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Integrating cytosolic phospholipase A2 with oxidative/nitrosative signaling pathways in neurons: a novel therapeutic strategy for AD

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Abstract

The pathophysiology of Alzheimer's disease (AD) is comprised of complex metabolic abnormalities in different cell types in the brain. To date, there are not yet effective drugs that can completely inhibit the pathophysiological event and efforts have been devoted to prevent or minimize the progression of this disease. Much attention has focused on studies to understand aberrant functions of the ionotropic glutamate receptors, perturbation of calcium homeostasis, and toxic effects of oligomeric amyloid beta peptides (A β) which results in production of reactive oxygen and nitrogen species (ROS/RNS), and signaling pathways leading to mitochondrial dysfunction and synaptic impairments. Aberrant phospholipase A₂ (PLA₂) activity has been implicated to play a role in the pathogenesis of many neurodegenerative diseases, including AD. However, mechanisms for their modes of action and their roles in the oxidative and nitrosative signaling pathways have not been firmly established. In this article, we review recent studies providing a metabolic link between cytosolic PLA₂ (cPLA₂) and neuronal excitation due to stimulation of ionotropic glutamate receptors and toxic A β peptides. The requirements for Ca²⁺ binding together with its post-translational modifications by protein kinases and possible by the redox-based S-nitrosylation provide strong support for a dynamic role of cPLA₂ in serving multiple functions to neurons and glial cells under abnormal physiological and pathological conditions. Therefore, understanding mechanisms for cPLA₂ in the oxidative and nitrosative pathways in neurons will allow development of novel therapeutic targets to mitigate the detrimental effects of AD.

Keywords

Alzheimer's disease; phospholipase A₂; reactive oxygen species (ROS); nitric oxide (NO); NADPH oxidase; mitochondria

Introduction

Alzheimer's disease (AD) is one of the most devastating age-related neurodegenerative diseases affecting more than 5 million people in the United States alone. With the rapid increase in aging population in the next two decades, the number of AD patients is expected

to double, causing a huge economic burden to the society. AD pathophysiology is comprised of complex metabolic changes in neurons, glia and neurovascular cells; besides the increased deposition of amyloid plaques and fibrillary tangles in neurons, other hallmarks for the disease include oxidative stress, glial cell inflammation, cerebrovascular abnormalities, and most importantly, synaptic failure (Selkoe 2002). However, despite these neurochemical and physiological manifestations, the biochemical mechanisms underlying the decline in memory and other cognitive functions associated with the progression of AD remain undetermined. Recent studies have provided strong support for the involvement of aberrant oxidative/nitrosative signaling pathways which result in a progressive damage of the neuronal circuitry, especially those associated with cholinergic and glutamatergic neurons. Excessive stimulation of ionotropic glutamate receptors, e.g., the N-methyl-D-aspartic acid (NMDA) receptors, has been shown to cause disturbance of neuronal calcium homeostasis leading to activation of proteases, nucleases and phospholipases, and trigger signaling pathways for production of reactive oxygen/nitrogen species (ROS/RNS). In turn, these events become the basis of mitochondrial dysfunction and neuronal apoptosis.

Phospholipases A₂ (PLA₂s) are enzymes for hydrolysis of fatty acids in membrane phospholipids. Among many types of PLA₂s known to occur in the central nervous system, there is increasing recognition for the role of Ca²⁺-dependent cytosolic phospholipase A₂ (cPLA₂) in the pathophysiology of AD. cPLA₂ is present ubiquitously in most brain cells including neurons, astrocytes, and microglial cells. Besides Ca²⁺, this enzyme is regulated by receptor-mediated signaling pathways including phosphorylation by protein kinases and S-nitrosylation, i.e., covalent reaction of NO with specific protein thiol groups. Earlier studies have demonstrated the role of PLA₂s in neurodegenerative diseases (Sun *et al.* 2004), and that their damaging effects are attributed to involvement in oxidative/nitrosative signaling pathways (Sun *et al.* 2007). In particular, attention has been focused on the cytosolic PLA₂ (cPLA₂) in neurons; its activation linked to glutamate excitotoxicity and in neuronal damage after exposure to cytotoxic beta amyloid peptides (Aβ). Since studies to link cPLA₂ to the oxidative/nitrosative pathways in neurons and its role in AD pathology have not been extensive, an important goal for this paper is to gather recent information on glutamate excitation and ascertain the role of cPLA₂ in oxidative/nitrosative pathways associated with AD pathology.

