Integration of cytokine biology and lipid metabolism in stroke

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1. ABSTRACT

Cytokines regulate the innate and adaptive immune responses and are pleiotropic, redundant and multifunctional. Expression of most cytokines, including TNF-alpha and IL-1alpha/beta, is very low in normal brain. Metabolism of lipids is of particular interest due to their high concentration in the brain. Inflammatory response after stroke suggests that cytokines (TNF-alpha, IL-1alpha/beta, IL-6), affect the phospholipid metabolism and subsequent production of eicosanoids, ceramide, and ROS that may potentiate brain injury. Phosphatidylcholine and sphingomyelin are source for lipid messengers. Sphingomyelin synthase serves as a bridge between metabolism of glycerolipids and sphingolipids. TNF-alpha and IL-1alpha/beta can induce phospholipases (A_2, C, and D) and sphingomyelinases, and concomitantly proteolyse phosphatidylcholine and sphingomyelin synthesizing enzymes. Together, these alterations contribute to loss of phosphatidylcholine and sphingomyelin after stroke that can be attenuated by inhibiting TNF-alpha or IL-1alpha/beta signaling. Inflammatory responses are instrumental in the formation and destabilization of atherosclerotic plaques. Secretory PLA_2 IIA is found in human atherosclerotic lesions and is implicated in initiation, progression and maturation of atherosclerosis, a risk factor for stroke. Lipoprotein-PLA_2, part of apolipoprotein B-100 of LDL, plays a role in vascular inflammation and coronary endothelial dysfunction. Cytokine antagonism attenuated secretory PLA_2 IIA actions, suggesting cytokine-lipid integration studies will lead to new concepts contributing to bench-to-bedside transition for stroke therapy.

2. STROKE

2.1. Stroke or "brain attack": a problem of vast clinical importance

Cerebral ischemia or stroke is characterized by an obstruction of blood flow to the brain and is the leading cause of long-term disability, third leading cause of death. It is estimated that about 700,000 Americans suffer from stroke each year, and approximately 4.7 million Americans are stroke survivors. The economic impact of stroke amounts to more than $62.7 billion in direct health care costs and due to loss of productivity annually (1).

Stroke generally refers to a local interruption of blood flow to the brain due to blockage of a cerebral artery. Approximately 12% of strokes are hemorrhagic (rupture of a cerebral blood vessel; 9% intracranial, 3% subarachnoid), whereas the remaining 88% are ischemic and result from occlusion of a cerebral artery (either thrombolic or embolic). Thrombolic stroke results from the formation of a clot or thrombus in a cerebral artery that blocks blood flow at the site of formation. Embolic stroke occurs when a cerebral artery is blocked by a clot that formed elsewhere and was carried to the brain through the circulation. Cerebral ischemia can also be global. i.e., total loss of blood flow to the brain caused by events such as cardiac arrest.

Stroke results in disruption of glucose and oxygen supply that ultimately leads to apoptotic and necrotic cell death, and development of an infarction. Focal ischemia is characterized by an ischemic core surrounded by a "penumbra" region that has partial reduction in blood flow due to presence of collateral arteries. If left untreated, the infarct can propagate into the penumbra. The ischemic core is generally considered unsalvageable, whereas the penumbra may be rescued by timely intervention and poses a target for the development of therapeutic treatment. Inflammation poses as one the high risk factors for stroke in the initiation, progression and maturation of atherosclerosis. CNS inflammation plays a dual role in the patho-physiological processes of stroke: deleterious during the early acute phase while participating in repair processes during the late stage.

2.2. Atherosclerosis is a risk factor for stroke

Atherosclerosis has traditionally been viewed as simply the deposition and accumulation of lipids and cellular debris within the wall of medium to large arteries, resulting in plaque formation and disturbance of blood flow. The role of cholesterol in atherosclerosis is well established and has been elegantly reviewed elsewhere (2). In atherosclerosis, it is now believed that a complex endothelial dysfunction induced by a variety of factors results in an inflammatory response that is instrumental in the formation and destabilization of plaques, one of the greatest risk factors for ischemic stroke (3, 4). Gene expression profile and understanding dynamic nature of atherosclerotic vascular wall will pave path for genomics, proteomics, and metabolomics ultimately leading to therapeutics and makes bench-to-bedside transition (201). Inflammatory cytokines have a central role in atherogenesis and plaque rupture (202, 205). Increased levels of tumor necrosis factor-alpha (TNF-alpha) and interleukin-1 (IL-1) up-regulate expression of adhesion molecules and promote monocyte recruitment into developing atherosclerotic lesions. Expression of macrophage matrix metalloproteinase 9 (MMP-9) degrades extracellular matrix components including the fibrous cap of atheromatous plaques. Destabilization of this aggregate can result in release of an embolus and induction of ischemic stroke (3-8). Statins inhibit cytokine expression independent of cholesterol lowering in both *in vivo* and *in vitro* models, suggesting that they might attenuate the pathogenesis of chronic progressive diseases (coronary diseases, AD, diabetes, MS, osteoporosis, chronic pancreatitis, pulmonary fibrosis and much more) that generally accompany atherosclerosis (202). Data from clinics provide the basis to hypothesize that statins, given for cardiovascular disease, might have the secondary benefit of inhibiting these chronic progressive diseases. A systemic inflammation also aggravates brain injury after stroke (9).

