface receptors (e.g., CD95/APO-1/Fas; TNF receptor) and their ligands (CD95-L; TNF) as well as evolutionarily conserved mechanisms involving mitochondrial dysfunction, p53, and enzymes associated with degradation of glycerophospholipids and sphingolipid participate in the modulation and execution of cell death. Effectors for neural cell death include oxidative stress, inflammatory processes, calcium-mediated toxicity, and survival factor deficiency. Many processes and enzymes that promote and maintain excitotoxicity, inflammation, and oxidative stress contribute to apoptotic and necrotic cell death in brain tissue. These enzymes include caspases and calpains, poly (ADP-ribose) polymerase, phospholipases, sphingomyelinases, and kinases. Caspase and calpain activities provide molecular basis of apoptotic
morphology, whereas phospholipases, sphingomyelinases, and kinases modulate the levels of lipid mediators and thus the intensity of apoptotic and necrotic cell death. Collective evidence suggests that stimulus-mediated changes in activities of caspases and calpains, poly (ADP-ribose) polymerase, phospholipases, sphingomyelinases, and kinases along with loss of ion homeostasis and depletion of ATP are closely are associated with pathogenesis of acute neural trauma and chronic neurological disorders (Farooqui and Horrocks, 1994; Phillis and O'Regan, 2004; Farooqui et al., 2007a,b).

For the successful treatment of acute neural trauma and neurodegenerative disorders, a timely delivery of well-tolerated, chronically active, and specific inhibitors of excitotoxicity, inflammation, and oxidative stress that can bypass or cross the blood–brain barrier without harm is necessary. Glutamate receptor antagonists, antioxidants, anti-inflammatory agents, caspase, calpain, PLA₂, SMase, and nitric oxide synthase inhibitors have been successfully used for the treatment of acute neural trauma and neurodegenerative disorders in cell culture and animal models (Fig. 10.7) (Farooqui et al., 2006; Ray et al., 2003; Claus et al., 2000; Soeda et al., 2004; Goadsby, 2007).

10.7.1 Glutamate Receptor Antagonists

Glutamate produces apoptotic neuronal cell death at high concentrations in neural cell cultures. Depending on the neural cell type, glutamate-mediated



Fig. 10.7 Prevention of apoptosis by enzyme inhibitors, glutamate antagonists, and endogenous inhibitors

apoptotic cell death is also accompanied by regulation of genes such as Bcl-2, Bax, and/or caspase-3 and mitochondrial cytochrome c. Glutamate receptor antagonists have neuroprotective effects in animal model and preclinical models of stroke, head injury, spinal cord trauma, epilepsy, and many neurodegenerative diseases (Sonkusare et al., 2005; Chen and Lipton, 2006; Ratan et al., 1994). They retard both apoptotic and necrotic modes of cell death in cell cultures and animal models of neurodegenerative diseases. Both non-competitive and competitive antagonists have undergone tolerability studies in acute stroke and traumatic brain injury in humans. They not only antagonize glutamate-mediated toxicity but also block normal neuronal function. Based on these findings, their clinical trials have been abandoned owing to concerns about drug toxicity, particularly in stroke and head injury (Lees, 1997); Sacco et al., 2001; Labiche and Grotta, 2004). Memantine, a low-affinity NMDA receptor antagonist, produces beneficial effects in moderate-to-severe Alzheimer disease (Fig. 10.8). It is the only drug that has been currently approved by FDA for the treatment of more advanced stages of AD (Sonkusare et al., 2005; Chen and Lipton, 2006; Tanovic, and Alfaro, 2006).



Fig. 10.8 Chemical structures of glutamate antagonists, antioxidants that have been used for the treatment of acute neural trauma and neurodegenerative diseases. Memantine (a); selfotel (b); eliprodil (c); tirilazad mesylate (d); edaravone (e); and NXY-095 (f)

10.7.2 Antioxidants and Anti-inflammatory Agents

An unbalanced overproduction of reactive oxygen species (ROS) induces oxidative stress that causes neuronal damage and ultimately leading to neuronal death by apoptosis or necrosis. Oxidative stress is closely associated with the pathogenesis of ischemia, spinal cord trauma, head injury, AD, PD, and ALS. Nutritional antioxidants (especially vitamin E and polyphenols) can block neuronal death in vitro and may have therapeutic properties in animal models of neurodegenerative diseases including AD, PD, and ALS. Although experimental data on cell culture and animal model are consistent with neuroprotective effects of antioxidants, the clinical evidence that antioxidants agents may prevent or slow the course of these chronic diseases is controversial and relatively unsatisfactory. Neurons are more susceptible to direct oxidative injury by ROS than glial cells. In brain, ROS contribute to apoptosis and necrosis not only by modulating the expression of inflammatory and stress-sensitive genes (genes for cytokines) (Farooqui and Horrocks, 2006), but also by activating mechanisms that result in a glia cell-mediated inflammation associated with secondary neuronal damage (Block and Hong, 2005; Farooqui and Horrocks, 2007; Farooqui et al., 2007b). These activated glial cells are histopathological hallmarks of acute neural trauma and neurodegenerative diseases (Farooqui et al., 2007b). Even though direct contact of activated glia with neurons per se may not necessarily be toxic, the immune mediators released by activated glial cells are endogenous neurotoxins (e.g., nitric oxide and reactive oxygen species, pro-inflammatory cytokines, and chemokines) that facilitate apoptotic or necrotic cell death. Therefore, the use of a cocktail of anti-excitotoxic, antioxidant, anitiinflammatory compounds (Fig. 10.8) for correcting the fundamental oxidant/antioxidant imbalance in patients suffering from acute neural trauma and neurodegenerative diseases is important vistas (Gilgun-Sherki et al., 2002, Gilgun-Sherki et al., 2001, Gilgun-Sherki et al., 2006; Wang et al., 2006).

The molecular mechanism of the neuroprotective effects of anti-inflammatory and antioxidant agents may depend not only on the general free radical trapping or antioxidant activity per se in neurons but also on the downregulation of NF- κ B activity (Shen et al., 2003), suppression of genes induced by pro-inflammatory cytokines, and other mediators released by glial cells (Gilgun-Sherki et al., 2006; Wang et al., 2006). The effectiveness of a mixture of anti-inflammatory and antioxidant agents in protecting against acute neural trauma and neurodegenerative diseases depends on their ability to cross the blood-brain barrier, their potential in terms of subcellular distribution in mitochondria, plasma membrane, and cytoplasm and their multifunctional capacity as well as their synergistic actions (Gilgun-Sherki et al., 2001, 2006; Tan et al., 2003; Gilgun-Sherki et al., 2002). Consideration of these factors in a cocktail along with agents that increase the production of ATP in degenerating neurons can improve the therapeutic outcome of acute neural trauma and neurodegenerative diseases. A clearer appreciation of the potential therapeutic ability of anti-inflammatory and antioxidant cocktail would emerge only when in vivo importance of interactions among excitotoxicity, neuroinflammation, and oxidative stress is realized and is fully understood at the molecular level (Farooqui and Horrocks, 2007; Farooqui et al., 2007b).