Cytotoxic effects of beta amyloid peptides

While accumulation of amyloid plaques has been regarded as one of the pathophysiological landmarks of AD, the “amyloid hypothesis” has been under challenge because a direct correlation between the amount of amyloid plaques deposition and the severity of the disease has not been firmly established (Terry *et al.* 1991). In fact, amyloid plaques in the brain have been regarded as “tomb stones” without obvious functions. Nevertheless, amyloid plaques are enriched in Aβ peptides which are produced from the amyloid precursor protein (APP) through cleavage by beta and gamma secretases. In recent years, special attention has been placed on studies to investigate mechanism(s) of aberrant Aβ production from APP and their ability to aggregate and cause toxic effects on neurons, glia and cerebral endothelial cells. In neurons, toxic Aβ oligomers have been shown to down regulate NMDA receptor trafficking (Snyder *et al.* 2005), alter neuronal circuitry, and impair synaptic activity (Palop & Mucke 2010). Studies by Selkoe’s group detected the release of soluble Aβ oligomers in culture medium of neurons and hippocampal slices over-expressing human mutant APP, and Aβ oligomers recovered from the conditioned media could increase NMDA-induced Ca²⁺ influx into synaptic spines (Shankar *et al.* 2007, Shankar *et al.* 2008). Oligomeric Aβ has been shown to perturb Ca²⁺ homeostasis in neurons, alter Ca²⁺-dependent enzymes (Paul & Connor 2010, Demuro *et al.* 2010, Alberdi *et al.* 2010), and inhibit hippocampal long-term potentiation (LTP), a form of synaptic plasticity (Ondrejcek

et al. 2010). Studies using antibodies specifically detecting oligomeric form of A β also support the presence of A β oligomers in the AD brain. Furthermore, the abundance of A β oligomers in AD brain was positively correlated with the degree of synaptic loss and severity of cognitive impairment (Pham *et al.* 2010). In fact, numerous studies have successfully used *in vitro* protocols for preparation of A β oligomers to demonstrate their detrimental effects on neurons (Stine *et al.* 2003, Stine *et al.* 2010). Consequently, more studies are needed to better understand aberrant A β aggregation in brain and mechanisms whereby these oligomers impair synaptic functions (Wilcox *et al.* 2011).

NMDA receptor-mediated glutamatergic signaling pathways induce Ca²⁺ influx and generation of RNS/ROS

It is well known that excitatory neurotransmission is necessary for normal development and plasticity of synapses, and some forms of learning or memory. However, excessive activation of glutamate receptors has been implicated in neuronal damage in many neurological disorders. Glutamate is the major excitatory neurotransmitter in the brain and is rapidly released (in milliseconds) from nerve terminals in a Ca²⁺-dependent manner. Released glutamate can diffuse across the synaptic cleft to interact with postsynaptic receptors in adjacent neurons. It is currently thought that overstimulation of extrasynaptic NMDA receptors can result in neuronal damage, whereas, activation of synaptic NMDA receptor can mediate the survival pathways (Hardingham *et al.* 2002, Papadia *et al.* 2005, Papadia *et al.* 2008, Okamoto *et al.* 2009). The NMDA receptor has attracted attention for a long period of time because it has specific properties that set it apart from other ionotropic glutamate receptors, e.g., the (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid (AMPA) and kainate receptors. One such characteristic is that the NMDA receptor channels are highly permeable to Ca²⁺, thus permitting Ca²⁺ entry after ligand binding. Subsequent binding of Ca²⁺ to various intracellular molecules can lead to many significant consequences. In particular, excessive activation of NMDA receptors leads to the production of damaging free radicals (e.g., NO and ROS) and other enzymatic processes contributing to cell death (Lipton & Rosenberg 1994).

Increased levels of neuronal Ca²⁺, in conjunction with the Ca²⁺-binding protein calmodulin, trigger the activation of nNOS and subsequent generation of NO from the amino acid L-arginine (Bredt *et al.* 1991, Abu-Soud & Stuehr 1993). NO is a gaseous radical (thus highly diffusible) and a key molecule that plays a vital role in normal signal transduction but in excess can lead to neuronal cell damage and death. Three subtypes of NOS have been identified; two constitutive forms – neuronal nNOS, endothelial eNOS, and one inducible form, iNOS. Constitutive and inducible NOSs are also further distinguished by CaM binding: nNOS and eNOS bind calmodulin in a reversible Ca²⁺-dependent manner. In contrast, iNOS binds calmodulin so tightly at resting intracellular Ca²⁺ concentrations that its activity does not appear to be affected by transient variations in Ca²⁺ concentration. Interestingly, in order to terminate iNOS-mediated NO production, microglia may redistribute iNOS to the aggresome for inactivation (Kolodziejska *et al.* 2005).