10.7.3 Prevention of Apoptosis by Inhibitors of Caspases, Calpains, PLA₂, Nitric Oxide Synthase, and SMase

Studies on animal models of ischemia, spinal cord trauma, head injury, HD, and ALS indicate that caspase-1 and -3 inhibitors reduce neuronal damage by directly inhibiting apoptotic cell death (Holtzman and Deshmukh, 1997). Caspase-1 and -3 have been implicated in pathogenesis of ischemia, spinal cord trauma, and head injury and that caspase inhibition reduces post-traumatic lesion size and improves motor performance (Li et al., 2000a; Rami et al., 2000; Hara, 1999). Similarly, caspase inhibitors also provide protection against AD, PD, HD, and ALS in cell culture and animal models (Li et al., 2000b; Tokuda et al., 2007; Kim et al., 1999; Holtzman and Deshmukh, 1997).

Despite concerns that targeting caspases alone may prove insufficient to provide complete neuroprotection from acute neural trauma and neurodegenerative diseases, in vivo studies indicate that caspase inhibition promotes survival and functional outcome in a variety of neurological disease models (Rideout and Stefanis, 2001). In addition, studies of human postmortem brain tissues suggest that caspases are activated in chronic human diseases and are target for the treatment of neurodegenerative diseases. Caspases, through the production of toxic fragments of critical protein substrates, may also be involved in earlier steps of neuronal dysfunction, such as protein aggregation in HD and AD. Many synthetic caspase inhibitors have been developed to prevent apoptotic cell death in cell cultures and animal models of ischemia and neurodegenerative diseases and attempts are underway to bring to the clinic, where they can be of tremendous benefit for reducing apoptosis and necrosis in ischemic vascular diseases, in neurodegenerative diseases, and in organ transplant surgery (Callus and Vaux, 2007). Idun and Pfizer have produced a number of broad-spectrum caspase inhibitors (IDN-8066, -7503, -7436, -1965, -6556) and have tested their efficiency for blocking apoptosis. IDN-6556 is a potent, irreversible, broadspectrum caspase inhibitor that strongly blocks Fas-induced apoptosis in vitro and prevents liver injury in the mouse in vivo. In a phase I clinical trial, oral administration of IDN-6556 reduces levels of liver enzymes in the blood in patients with liver diseases, presumably by reducing apoptosis of liver cells. It is now in phase II trials in patients undergoing liver transplantation. It is hoped that it will reduce ischemia-reperfusion injury-mediated apoptosis. At present, nothing is known about blood-brain barrier permeability and half-lives of these inhibitors. So they have not been used for the treatment of acute neural trauma and neurodegenerative diseases.

Calpains are a family of calcium-dependent cysteine proteases that exhibit broad substrate specificity influencing cell proliferation and migration. These enzymes have also been implicated in apoptotic cell death in ischemia, spinal cord injury, and neurodegenerative disease (Ray et al., 2003; Buki et al., 2003; Ray, 2006; Carragher, 2006). Calpastatin, an endogenous protein inhibitor, regulates calpain activity. Overactivation of calpains degrades calpastatin, limiting its regulatory efficiency. Although the precise physiological function of calpains remains elusive, association of calpains with acute neural trauma and neurodegenerative diseases suggests that calpains participate in neurodegenerative process via increase in intracellular free Ca^{2+} , which promotes the degradation of key cytoskeletal and membrane proteins. Cleavage of these key proteins by calpain is an irreversible process that perturbs the integrity and stability of neural cells, leading to apoptosis. It is proposed that calpain in conjunction with caspases promotes neuronal apoptosis in brain tissue. Many cell-permeable calpain inhibitors such as peptide epoxide, aldehyde, and ketoamid inhibitors target the active site of calpains and are under evaluation in animal models of human neurological diseases. Some calpain inhibitors have shown significant neuroprotection in animal models of spinal cord trauma and head injury indicating their therapeutic potential (Ray and Banik, 2003; Buki et al., 2003).

Calpains contribute to the pathogenesis of AD not only by impairing synaptic transmission and increasing neurotransmitter release but also by modulating processes that govern the function and metabolism of proteins closely associated with AD, including tau and amyloid precursor protein (Battaglia et al., 2003). Studies are underway for testing the hypotheses that a treatment with calpain inhibitors may restore normal cognition and synaptic transmission in a transgenic model of AD (APP, K670N:M671L mouse) and PS1 (M146L) mouse (Di Rosa et al., 2002; Battaglia et al., 2003).

Studies on PLA_2 and SMase inhibitors in cell culture and animal models of acute neural trauma and neurodegenerative have been performed, and results have been encouraging (Farooqui et al., 2006; Taguchi et al., 2003; Yokomatsu et al., 2003). PLA_2 inhibitors, quinacrine, and arachidonoyl trifluoromethylketone (Fig. 10.9) have neuroprotective effects in animal models of ischemia, spinal cord injury, AD, PD, and prion diseases (Arai et al., 2001; Adibhatla et al., 2002; Yoshinaga et al., 2000; Tariq et al., 2001; Estevez and Phillis, 1997; Dubin et al., 1982; Love, 2001; Stewart et al., 2001; Farooqui et al., 2006; Malaviya et al., 2006).

Elucidation of the mechanism of action of PLA_2 inhibitors in vivo is a critical area of research due to the potential pharmacologic benefits of these compounds as therapeutic agents for the treatment of inflammation and oxidative stress in neurotrauma and neurodegenerative diseases (Farooqui et al., 1999). Collective evidence suggests that PLA_2 inhibitors have emerged as major drugs for preventing inflammation and oxidative stress (Farooqui et al., 2006; Malaviya et al., 2006). Inhibitors of $cPLA_2$ modulate the expression of cytokines, growth factors, nuclear factor- κ B, and adhesion molecules and thus can



Fig. 10.9 Chemical structures of PLA_2 and SMase inhibitors that protect from cell death in cell cultures. Long-chain oxomide (**a**); quinacrine (**b**); arachidonyltrifluoromethylketone (**c**); scyphostatin (**d**); bromoenol lactone (**e**); analog of scyphostatin (**f**); and difluoromethylene analog of sphingomyelin (**g**)

be used for the treatment of endogenous oxidative stress and neuroinflammation in ischemia, spinal cord injury, and AD in animal models. Specificity, selectivity, harmlessness, and the ability of a PLA₂ inhibitor to cross the blood-brain barrier are important qualities of a PLA₂ inhibitor as a potential therapeutic agent for neurological disorders.

Advanced molecular biology procedures have been used to overcome problems associated with the specificity of chemical inhibitors of PLA₂. For example, antisense oligonucleotides that specifically inhibit cPLA₂ or iPLA₂ have been synthesized. Antisense for cPLA₂ has been used for lowering lipopolysaccharide neurotoxicity in glial cell cultures (Won et al., 2005). iPLA₂ antisense oligonucleotides have been used to study cortex-striatum-thalamuscortex circulatory associated with vacuous chewing movements in an animal model of Parkinson tremor (Lee et al., 2007). RNAi for iPLA₂ has also been developed. Transfection studies with RNA interference (RNAi) of iPLA₂ indicate that the levels of iPLA₂ protein and iPLA₂ activity are decreased in a dosedependent manner in transfected non-neural cells (Shinzawa and Tsujimoto, 2003). Collective evidence suggests that the development of specific inhibitors for different PLA_2 isoforms should be an important goal for future research on brain PLA_2 activities. The chemical approaches along with development of RNAi and antisense for various isoforms may provide the important information needed to develop specific PLA_2 inhibitors that can be used to retard oxidative stress and inflammatory reactions during neurodegeneration in neurological disorders.

Sphingomyelinase inhibitors block apoptotic cell death in cell culture (Ohanian and Ohanian, 2001). Injections of a difluoromethylene analog of sphingomyelin (SMA-7) (Fig.10.9) to mice with occluded middle cerebral arteries significantly reduce the size of cerebral infarcts, when compared to the control mice. These observations suggest that generation of ceramide and N-SMase contributes to apoptotic cell death and that inhibiting neutral SMase activity is an important strategy to prevent neuron death in ischemic injury (Soeda et al., 2004). Other sphingomyelin analogs (Taguchi et al., 2003; Yokomatsu et al., 2003) also show protection against apoptotic cell death in various types of cell cultures (Levade et al., 2002; Luberto et al., 2002). Collectively, these studies suggest that ceramide generation in brain tissue can be used as a target for treating neurological disorders and malignant tumors.

At least three nitric oxide synthase (NOS) isoforms have been identified by molecular cloning and biochemical studies: a neuronal NOS or type 1 NOS (nNOS), an immunologic NOS or type 2 NOS (iNOS), and an endothelial NOS or type 3 NOS (eNOS). The enzymic activities of eNOS or nNOS are induced by phosphorylation triggered by Ca^{2+} entering cells and binding to calmodulin. In contrast, the regulation of iNOS seems to depend on de novo synthesis of the enzyme in response to a variety of cytokines, such as interferon- γ and lipopolysaccharide. Acute neural trauma and neurodegenerative diseases are accompanied by an upregulation in nNOS activity in neurons and eNOS activity in glial cells and vascular endothelium and later an increase in iNOS activity in a range of cells including infiltrating neutrophils and macrophages, activated microglia, and astrocytes. These effects on the NOS activities are mediated by the reversal of glutamate reuptake at synapses, activation of NMDA receptors, and elevation in intracellular Ca^{2+} (Love, 1999). The role of nitric oxide is very complex, as it can be cytotoxic or cytoprotective in relation to sources, time of synthesis, and medium redox state. Animal gene studies indicate that nitric oxide production by endothelial nitric oxide synthase may be advantageous, while nitric oxide generation by neuronal and inducible nitric oxide synthase is disadvantageous. Excessive amounts of nitric oxide in neural cells give rise to highly toxic oxidants (peroxynitrite, nitric dioxide, nitron ion) that cause apoptotic and necrotic cell death in ischemic injury, PD, AD, and HD (Ovbiagele et al., 2003). The inducible nitric oxide synthase (iNOS) isoform is a mediator in inflammatory reactions that involve the synthesis of nitric oxide in the injured brain. Studies on the analysis of neuronal degeneration and survival, cellular apoptosis, and formation of nitrotyrosine following treatment with the iNOSinhibitor L-N-iminoethyl-lysine (L-NIL) in a model of brain contusion have indicated that iNOS activity, but not iNOS immunoreactivity, is significantly reduced in animals that received L-NIL. This inhibitor reduces apoptotic cell death. It is proposed that L-NIL protects the injured brain by limiting the production of peroxynitrite at the injury site (Gahm et al., 2006). Novel therapies have been directed at genes mediating apoptosis, but no conclusive data concerning the safety and efficacy of neuroprotectants in humans have emerged (Chen et al., 2002). In addition to the above inhibitors, use of agents that block the release of cytochrome c from mitochondria has also been proposed (Williams et al., 2006). Roche has synthesized agents that target the voltagedependent anion channel component of the mitochondrial permeability transition. For example, 2-aminoethoxydiphenyl borate prevents Ca²⁺ release and tricyclic antidepressants block mitochondrial permeability transition. Serono has developed substituted carbazoles that inhibit Bax channel formation and the release of cytochrome c in isolated mitochondria. GMI PHARMA designed GP19410, which retards mitochondrial depolarization by preventing t-Bid-mediated cytochrome c release (Williams et al., 2006). These inhibitors can now be tried in animal models of neurological disorders for their beneficial effects, ability to cross blood-brain barrier, and half-life in circulation.

As stated above the problem with available caspases, calpains, PLA₂, SMase, and nitric oxide synthase inhibitors has been the occurrence of multiple forms of these enzymes, lack of information on their specificity, blood-brain barrier permeability, and half-life. Many caspase, calpain, PLA₂, SMase, and nitric oxide inhibitors originally thought to be selective for a specific isoform of these enzymes are now known to inhibit other isoforms of caspases, calpains, PLA₂ isoforms, and SMases (Farooqui et al., 1999; Cummings et al., 2000; Fuentes et al., 2003). Furthermore, the effect of inhibitors on the physical state of substrate aggregates in neural membranes remains unknown. In searching for good caspase, calpain, cPLA₂, or SMase inhibitors, kinetic properties of these enzymes are not enough to evaluate whether inhibitors are suitable for clinical use because interfacial quality of lipid bilayer also plays an important role in modulation of their activities. An ideal inhibitor should not only block caspase, calpain, PLA_2 , SMase, and nitric oxide synthase activities, but have regional specificity and should be able to reach the site where cells are under oxidative stress and neurodegenerative processes are taking place. I urge caution when considering the inhibitors of caspases, calpains, PLA₂, SMase, and NOS as a direct therapeutic method for the treatment of acute neural trauma and chronic neurodegenerative diseases.