Excitatory glutamate receptors stimulate ROS production through NADPH oxidase

Increase in oxidative/nitrosative stress is an important characteristic feature underlying the pathophysiology of AD (Butterfield *et al.* 2011). The oxidative/nitrosative hypothesis is supported by detections of increases in markers, such as protein carbonyls, 3-nitrotyrosine, hydroxynonenal and isoprostanes, in mild cognitive impaired (MCI) brains (Keller *et al.* 2005, Mattson 2009, Sultana & Butterfield 2010). In the brain, neurons in hippocampus are

especially susceptible to oxidative stress. Uncontrolled stimulation of ionotropic glutamate receptors, especially the NMDA receptor subtype, is known to cause Ca^{2+} influx and trigger Ca^{2+} -dependent enzymes including protein kinases, proteases, nucleases and phospholipases, leading to oxidative stress, mitochondrial dysfunction and apoptosis. In recent studies, oxidative stress in this form of neuronal excitation is linked to ROS production through the activation of NADPH oxidase (Brennan *et al.* 2009, Shelat *et al.* 2008).

NADPH oxidase is a redox active enzyme occurring widely in all cell types in the brain (Sorce & Krause 2009). The prototypic NADPH oxidase (NOX2) is comprising of membrane-subunits, i.e., gp91phox and p22phox, and cytosolic subunits p47phox, p67phox, p60phox, and rac 1 or rac 2 (Bedard & Krause 2007). Recognition of ROS production from NADPH oxidase in neurons and glial cells in the central nervous system has been an important finding. Due to possible physiological and pathological processes involving ROS in the CNS, there is intense interest to explore receptor signaling pathways linking to NADPH oxidase and inhibitors regulating the ROS production process (Jaquet *et al.* 2009).

A number of NADPH oxidase (NOX) isoforms are present in the mammalian system. Besides NOX2 which is the phagocytic form of NADPH oxidase, other isoforms of NOXs have been identified in brain, e.g. NOX1, NOX4 and NOX5 (Sorce & Krause 2009, Brown & Griendling 2009). NOX1 is shown to play a role in ischemia/reperfusion injury (Kahles *et al.* 2010) and paraquat-induced oxidative damage in dopaminergic neurons (Cristovao *et al.* 2009). On the other hand, NOX4 is involved in chronic glutamate toxicity in neurons (Ha *et al.* 2010). The synaptic localization of NOX2 in neurons provides support for the role of this enzyme in mediating physiological functions in the synapse. Recent studies showed an increase in NOX2 expression and activity in cortex of AD patients (Ansari & Scheff 2011, Bruce-Keller *et al.* 2010, Shimohama *et al.* 2000). The role of NOX isoforms in regulating redox reactions in brain gives strong indication that these enzymes are important in neurodegenerative diseases including AD, Parkinson's disease (PD) and stroke (Zekry *et al.* 2003, Yoshioka *et al.* 2011).

Nitrosative stress regulates protein misfolding and neuronal cell death

Oxidative/nitrosative stress can facilitate protein misfolding and aggregation, and is thought to play a role as a pathogenic trigger of neurodegenerative diseases. Recent scientific advances, however, support the notion that NO-related species may participate in the process of protein misfolding through protein S-nitrosylation under degenerative conditions.

Early investigations indicated that NO mediated signaling pathways can regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death (Dawson *et al.* 1991, O'Dell *et al.* 1991, Bredt & Snyder 1994, Schuman & Madison 1994). In general, NO can exert physiological and pathophysiological effects via stimulation of guanylate cyclase to form cyclic guanosine-3', 5'-monophosphate (cGMP) or through S-nitros(yl)ation of regulatory protein thiol groups (Lei *et al.* 1992, Stamler *et al.* 2001). S-Nitrosylation is the covalent addition of an NO group to a critical cysteine thiol/sulfhydryl (RSH or, more properly, thiolate anion, RS^-) to form an S-nitrosothiol derivative (R-SNO). Over the past decade, accumulating evidence has suggested that S-nitrosylation can regulate the biological activity of a great variety of proteins, in some ways akin to phosphorylation (Yao *et al.* 2004, Uehara *et al.* 2006, Gu *et al.* 2002). Analyses of mice deficient in either nNOS or iNOS confirmed that NO is an important mediator of cell injury and death after excitotoxic stimulation (Huang *et al.* 1994, Iadecola *et al.* 1997). In addition, inhibition of NOS activity ameliorates the progression of disease pathology in animal models of PD, AD, and Amyotrophic lateral sclerosis (ALS), suggesting that excess

generation of NO plays a pivotal role in the pathogenesis of several neurodegenerative diseases.