10.8 Conclusion

Apoptosis and necrosis are two mechanisms of cell death that occur during brain development, acute neural trauma, and neurodegenerative diseases. They are triggered by the disruption of cell cycle, withdrawal of neurotrophic factors, release of excitatory amino acids, treatment with $A\beta$ peptide, inflammatory reactions, and oxidative stress. Apoptosis is characterized by nuclear chromatin condensation, cell shrinkage, bleb formation, and externalization of PtdSer. In contrast, necrosis is characterized by cellular lysis and inflammatory reactions. Biochemical changes during apoptosis include minor alterations in calcium ions, stimulation of isoforms of PLA₂, SMases, and production of low levels of ROS and RNI. Proteolytic cleavage of PARP by caspases is a hallmark of apoptosis. Hydrolysis of arachidonic acid and its metabolites, ceramide and its metabolites, and cholesterol and 24-hydroxycholesterol is closely associated with apoptotic cell death. Interactions among these lipid mediators modulate the intensity and duration of apoptotic cell death. In contrast, necrosis is characterized by ATP depletion, high levels of ROS, peroxynitrite, and 4-HNE. Since ATP levels modulate the mode of neural cell death in brain tissue, apoptosis can be switched to necrosis or necrosis can be transformed into apoptosis depending on energy status of the cell undergoing demise.

The successful treatment of acute neural trauma and neurodegenerative diseases requires a timely delivery of well tolerated, chronically active, and specific inhibitors of excitotoxicity, inflammation, and oxidative stress that can bypass or cross the blood-brain barrier without harm. Glutamate receptor antagonists, antioxidants, anti-inflammatory agents, caspase, PLA₂, and SMase inhibitors have been successfully used for the treatment of acute neural trauma, and neurodegenerative disorders in cell culture and animal models. Their tolerance, blood brain barrier permeability, and effectiveness should be thoroughly tested in animal models that are close to human on evolutionary tree. They can then be ready for testing in human subjects.

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Chapter 11 Perspective and Directions for Future Developments on Glycerophospholipid-, Sphingolipid-, and Cholesterol-Derived Lipid Mediators

11.1 Introduction

Neural membranes not only serve as barrier between extracellular and intracellular compartments, but act as storage depots for the generation of lipid mediators. Lateral and transverse forces that stabilize neural membrane are internally modulated through interactions between the two leaflets of lipid bilayer. These forces are also modulated by receptor agonist interactions. The intensity of these interactions changes rapidly as the membrane is bent or stretched and as new constituents are added, removed, or chemically modified (Janmey and Kinnunen, 2006; Farooqui and Horrocks, 2007). Stimulation of various receptors results in enhancement of neural membrane glycerophospholipid, sphingolipid, and cholesterol metabolism by phospholipases, sphingomyelinases, and cytochrome P450-dependent oxygenases, and generation of lipid mediators.

These lipid mediators differ in structural composition and exert a diverse array of effects on cellular functional activities including those linked to neural cell homeostasis, immune responsiveness, oxidative stress and neuroinflammation (Farooqui and Horrocks, 2007).

The generation of glycerophospholipid, sphingolipid, and cholesterol-derived lipid mediators is necessary for normal cellular function. Enzymically derived lipid mediators derived from glycerophospholipids include eicosanoids and docosanoids. The non-enzymic lipid mediators of glycerophospholipid metabolism are isoprostanes (isoP) and neuroprostanes (NP), and (c) 4-hydroxynonenal (4-HNE) and 4-hydroxyhexanal (4-HHE) (Phillis et al., 2006; Farooqui and Horrocks, 2006; Bazan, 2005a,b; Serhan, 2005a,b,c). Sphingolipid-derived lipid mediators include ceramide, ceramide 1-phosphate, and sphingosine 1-phosphate. Cholesterol metabolites include 7-ketocholesterol and 24-hydroxycholesterol (Farooqui and Horrocks, 2006; Guan et al., 2006). These mediators play a crucial role in the regulation of cell proliferation, survival, and cell death (Fig. 11.1) (Farooqui and Horrocks, 2006; Ong et al., 2003; He et al., 2006).

Levels of glycerophospholipid-derived lipid mediators in neural and nonneural tissues are partly regulated by diet (Farooqui and Horrocks, 2004). The high intake of food enriched in arachidonic acid (vegetable oils) elevates levels



Fig. 11.1 Involvement of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in neural cell survival and death. Plasma membrane (PM); agonist (A1); receptor (R1); phospholipases A₂, sphingomyelinases, and cytochrome P450-dependent oxygenases (1)

of eicosanoids and upregulates the expression of pro-inflammatory cytokines. Arachidonic acid-enriched diet can also increase levels of isoprostanes, which have vasoconstrictive effects in pulmonary artery, coronary arteries, cerebral arterioles, retinal vessels, and portal vein (Montuschi et al., 2007). Arachidonic acid and its metabolites (eicosanoids) have prothrombotic, proaggregatory, and pro-inflammatory properties. In contrast, diet enriched in DHA (fish and fish oil) generates docosanoids, which not only downregulates pro-inflammatory cytokines, but also have anti-inflammatory, antithrombotic, anti-arrhythmic, hypolipidemic, vasodilatory, and anti-excitotoxic effects (Farooqui et al., 2007a,b; Simopoulos, 2002; Hogyes et al., 2003). As stated in Chapter 8, polyunsaturated fatty acids interact with the genome through several mechanisms. They regulate the activity or nuclear abundance of several transcription factors, including PPAR, LXR, HNF-4, NF-*k*B, and SREBP (Jump, 2004). Fatty acids or their metabolites bind directly to specific transcription factors to regulate gene transcription. Fatty acids also modulate gene expression indirectly through their interactions with COX, LOX, PKC, or sphingomyelinase signal transduction pathways as well as pathways that involve changes in membrane lipid/lipid raft composition that affect G-protein receptor or tyrosine kinase-linked receptor signaling (Horrocks and Farooqui, 2004). This suggests that the ratio between n-6 and n-3 fatty acids must be properly maintained in human diet. The primary goal of future research on essential fatty acids should be how to increase the levels of n-3 fatty acids in human tissue including brain through diet and avoid neuropsychiatric and neurodegenerative diseases.

Ceramide and sphingosine usually inhibit proliferation and promote apoptosis, while sphingosine 1-phosphate promotes growth and suppresses apoptosis. Because these metabolites are inter-convertible (Vaena de Avalos et al., 2004), it has been suggested that it is not the absolute amounts of these metabolites but rather their relative levels that determine neural cell death or survival. Although the molecular mechanism of ceramide action is not fully understood, many studies suggest that ceramide modulates activities of many enzymes including caspases, protein and lipid kinases, phospholipases A_2 and D, and phosphatases (Cutler and Mattson, 2001; Luberto et al., 2002; Levade et al., 2002; Farooqui et al., 2007a,b).

7-Ketocholesterol, 24-hydroxycholesterol, and 25-hydroxycholesterol are cholesterol-derived enzymic and non-enzymic metabolites with diverse biological activities. They not only have direct affects on neural cell survival, but also have many indirect affects on sphingolipid metabolism, platelet aggregation, apoptosis, and protein prenylation (Schroepfer, 2000; Ong et al., 2003; He et al., 2006; Chia et al., 2008; Vaya and Schipper, 2007). The most important function of oxycholesterol is the regulation of cholesterol homeostasis, which is controlled in part by a complex series of interactions of oxysterol ligands with various receptors, such as the oxysterol-binding protein, the cellular nucleic acid-binding protein, the sterol regulatory element-binding protein, the LXR nuclear orphan receptors, and the low-density lipoprotein receptor (Schroepfer, 2000; Vaya and Schipper, 2007). Collective evidence suggests that in brain cellular homeostasis is maintained through a complex network of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators, transporters, and enzymic pathways established among plasma membrane, subcellular organelles, cytoplasm, and nucleus. This composite network responds to environmental changes by not only modulating genes for enzymes associated with synthesis and degradation of lipid mediators but also inducing the synthesis of survival factors such as neurotrophins.

11.2 Association of Lipid Mediators with Neural Cell Death

At present, in vivo threshold concentrations of glycerophospholipid-, sphingolipid-, and cholesterol-derived mediators that promote and facilitate neural cell injury and death are not known. In neurological disorders, cell death depends not only upon elevated levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators but also on cross talk (interplay) among glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators (Farooqui et al., 2004, 2007c). Neurodegeneration (apoptosis and necrosis) in kainic acid neurotoxicity, acute neural trauma (ischemia), and neurodegenerative diseases (Alzheimer disease, AD) are accompanied by markedly increased activities of caspases, phospholipases A₂, cyclooxygenases, sphingomyelinases, and cholesterol-metabolizing enzymes along with elevated levels of lipid mediators, oxidative stress, and neuroinflammation

| Lipid mediator | AD | PD | ALS | References |
|--------------------------------|-----------|-----------|-----------|--|
| FFA | Increased | Increased | Increased | Bazan et al. (2002), Farooqui and Horrocks (2007) |
| Prostaglandins | Increased | Increased | Increased | Bazan et al. (2002), Farooqui and Horrocks (2007) |
| 4-HNE | Increased | Increased | Increased | Farooqui and Horrocks (2006), Farooqui and Horrocks (2007), Zarkovic (2003) |
| Isoprostanes | Increased | Increased | Increased | Irizarry et al. (2007), Montine et al. (2004), Montuschi et al. (2007) |
| Ceramide | Increased | Increased | Increased | Cutler et al. (2002), Yu et al. (2000), France- Lanordo et al. (1997) |
| 24-Hydroxycholesterol | Increased | _ | _ | Vega and Weiner (2007) |
| 7β-Keto- hydroxycholesterol | _ | _ | _ | _ |
| 7-ketocholesterol | - | _ | _ | - |

 Table 11.1
 Alterations in levels of phospholipid, sphingolipid, and cholesterol-derived lipid

 mediators in kainic acid-induced neurotoxicity and neurological disorders

(Table 11.1) (Farooqui and Horrocks, 2006; Park et al., 2000). The role of glycerophospholipid-, sphingolipid- and cholesterol-derived lipid mediators is not only to convey the messages from cell surface to the nucleus, but also to interact with each other and modulate the intensity of signal transduction processes to control neural cell survival (Farooqui et al., 2007a). As stated above, their physiological levels are needed for optimal signal transduction processes in healthy neurons and glial cells, but their abnormally high levels in neurological disorders are associated with apoptotic or necrotic cell death in acute neural trauma and neurological disorders. Accumulation of lipid mediators, oxidative stress, neuroinflammation, along with changes in cellular redox, ion homeostasis, depletion of endogenous antioxidants, reduced expression of trophic factors, and mitochondrial dysfunction may be associated with neurodegeneration in acute neural trauma and neurodegenerative diseases (Shaw et al., 2007; Farooqui and Horrocks, 2007; Farooqui et al., 2007b). Whether these processes are early events or consequences of neurodegenerative process or initiated by some other genetic factors remains an open question. It remains to be seen whether lipid mediators can be used as biomarkers for neurodegenerative diseases? It is proposed that combination of biochemical methods and neuroimaging profiling may facilitate early diagnosis (see below) and evaluation of disease progression for therapeutic trials. To date only few biomarkers have been identified. They include phosphorylated tau protein and aggregated A β peptide for AD, synuclein for PD, SOD mutations for familial ALS, and CAG repeats in HD. It is obvious that the number of valid biomarkers is limited, and despite immense efforts to discover novel biomarkers over the last decade, the success rate has been very low. However, it is proposed that newly discovered biomarkers may have great potential in predicting chances for neurodegenerative diseases, aiding in early diagnosis, and setting standards for the development of new therapies to treat neurodegenerative diseases (Shaw et al., 2007; Farooqui and Horrocks, 2007; Farooqui et al., 2008). Furthermore, FDA and clinical laboratories researchers have to develop processes and systems for the validation of the use of lipid mediators as biomarkers. Newly discovered biomarker (lipid mediator) will have to complete the journey through clinical trials and FDA validation and approval. At the present, of prime importance is the increasing awareness that neurodegenerative diseases are multifactorial conditions associated with excitotoxicity, oxidative stress and neuroinflammation along with altered levels of lipid mediators in biological fluids (Shaw et al., 2007; Farooqui and Horrocks, 2007, 2006). With detection of enzymic lipid mediators by lipidomics, it may be possible to establish a relationship between the etiology of neurodegenerative diseases and levels of specific lipid mediators, which may not only help in an early detection, but can also be used to follow treatment and recovery processes. An ideal lipid mediator for the detection of neurodegenerative disease should be precise and reliable, distinguishable between biological fluid from normal and neurodegenerative diseases. It should be easy to quantify and reproducible. It should not be subjected to wide variation in the general population and not affected by co-morbid factors. To evaluate the effect of medication, the biomarker should change linearly with disease progression (Henley et al., 2005) (Table 11.2).