Neuronal ROS production and mitochondrial dysfunction in AD

Besides NADPH oxidase, other redox reactions in subcellular organelles, e.g., mitochondria, can also produce ROS and contribute to synaptic impairments (Pagani & Eckert 2011). Studies in our laboratory provided evidence that both NMDA and A β can induce rapid production of ROS from NADPH oxidase in neurons (Shelat *et al.* 2008). Furthermore, ROS from NADPH oxidase can trigger signaling pathways leading to mitochondrial dysfunction (He *et al.* 2011a). The role of NADPH oxidase as an initial source of ROS could be demonstrated by pretreatment of neurons with gp91ds-tat, a specific peptide inhibitor for NOX2, which abrogated the NMDA- and A β -mediated ROS production and mitochondrial dysfunction (He *et al.* 2011b). In another study, exposure of A β to hippocampal slice culture also induced an increase in mitochondrial superoxide production and a concomitant decrease in long term potentiation (LTP); inhibition of mitochondrial ROS production protected against A β -induced hippocampal synaptic damage (Ma *et al.* 2011). Taken together, these results are in line with the notion that excessive neuronal excitation or exposure of neurons to toxic A β can induce ROS from both NADPH oxidase and mitochondria; together, these oxidative events form the basis of neuronal apoptosis and cell death, a crucial mechanism underlying the pathophysiology of AD (Simonyi *et al.* 2010).

NADPH oxidase/ROS signaling pathway - activation of MAPK and cPLA₂

Production of ROS from NADPH oxidase is known to trigger changes in a number of signaling pathways including activation of mitogen-activated protein kinases (MAPK). In neurons, NMDA and A β induce ROS production through NADPH oxidase and in turn this leads to activation of MEK1/2/ERK1/2, and cPLA₂ (Shelat *et al.* 2008). cPLA₂ is a 87 kDa protein comprised of a C2 domain with binding sites for intracellular Ca²⁺, a catalytic domain with three putative phosphorylation sites: Ser505, Ser727, Ser515 (Murakami *et al.* 2011), and at least one cysteine residue is receptive for S-nitrosylation by NO (Xu *et al.* 2008). In human epithelial cells, S-nitrosylation of cPLA₂ by NO from iNOS led to a six-fold increase in enzyme activity (Xu *et al.* 2008). With these properties, activation of cPLA₂ has been linked to a number of cell surface receptors and ion channels, including the G-protein-coupled P2Y₂ receptor in astrocytes (Xu *et al.* 2002) and the ionotropic glutamate receptors in neurons (Shelat *et al.* 2008). cPLA₂ is known to target membrane phospholipids for the release of arachidonic acid (AA), a lipid mediator serving as a precursor for prostaglandin synthesis. In neurons, AA can also serve as a retrograde messenger, an event implicated in memory functions (Bolshakov & Siegelbaum 1995, Leu & Schmidt 2008, Rapoport 2008). Studies *in vitro* indicated that AA could also exert toxic and trophic effects on neurons depending on the concentration presented (Okuda *et al.* 1994). Activation of cPLA₂ also induces the transient release of lysophospholipids, which because of their detergent-like properties, can perturb membrane micro-environment and alter protein function (Lee *et al.* 2010).

Role of cPLA₂ in A β -mediated neuronal apoptosis

There is increasing evidence suggesting a role for cPLA₂ in A β -induced neuronal apoptosis. In the study by Pillot's group, A β was shown to cause neuronal apoptosis through signaling pathways involving PKC, p38 and MEK/ERK, and subsequently cPLA₂ and COX (Kriem *et al.* 2005). Our study with primary neurons further showed the ability for oligomeric A β to activate NADPH oxidase and MAPK (ERK1/2) prior to cPLA₂ activation and release AA (Fig. 1) (Shelat *et al.* 2008). In astrocytes, a study by Zhu *et al.* (2006) also showed the ability of oligomeric A β to activate NADPH oxidase and decrease mitochondrial membrane

potential, a signaling event involving both cPLA₂ and iPLA₂ (Zhu *et al.* 2006). In support of the role of cPLA₂ in mediating the toxic events of A β , neurons isolated from mice deficient in cPLA₂^{-/-} showed resistance to the toxic effects of A β (Desbene *et al.* 2011). Besides the response to cell injury, cPLA₂ was shown to play an important role in mediating neuronal homeostatic processes related to growth cone repulsion and collapse, and neurite outgrowth and differentiation during neuro-development (Schaeffer *et al.* 2009, Sanford *et al.* 2012). In an *in vivo* study, intracerebroventricular injection of A β oligomer to wild type mice caused activation of cPLA₂, which in turn led to a decline in cognitive function and reduction of synaptic markers (Desbene *et al.* 2011). In other studies, activation of cPLA₂ in non-neuronal cells can trigger neuroinflammatory responses (Sundaram *et al.* 2012). In microglial cells, aggregated A β was shown to induce superoxide production and stimulate cPLA₂ and AA release; and the effects of toxic A β were abrogated by cPLA₂ inhibitors and antisense oligonucleotides (Szaingurten-Solodkin *et al.* 2009).

Evidence for the role of cPLA₂ in AD

In agreement with activation of cPLA₂ through the protein kinases, an increase in p-cPLA₂ was observed in the hippocampus of Tg (transgenic) mice expressing hAPP as well as in human AD brain (Sanchez-Mejia *et al.* 2008). This study further showed that decreasing cPLA₂ expression by crossing AD Tg mice with cPLA₂ deficient mice resulted in better neurochemical and behavioral outcome as compared with the Tg mice with full copies of cPLA₂. Although several cPLA₂ isoforms are present in the brain, most studies have demonstrated involvement of cPLA₂ alpha. Few studies have investigated involvement of other cPLA₂ isoforms. Besides neurons, whether cPLA₂ subgroups contribute to AD pathology in other cell types remain to be further investigated (Schaeffer *et al.* 2010).

Despite evidence for increased phosphorylation of cPLA₂ in Tg animal models and in AD brain, studies by Gattaz's group demonstrated a reduction in PLA₂ activity in human AD brain, and the reduction in activity correlated with memory impairment and neuropathology (Schaeffer *et al.* 2009). Since studies were carried out with brain tissue, it is difficult to differentiate different types of PLA₂ and thus the mechanism underlying the decrease in PLA₂ activity in AD brain remains elusive. Based on evidence that multiple receptor-pathways and ATP-dependent protein kinases are needed for activation of cPLA₂, it is possible that the decrease in PLA₂ activity is linked to the decline in mitochondrial function and ATP production, which are characteristic features for AD brains (Hirai *et al.* 2001, Moreira *et al.* 2010). Since AD pathology is associated with chronic impairment of neuronal function, it is also possible that a diminished response to neuronal excitation may result in decrease in the ERK pathway for phosphorylation of cPLA₂ (He *et al.*, 2011). Recent lipidomic studies have identified changes in lipids and lipid mediators in the AD brain membranes, including elevation of AA and production of prostaglandins (Igarashi *et al.* 2010). However, although a change in lipid mediators and lipid membrane environment may explain the alterations of neuron and glial cell function, more studies are needed to further elucidate the mechanism leading to these changes (Sanchez-Mejia & Mucke 2010). Taken together, these studies provide strong evidence for the critical role of cPLA₂ in mediating physiological and pathological functions in the brain.

Regulation of cPLA₂ through the nNOS pathways in neurons

While there is evidence for an upregulation of iNOS in AD brain, the role of eNOS and nNOS in the pathology of this disease remains largely unknown. Study by Masliah's group reported a decrease in nNOS expression in neurons in the entorhinal cortex of AD patients, a finding correlating nNOS in neurons to neurodegeneration (Thorns *et al.* 1998). As shown in Fig. 1, a number of proteins in the NMDA receptor pathway, including p47phox, nNOS,

COX-2 and caspases, are susceptible to post-translational modifications through S-nitrosylation. There is further evidence that NMDA-mediated ROS production by NADPH oxidase in neurons is mediated by NO from nNOS (Girouard *et al.* 2009). Since NMDA receptor and NADPH oxidase is known to induce ERK1/2, cPLA₂ and AA release (Shelat *et al.* 2008), it is possible that regulation of ROS by the NO/nNOS pathway may also alter cPLA₂/AA, and COX-2 in neurons. Interestingly, high levels of COX-2 are found in the post-synaptic neurons, and NMDA receptor-induced nNOS can cause S-nitrosylation and activation of COX-2, as demonstrated by the production of high levels of PGE₂ (Tian *et al.* 2008). In fact, inhibition of COX-2 in neurons can ameliorate the toxic effects of A β (Kriem *et al.* 2005).