Only few enzymes associated with glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediator metabolism have been identified, but they have not been purified and characterized from brain tissue (Farooqui and Horrocks, 2006, 2007; Farooqui et al., 2007a,b). For example, at present very little information is available on resolvins, protectins, and neuroprotectin synthesizing and degrading enzymes in brain. Regulation of the activity of these enzymes may be a very important area for future research work. Receptors for resolvins and protectins have been identified in non-neural cells, but no information is available on factors that regulate their movement across the neural cell membranes (Bazan, 2005a,b; Serhan, 2005a,b,c, 2006). Very little information is available on non-enzymic lipid mediators of arachidonic acid metabolism (isoprostanes) and docosahexaenoic acid metabolism (neuroprostanes) in brain tissue (Fam and Morrow, 2003; Roberts et al., 1998). Collective

 Table 11.2
 Characteristics of an ideal lipid mediator that can be used as biomarker for the detection of neurodegenerative diseases

| Criteria | Lipid mediator with biomarker properties |
|----------|--|
| 1 | High specificity of lipid mediator |
| 2 | Availability of lipid mediator in blood, CSF, and urine |
| 3 | Well establish relationship of lipid mediator with neurodegeneration |
| 4 | Inexpensive detection of lipid mediator |

evidence suggests that more information is needed on isolation, characterization, and quantification of enzymic and non-enzymic lipid mediators in normal brain and brains from patients with neurological disorders.

11.3 Detection and Levels of Lipid Mediators in Neurological Disorders by Lipidomics

Earlier studies on levels of lipid mediators in biological fluids, mainly cerebrospinal fluids (CSF), using classical biochemical procedures were unsuccessful in AD, PD, HD, and ALS patients (Kim et al., 2004; Migliore et al., 2005; Floyd and Hensley, 2002). In recent years, investigators are developing technologies related to lipidomics, proteomics, and genomics. These procedures can detect minute amounts of lipid mediators in biological fluids (German et al., 2007; Watson, 2006; Soule et al., 2006; Bowers-Gentry et al., 2006). Lipidomics analysis has been used for detection and characterization of F₂-isoprostanes, prostaglandins, leukotrienes, lipoxins, hydroxyeicosatetraenoic acids, nitrotyrosine, carbonyls in proteins, oxidized DNA bases, and 4-HNE in CSF (Serhan, 2005a; Serhan et al., 2006; Adibhatla et al., 2006; Milne et al., 2006; Hunt and Postle, 2006; Morrow, 2006; Lu et al., 2006; Perluigi et al., 2005). Lipidomics has also been used to detect the levels of lipid mediators in small tissue samples from neurotoxin-injected rat brain, animal models of neurodegenerative diseases, and brain samples from patients with neurodegenerative diseases (Yoshikawa et al., 2006; Butterfield et al., 2006). Using proteomics more studies are required on the determination of levels of enzymes responsible for the generation of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in CSF and biopsy and autopsy samples of brain tissue not only from animal models but from patients with acute neural trauma and neurodegenerative diseases. Establishment of automatic systems including databases and accurate analyses of lipid mediators derived from enzymic and non-enzymic metabolism of neuronal membrane glycerophospholipids, sphingolipids, and cholesterol will facilitate the identification of key biomarkers associated with neurodegenerative diseases (Lu et al., 2006).

Microarray analysis of tissue samples from brain regions associated with AD, PD, HD, ALS, MS, CJD, and schizophrenia can provide information on candidate genes that influence levels of lipid mediators, oxidative stress, and neuroinflammatory responses. Gene expression analysis can also provide information on vulnerable brain regions in neurodegenerative diseases that share putative pathogenetic alterations in common classes of genes, including decrements in synaptic transcript levels and increments in immune response transcripts (Mufson et al., 2006). Thus, gene expression profiles of susceptible neuronal populations may reveal mechanistic clues to the molecular mechanism underlying various neurological diseases. This not only would help in understanding molecular mechanisms associated with the development of

neurodegenerative diseases, but also would facilitate molecular diagnostics and targets for drug therapy based on gene expression in body fluids such as CSF and blood (Facheris et al., 2004). Levels of lipid mediators can then be used to monitor responses to drug therapy.

11.4 Detection of Lipid Mediators by Positron Emission Tomography

Positron emission tomography (PET) and single photon emission tomography (SPECT) are molecular imaging procedures that use radiolabeled molecules to image molecular interactions of biological processes in vivo. PET and SPECT imaging technologies for brain tissue have been developed to facilitate a novel way for detecting the neurodegeneration site using low mass amounts of radiolabeled compound to image the whole brain (Phelps, 2000). PET has been used to titrate drugs to their sites of action within specific tissue region in vivo and to assay biological outcomes of the processes being modified in animals and patients (Phelps, 2000; Masters et al., 2006). The goal of PET technology is to provide a novel way to improve the rates of discovery and approval of radiopharmaceuticals. Extending this relationship into clinical practice can improve drug use by providing molecular diagnostics in concert with molecular therapeutics. Diseases are biological processes, and molecular imaging with PET is sensitive and informative to these processes. Collectively, these studies suggest that PET and SPECT are more sensitive than structural imaging procedures. They are capable of identifying subtle neurochemical alterations in brain tissue before the commencement of structural changes that facilitate early diagnosis, monitoring disease progression, and better treatment follow-up (Phelps, 2000; Masters et al., 2006). These procedures have emerged as powerful tools for quantifying the neurobiological correlates of dementia with neurodegenerative changes in brains of AD, PD, HD, and ALS patients (Kimberley and Lewis, 2007; Thobois et al., 2005; Ono, 2007; Masters et al., 2006). Selective labeling of lipid mediator precursor offers unique means for investigating the dynamic relationships between various lipid mediators in neurodegenerative process in vivo. This procedure has many advantages over studies on platelet and cerebrospinal fluid, neuroendocrine challenge studies, animal models, and postmortem receptor-binding assays (Farde, 1996; Halldin et al., 2001). These advantages include determination of subpicomolar concentrations of drug $(10^{-12}M)$ at high ratio of radiolabeled to unlabeled drug molecules. Consequently, intravenous injection of less than a microgram of the radioactive drug is sufficient to perform a PET study in human. Thus, recent advances in tracer kinetic modeling, magnetic resonance imaging (MRI) to PET registration, instrumentation, and image processing have paved the way for increased emphasis on functional imaging studies on patients with neurological disorders. These techniques provide important information on rates of generation of lipid mediators as quantitative autoradiography or PET. Thus, PET studies provide data that may aid in selecting the best drug candidates, determining optimal dosing regimens, clearing regulatory hurdles, and lowering risks of failure (Farde, 1996; Halldin et al., 2001).