Proinflammatory cytokines can stimulate the production of NO from iNOS in microglial cells (Sheng *et al.* 2011). There is increasing evidence for the pleiotropic effects of NO; neuronal degeneration can be resulted from excess NO produced by microglial cells (Brown & Neher 2010, Brown 2010). Exogenous NO can cause damage to neurons and trigger intracellular signaling pathways. In a study with PC-12 cells, exposure of cells to sodium nitroprusside, an NO donor, was shown to activate cPLA₂ and AA release (Chalimoniuk *et al.* 2007). A β (25–35) can also induce NO release in rat temporal cortex and this event was attributed to memory impairment (Limon *et al.* 2009). Taken together, these studies suggest a synergism among nNOS, cPLA₂ and COX-2 in A β toxicity in neurons.

Studies with cPLA₂ inhibitors

A number of studies (mainly with immune cells in the peripheral system) have demonstrated a role for PLA₂ or its metabolic products to regulate ROS production induced by NADPH oxidase (Henderson *et al.* 1989, Dana *et al.* 1994, Levy *et al.* 2000, Chenevier-Gobeaux *et al.* 2007, Chatterjee *et al.* 2011, Shmelzer *et al.* 2003). However, although our studies with neurons have demonstrated the signaling pathway linking NMDA-induced NADPH oxidase/ROS to cPLA₂ through ERK1/2 (Shelat *et al.* 2008), a retrograde interaction between cPLA₂ and/or its metabolic products with NADPH oxidase has not been examined in detail. Nevertheless, studies with PLA₂ inhibitors have demonstrated pleiotropic action of this enzyme in regulating cell functions. In prion disease, the toxic prion-derived peptide (PrP^{Sc}106–126) was attached to the glycosylphosphatidylinositol anchor and localized to the cholesterol-rich lipid raft region of neurons (Bate *et al.* 2011). Treatment with PLA₂ inhibitors (AACOCF₃ or MAFP) prevented the entry of the prion peptide to the lipid rafts of cortical neurons. These events were attributed to the production of platelet-activating factor (PAF), a lipid mediator formed as a result of cPLA₂ activation and acetylation of the lysophospholipid (Bate *et al.* 2010a). There is also indication that inhibition of PLA₂ through AACOCF₃ can render protection against A β -mediated degeneration of prion protein in the synapse (Bate *et al.* 2010b). In this regard, PAF receptor antagonists such as ginkgolide B, Hexa-PAF and CV6029 are beneficial and can protect against synaptic degeneration induced by the toxic prion and A β peptides (Bate *et al.* 2010b). Besides synthesis of PAF, cPLA₂-induced production of PGE₂ can also play a role in mediating synaptic degeneration, because treatment with the PGE₂ receptor antagonist AH13205 can also protect against synapse degeneration induced by A β and prion protein.

Other studies demonstrated defects in neurite outgrowth in cortical neurons upon sustained inhibition of cPLA₂ with MAFP (Mendes *et al.* 2005, Forlenza *et al.* 2007). Studies with cPLA₂ inhibitors further demonstrated involvement of cPLA₂ in mediating secondary effects in spinal cord injury (Liu *et al.* 2006), and in clinical symptoms in experimental autoimmune encephalomyelitis (EAE), an *in vivo* model for multiple sclerosis (Vana *et al.* 2011, Marusic *et al.* 2008). In this latter study, however, the effect of cPLA₂ inhibitor was attributed to its ability to block peroxynitrite formation in the spinal cord white matter (Li *et*

al. 2011). In human monocytes, down regulation of cPLA₂ (by siRNA) was shown to block the stimulation-induced translocation of p47phox and p67phox, further suggesting a relationship between cPLA₂ and NADPH oxidase (Zhao *et al.* 2002). In microglial cells, LPS can induce up-regulation of cPLA₂, iNOS and NADPH oxidase, and pretreatment of AACOCF₃ significantly attenuated iNOS induction, NO production, ROS production, and ability for microglial cells to confer cytotoxic effects on oligodendroglial cells (Li *et al.* 2011).

There is evidence for the role of PLA₂ in modification of cholinergic and glutamatergic pathways during the early stages of AD (Schaeffer & Gattaz 2008). Studies *in vivo* (Schaeffer *et al.* 2011a) demonstrated the ability of MAFP (by i.c.v. infusion for 3 days) to decrease the Tau protein levels in the frontal cortex and hippocampus. Measurement of physical properties (anisotropy) of brain membranes showed a reduction of flexibility of fatty acyl chains and increased fluidity of the lipid-water interface after MAFP treatment. Therefore, besides the release of AA and synthesis of eicosanoids, PLA₂ itself can also play a role in modulating membrane physical properties leading to altered neuronal excitation, glucose metabolism, memory and cognitive function, factors important in the pathophysiology of neurological diseases including AD (Schaeffer *et al.* 2011b).

Botanical phenolics inhibit glutamate excitotoxicity and oxidative/nitrosative stress

The ability to link the NMDA- and A β -induced ROS production through NADPH oxidase to signaling pathways leading to activation of ERK and cPLA₂ provided a strong indication for the involvement of oxidative stress due to multiple actions of cPLA₂ (Shelat *et al.* 2008). With prolonged exposure, oligomeric A β may gradually perturb neurons leading to mitochondrial dysfunction (He *et al.* 2011a). Besides neurons, A β can also stimulate other receptor pathways leading to cPLA₂ activation (Askarova *et al.* 2011). Results of these and other studies provide a link between NADPH oxidase/ROS and cPLA₂ in neuronal damage as elicited by excitotoxic NMDA and cytotoxic A β . Although how this pathway is linked to neuronal impairment remains to be further investigated, it is reasonable that efforts to inhibit the oxidative events can be beneficial in retarding the progression of neurodegenerative diseases. Our laboratory has successfully demonstrated protective effects of some botanical polyphenols against neuronal damage due to global cerebral ischemia (Simonyi *et al.* 2005, Sun *et al.* 2008, Sun *et al.* 2010). Neuroprotective effects were obtained after treating animals with resveratrol, curcumin and apocynin (Wang *et al.* 2005a, Wang *et al.* 2005b, Wang *et al.* 2006, Simonyi *et al.* 2012). In the AD field, there is special interest towards understanding protective effects of curcumin which has been shown to possess pleiotropic properties including inhibition of A β fibrillation, accumulation of amyloid plaques and ameliorating behavioral deficits in animal models (Begum *et al.* 2008, Ringman *et al.* 2005, Frautschy & Cole 2010, Cole *et al.* 2007). Besides *in vivo* studies, other botanicals have been shown to inhibit the induction of iNOS and other inflammatory responses induced by pro-inflammatory cytokines, LPS and A β in microglial cells (Capiralla *et al.* 2012, Sheng *et al.* 2011). In a study with spinal cord neurons, treatment with Ginkgo biloba (EGb761) could inhibit glutamate excitotoxicity and subsequent increase in phosphorylation of cPLA₂ (Zhao *et al.* 2011).

In recent years, there is substantial interest to investigate protective effects of green tea polyphenols, compounds exhibiting strong antioxidant and iron chelating properties (Cabrera *et al.* 2006, Weinreb *et al.* 2004, Higdon & Frei 2003). Many studies have demonstrated the ability for epigallocatechin-gallate (EGCG), a major ingredient of the green tea polyphenols, to delay AD progression (Dragicevic *et al.* 2011) through a variety of mechanisms including inhibition of fibrillation of A β (Lee *et al.* 2009a) and inhibition of A β

production in neurons isolated from TgAPPsw mice (Rezai-Zadeh *et al.* 2005, Obregon *et al.* 2006, Smith *et al.* 2010), and decrease levels of A β oligomers in the hippocampus of senescence-accelerated mouse prone-8 (SAMP8) (Li *et al.* 2009). EGCG was also shown to inhibit cerebral amyloidosis in TgAPPsw mice through activating α -secretase, the enzyme mediating the non-amyloidogenic pathway (Rezai-Zadeh *et al.* 2005, Lee *et al.* 2009a). Studies from our laboratory as well as others have demonstrated effects of EGCG to mitigate A β -induced ROS production, lipid peroxidation and mitochondrial dysfunction in neurons (Shimmyo *et al.* 2008, He *et al.* 2011a, Choi *et al.* 2001, Choi *et al.* 2011, Dragicevic *et al.* 2011). Studies have demonstrated effects of EGCG to inhibit A β -induced NO production in BV-2 cells and decreased A β -induced nitrosative damage in rat hippocampus (Kim *et al.* 2009), and ROS production by NADPH oxidase (Nishikawa *et al.* 2007, Steffen *et al.* 2008), and LPS-induced expression of inflammatory proteins, iNOS and COX-2 *in vivo* (Lee *et al.* 2009b). Taken together, studies with botanical phenolic compounds have demonstrated ability to diminish oxidative/nitrosative stress and glial inflammatory responses in brain (Choi *et al.* 2011, Sun *et al.* 2008). Despite that more studies are needed to understand mechanisms for these effects, there is hope that some compounds may prove to be excellent therapeutic agents to combat neurodegenerative diseases.