Incorporation of radioactive or heavy isotope-labeled fatty acids in brain, followed by its determination in brain regions and lipid compartments as a function of time, provides a useful means of studying metabolism of glycerophospholipid in vivo (Rapoport, 1999, 2005; Hampel et al., 2002; Esposito et al., 2007). By measuring fluxes, turnover rates, half-lives, ATP consumptions, and fatty acid metabolism in vivo, one can determine the rate of lipid mediator generation that can be imaged. Thus, metabolism of fatty acids and fatty acidderived lipid mediators can be used to study neuroplasticity and neurodegeneration in normal human brain and brain from patients with neurodegenerative diseases. Initial experiments on animal models of Alzheimer and Parkinson disease with chronic unilateral lesions of nucleus basalis or substantia nigra indicate that PET and [1-¹¹C]arachidonic acid can be used with drug activation to image signal transduction (Giovacchini et al., 2002; Bhattacharjee et al., 2007). Detailed investigations are required on the use of MRI along with PET imaging to judge the severity and progression of dementia during the course of various neurodegenerative diseases in patients and normal human subjects (Rapoport, 2001; Hampel et al., 2002; Wendum et al., 2003; Masters et al., 2006; Esposito et al., 2007; Bhattacharjee et al., 2007). In neural membranes, PLA₂ enzymes are coupled with many receptors through different coupling mechanisms (Farooqui et al., 2006). Some receptors, like 5-HT2A/2C receptors, are linked to G-protein coupling, while others like NMDA receptors do not require G-proteins. Recently attempts have been made to quantitate the imaging of PLA2-mediated signal transduction processes in response to fluoxetine, a selective serotonin reuptake inhibitor, in unanesthetized rats (Qu et al., 2003). These studies indicate that olfactory cortex, basal ganglia, hippocampus, and thalamus that have high density of serotonin reuptake transporters and 5-HT2A/2C receptors strongly respond to fluoxetine, while brain stem, spinal cord, and cerebellum show no significant response. Neuroimaging studies on arachidonic acid signaling in rat model of PD indicate that in vivo imaging of AA signaling using dopaminergic drugs can identify pre- and postsynaptic DA changes in animal models of PD (Bhattacharjee et al., 2007). Furthermore, preliminary PET measurements of injected [1-¹¹C]AA in 16 healthy volunteers indicate that visual stimulation at flash frequencies 2.9 Hz or 7.8 Hz compared with the dark (0 Hz) condition can modify both K* for arachidonic acid and regional cerebral blood flow in visual and related areas of the human brain (Esposito et al., 2007). Similar studies are needed on in vivo imaging of other isoforms of PLA₂ that are coupled to different receptors that may be associated with pathogenesis of neurodegenerative conditions in human brain.

Studies on intravenous injections of radiolabeled arachidonic and docosahexaenoic acids indicate that about 5% of brain arachidonic and docosahexaenoic acids are lost daily through metabolism (Rapoport, 2003). These fatty acids are replaced from dietary sources through the plasma. Turnover rates of arachidonate and docosahexaenoate are independent of each other, and probably are regulated by independent sets of agonists, receptors, and enzymes that are linked to different receptors through various coupling mechanisms. Studies on quantitative imaging of [¹¹C]docosahexaenoic acid are needed to understand the importance of DHA in neuroprotection in neurological disorders. PET studies are also needed for sphingolipid- and cholesterol-derived lipid mediators. Care should be taken in choosing appropriate component of lipid mediator that can not only enter and distribute throughout the brain to avoid peripheral side effects but rapidly visualized, monitored, and quantified by PET.

11.5 Proteomics, Enzymes of Lipid Metabolism, and Neurodegenerative Diseases

Proteomics and genomics are facilitating the identification of specific genes involved not only in regulation of biosynthesis and degradation of individual molecular species of glycerophospholipid, sphingolipid, and cholesterol, but also in identification of genes related to its sorting and transport to various cellular and subcellular compartments (Voelker, 2003; Lee et al., 2005; Forrester et al., 2004). Proteomics and other molecular biological approaches such as cloning the cDNA for enzymes synthesizing and degrading glycerophospholipids, sphingolipids, and cholesterol and their functional expression in neurons, astrocytes, oligodendrocytes, and microglia will advance the understanding of lipidology at cellular and subcellular levels in brain tissue. Regular use of proteomics and genomics technologies in clinical laboratories may lead to the identification and expression profile of genes involved in the modulation of levels of lipid mediators in neurons and glial cells (Hovland et al., 2001). This will also facilitate the monitoring of alterations in lipid mediator levels in regions where pathogenesis of neurodegenerative diseases starts (Colangelo et al., 2002; Thomas et al., 2006). Using proteomics, it is clearly shown that levels of 4-HNE-modified proteins are markedly increased in the spinal cord tissue samples from G93A-SOD1 transgenic mice (an animal model of ALS) compared to nontransgenic mice (Perluigi et al., 2005). This observation supports the view that oxidative stress is closely associated with the pathogenesis of ALS.

Although, transcripts, activities, and immunoreactive proteins for enzymes synthesizing and degrading glycerophospholipids, sphingolipid, and cholesterol are widely expressed throughout the brain (Molloy et al., 1998; Kishimoto et al., 1999; Zanassi et al., 1998; Balboa et al., 2002; Nakajima et al., 2002; Luberto et al., 2002; Levade et al., 2002; Korade et al., 2007) very little is known about their interactions with each other at cellular and subcellular levels in neurons and glial cells. Some progress has been made on purification and characterization of enzymes associated with glycerophospholipid, sphingolipid, and cholesterol metabolism using classical techniques. Their purification by proteomics has

not been performed and genes that encode them have been identified and mapped to chromosomes by genomics and genetic analysis procedures. Similarly, factors that modulate activities of phospholipases A₂, SMases, and cytochrome P450-dependent oxygenase have not yet been identified. These factors include cytokines and chemokines. Expression of genes associated with cytokines and chemokines and their association with phospholipases A2 and SMasesmediated apoptosis and necrosis in neurons, macroglial and microglial cell should be studied in cell culture models of acute neural trauma and neurological disorders (Allan and Rothwell, 2003; Lucas et al., 2006; Rothwell, 1999). Phospholipases A₂ and SMase-mediated generation of ROS also results in activation and translocation of NF- κ B to the nucleus, where it mediates the transcription of many genes implicated in inflammatory and immune responses. These genes include COX-2, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, TNF- α , IL-1 β , IL-6, sPLA₂, inducible nitric oxide synthase (iNOS), and matrix metalloproteinases (MMPs). ROS-mediated NF- κ B activation also involves NADPH oxidase, which is an important component of the innate immune response against toxic agents, and is involved in shaping the cellular response to a variety of physiological and pathological signals in neurodegenerative diseases (Anrather et al., 2006; Zhang et al., 2004; Frey et al., 2006; Rubin et al., 2005; Miller et al., 2006; Sun et al., 2007).