Conclusion and perspective

The pathophysiology of AD is marked by complex molecular mechanism(s) underlying aberrant metabolic pathways in neurons and glial cells. The complexity underscores the difficulty to develop a single drug to effectively cure this disease. There is a general consensus for increased oxidative/nitrosative stress in many neurodegenerative diseases, including AD, and the aberrant oxidative/nitrosative pathways are common basis for glial cell inflammatory responses, neuronal damage and synaptic impairments. In recent years, NADPH oxidase has been recognized as an important player in ROS production in brain cells, including neurons, astrocytes, microglia and cerebrovascular endothelial cells. Although emphasis here is on neurons, more studies are needed to unravel the role of this enzyme in the oxidative and inflammatory pathways in other cell types.

Studies with neurons support an aberrant excitatory neurotransmitter signaling pathway leading to excessive activation of NADPH oxidase and ROS production. Activation of this pool of ROS further triggers downstream pathways leading to activation of protein kinases and phosphorylation of cPLA₂. Interestingly, toxic A β oligomers can also enhance ROS production in neurons through NADPH oxidase. Although the exact mechanism remains to be further investigated, these studies support the deleterious effects of protein misfolding and aggregation in mediating toxic effects to neurons and causing synaptic impairments. ROS produced by NADPH oxidase is linked to activation of ERK1/2, which in turn, leads to phosphorylation and activation of cPLA₂. cPLA₂ has pleiotropic properties and besides the release of AA for synthesis of eicosanoids, lysophospholipids may play a role in modulating membrane physical properties, and serving as substrate for synthesis of platelet activating factor (PAF). Thus, integrating PLA₂ into the oxidative signaling pathways underlying glutamate excitotoxicity and A β toxicity will have great impact on understanding the pathogenesis of AD, and will allow the development of novel therapeutic targets to mitigate the damaging effects of A β .

Activation of NMDA receptor is also linked to NO production through nNOS, and in turn, NO triggers downstream pathway for production of cGMP, a second messenger. NO has pleiotropic effects, and in addition to reacting with ROS to form peroxynitrite (ONOO⁻), it can also be the source of S-nitrosylation, a mechanism for posttranslational protein modification. There is increasing recognition about proteins associated with the NMDA

receptor and signaling pathways are S-nitrosylated (including cPLA₂). Although the mechanism is not yet understood, there is increasing evidence that the NMDA/nNOS pathway may impact the NMDA/NADPH oxidase pathway and that cPLA₂ plays a role in mediating both pathways. These studies further demonstrate the importance for developing specific inhibitors for cPLA₂ to suppress the detrimental effects resulting from excitotoxic and oxidative/nitrosative pathways common to AD. Since AD is developed over a long period of time, and the complexity of the disease progression prevail the use of single drugs, there is strong quest for a preventative action through dietary supplementations, e.g., using botanical antioxidants. Therefore, future studies should target on understanding factors causing A β to aggregate and become toxic in AD brain, better understanding of the toxic effects of these protein aggregates on neurons and glial cells, and developing novel strategies to suppress deleterious effects due to excessive oxidative/nitrosative stress in the brain. Since many botanical polyphenols have been shown to exhibit antioxidant and anti-inflammatory properties, future studies should focus on discovering novel botanicals to suppress oxidative/nitrosative stress in the degenerating brain.

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Fig. 1.

Aβ oligomers stimulate oxidative/nitrosative pathways involving NMDA receptor, nNOS and NADPH oxidase leading to production of ROS and activation of cPLA2. cPLA2 has multiple actions; besides the release of AA and synthesis of eicosanoids, it can also produce DHA and lysoPL. These products have been shown to disturb mitochondrial function and modulate ROS production by NADPH oxidase. Dotted arrows are pathways not yet clarified. Note that besides the NO/cGMP/PKG pathway, many proteins in these pathways can undergo post-translational modifications through S-nitrosylation.