The occurrence of isoforms of phospholipases, sphingomyelinases, and cytochrome P450-dependent oxygenases and their downstream enzymes in cytoplasm and plasma and nuclear membranes complicates the analyses of their function at cellular and subcellular levels. Future studies on this multiplicity of phospholipases, sphingomyelinases, and cytochrome P450-dependent oxygenases using lipidomics and proteomics may provide information on the diversity of their function and the selectivity of molecular species as substrates (Bazan, 2005b; Serhan, 2005c; Bazan, 2005a; Serhan, 2005b). Future studies should also address factors that determine the specificity of neural cell responses at cellular, subcellular, and microdomain levels and how much of this specificity is dictated by the interplay among second messengers generated by phospholipases, sphingomyelinases, and cytochrome P450-dependent oxygenases.

11.6 Antisense and RNAi as Neuroprotective Agents

The approach for designing inhibitors of enzymes of glycerophospholipid, sphingolipid, and cholesterol metabolism should be based on our rapidly emerging concept of signal transduction pathways in neurological disorders. Specific inhibitors, antisense oligonucleotides, and RNAi for enzymes associated with glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators should be synthesized (Bennett, 1998; Pirollo et al., 2003; Huang and Miller, 2005; Heng and Cao, 2004). Better drug delivery systems that target brain need to be developed to protect PLA₂, SMase, and cytochrome P450-

dependent oxygenases inhibitors, antisense oligonucleotides, and RNAi from in vivo degradation or detoxification. The delivery of inhibitors, antisense, and RNAi should be performed through drug delivery systems that not only protect them from detoxification but reach the site where neurodegenerative processes are taking place (Yoshikawa et al., 1999; Andresen and Jorgensen, 2005). This would enhance the efficacy of PLA₂, SMase, and cytochrome P450-dependent oxygenase inhibitors, antisense oligonucleotides, and RNAi. The effects of these drugs on genes expression can be monitored by microarray procedures (Colangelo et al., 2002; Bosetti et al., 2005). These studies can lead to better therapeutic agents for the treatment of neurological disorders involving glycerophospholipid, sphingolipid, and cholesterol metabolism alterations.

11.7 Significance of Developing Early Detection Procedures and Treatment for Neurodegenerative Diseases

As stated earlier, neurodegenerative diseases are characterized by chronic and progressive loss of neurons in discrete areas of the brain, producing debilitating symptoms such as dementia, loss of memory, loss of sensory or motor capability, decreased overall quality of life, and, eventually, premature death (Farooqui and Horrocks, 2007). There is no treatment for most neurodegenerative diseases. Available treatments are symptomatic in nature and do not prevent or slow the progression of disease. Understanding of molecular mechanism associated with neuropathological progression can promote promising therapeutic approaches (Farooqui and Horrocks, 2007; Farooqui et al., 2008). Intense efforts are made not only to slow or halt progression using inhibitors of phospholipases, kinases, proteases, and nitric oxide syntheses, but also to prevent the onset of neurodegenerative processes. Strategies for neurorepair or neuroprotection are also actively pursued using dietary factors such as increasing the ratio between n-3/n-6 fatty acids, antioxidants, and other natural remedies (Cole et al. 2005). The major objective of neurodegeneration research is to determine the primary mechanism(s) of neurodegeneration and factors that modulate pathophysiologic events in neurodegenerative diseases. It is becoming increasingly evident that neurodegenerative diseases are multifactorial diseases. This notion has led to the suggestion that drugs directed against a single target may not be suitable for the treatment of neurodegenerative diseases and a cocktail of drugs with pluripharmacological effects may be more effective (Farooqui et al., 2008). The effectiveness of drug cocktail in treating neurodegenerative diseases may depend upon several factors. These factors include drug cocktail ability to cross the blood-brain barrier, its potential in terms of subcellular distribution occurring in neural cell (especially in mitochondria), its multifunctional capacity, and synergistic action of drug cocktail ingredients. Thus, more studies are required on therapeutic aspects of neurodegenerative diseases using a cocktail of inhibitors of phospholipases, calpains, protein kinases, and various antioxidants.

We are in the midst of a national crisis. Our nursing homes are already packed with AD, PD, and ALS patients. As baby boomer generation grows older, enormous impact of neurodegenerative diseases will be felt by the American society (Brookmeyer et al., 1998; Cogan and Mitchell, 2003; Hodes, 2006; Trojanowski, 2008). The projected cost to Medicare for treating AD patients is estimated to be about 1 trillion dollars by 2050. This number does not include other neurodegenerative diseases. Such a budget will not only burst NIH budget, but will also affect US economy. Thus, developing strategies to prevent neurodegeneration and to promote a healthy nervous system is extremely important (Hodes, 2006; Trojanowski, 2008).

11.8 Conclusion

We have been empowered by lipidomics, proteomics, and genomics. These procedures can be used for not only identifying and determining levels of molecular species of glycerophospholipids, sphingolipids, and various metabolites of cholesterol metabolism, but also detecting levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in small brain tissue and biological fluid samples. Comprehensive identification and full characterization of molecular species of glycerophospholipids and sphingolipids and characterization of levels of their lipid mediators in normal brain and brain from patients with neurological disorders, characterization of enzymes associated with their metabolism by proteomics, and microarray for identification of their genes would provide complete information needed to understand the pathogenesis of acute neural trauma and neurodegenerative disorders. Use of neuroimaging procedures like PET can allow in vivo quantification of radiolabeled lipid mediator concentration in the subpicomolar range allowing detection of neurodegenerative process at asymptomatic stages when there is no indication on CT and MRI (Phelps, 2000). Identification of biomarkers for neurodegenerative diseases may lead to not only early diagnosis and follow-up of the progression of neurodegenerative diseases, but also advance monitoring of therapeutic responses.

It is hoped that the coming 50 years would not only witness the understanding of the role of glycerophospholipid and sphingolipid molecular species and their lipid mediators in metabolic processes in brain tissue, but also gain information on intracellular glycerophospholipid, sphingolipid, and cholesterol trafficking, sorting, and their metabolic regulation at cellular and subcellular levels in the brain tissue.

The determination of levels of lipid mediators by lipidomics in biological fluid for early detection of neurodegenerative disorders can also provide new insight for early detection. This process may shed some light on the polymorphism and facilitate the identification of variants of neurodegenerative disorders. This information can be used for developing treatment for neurodegenerative diseases.

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