2.2.1 Initiation of atherosclerosis: apolipoprotein B-100 (apoB-100) of low-density lipoprotein (LDL)

Atherosclerosis is defined by the accumulation in the arterial intima of mainly LDL-derived lipids along with apoB-100. LDL is the major carrier of cholesterol in the circulation and is composed of one apoB-100 together with phosphatidylcholine (PC), sphingomyelin (SM) and

unesterified cholesterol (500:200:400 molecules respectively) constituting a surface film surrounding a core of cholesteryl esters and triacylglycerols.

2.2.2. Lipoprotein-PLA$_2$ (Lp-PLA$_2$) is also known as platelet activating factor (PAF) acetylhydrolase

Lp-PLA$_2$, 45 kDa protein, is a member of PLA$_2$ family classified as group VIIA PLA$_2$ and is also known as plasma PAF acetylhydrolases (10). This enzyme is found in blood circulation in most animals, and in humans is associated with apoB-100 of LDL and is also found in atherosclerotic plaques (10, 11). Higher levels of Lp-PLA$_2$ are also associated with coronary heart disease, stroke and dementia (11, 12). Lp-PLA$_2$ is produced and secreted by cells of monocyte-macrophage series, T-lymphocytes and mast cells. The enzyme is best known for its PAF acetylhydrolase activity but also hydrolyzes oxidized phospholipids such as oxidized PC of LDL to generate oxidized fatty acids and lyso-phosphatidylcholine (lyso-PC) (13). Local coronary lyso-PC formation is also associated with endothelial dysfunction and supports the role of this enzyme in vascular inflammation and atherosclerosis in humans (11). Lp-PLA$_2$ apparently plays a dual role; the anti-inflammatory function arises by hydrolyzing PAF, which is known to activate platelets, monocytes and macrophages.

2.2.3. Sphingomyelinase (SMase) activity of LDL: A link between atherosclerosis and ceramide

LDL possesses SMase activity, which hydrolyzes SM to release ceramide. Sequence analogy with bacterial SMase suggests that this activity may be intrinsic to apoB-100. Ceramide is elevated in atherosclerotic plaques as well as in LDL isolated from these lesions. Aggregation of LDL within the arterial wall is considered to be a critical step in the initiation of atherosclerosis and ceramide is believed to play an important role in this process (14).

2.2.4. Atherosclerosis and group IIA secretory PLA$_2$ (inflammatory PLA$_2$)

Group IIA phospholipase A$_2$ (secretory PLA$_2$ also known as inflammatory PLA$_2$) has been found in human atherosclerotic lesions (15, 16). sPLA$_2$ IIA is implicated in chronic inflammatory conditions such as arthritis and may also contribute to atherosclerosis (17), one of the risk factors for stroke. sPLA$_2$ IIA is a pro-atherogenic factor and it has been suggested that this enzyme regulates collagen deposition in the plaque and fibrotic cap development (18). sPLA$_2$ is one of the enzymes responsible for the release of lyso-PC *via* its catalytic action and these two play a crucial role in the development of atherosclerosis (19). Non-catalytic (non-enzymatic) atherogenic effects of sPLA$_2$ II are thought to involve binding to a muscular-type (M-type) sPLA$_2$ receptor (see section 6.1.3. on sPLA$_2$ receptors).

2.3. Energy failure is the initial metabolic event in stroke

The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP. Most of the ATP generated is utilized in the brain in maintaining intracellular homeostasis and transmembrane ion gradients of sodium, potassium, and calcium. Energy failure results in rapid loss of ATP and uncontrolled leakage of ions across the cell membrane that results in membrane depolarization and release of the neurotransmitters such as glutamate and dopamine (20, 21). Excess glutamate release and stimulation of its receptors results in activation of phospholipases/sphingomyelinases (22-26), phospholipid hydrolysis and release of ceramide and free fatty acids (FFA) including arachidonic acid (ArAc) (26, 27). Ultimately these processes lead to apoptotic or necrotic cell death (28) (Figure 1).

2.4. Animal models for stroke

The majority of ischemic strokes in patients result from blockage of the middle cerebral artery (MCA). One commonly used animal model for stroke uses transient MCA occlusion (tMCAO) in rats or mice (29). In this model, a nylon monofilament is advanced through the internal carotid artery to occlude the MCA. The drop in blood flow in the brain is determined by Doppler flowmetry with a probe placed over the MCA territory. Restoration of blood flow (reperfusion) is achieved by withdrawing the occluding suture. Permanent MCAO (no reperfusion) is achieved usually by electro-coagulation or photochemically induced thrombosis of the MCA. Other animal models induce a global or forebrain ischemia by four-vessel occlusion in rat or bilateral common carotid artery occlusion in gerbil, which lacks the circle of Willis and thus has no collateral blood flow. Global models mimic the complete loss of blood flow to the brain that occurs during cardiac arrest.

3. THE BIOLOGICAL MEMBRANE: STRUCTURE AND FUNCTION

Cellular membranes are composed of glycerophospholipids (PC, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI)), sphingolipids (SM, ceramide and gangliosides), cholesterol and cholesterol esters, acylglycerols, and fatty acids. The phospholipid bilayer and associated lipids provide not only a permeability barrier but also a structured environment that is essential for the proper functioning of membrane-bound proteins (2). Cholesterol is one of the most important regulators of lipid organization as its structure allows it to fill interstitial spaces between hydrophobic fatty acid chains of phospholipids. The neutral lipids such as PC and SM predominantly reside on the outer or exofacial leaflet, whereas anionic phospholipids PS (exclusively inner leaflet), PE, and PI reside on the inner or cytofacial leaflet of the biological membrane. The transbilayer distribution of cholesterol between the leaflets determines membrane fluidity and can alter the membrane function. The asymmetric distribution of phospholipids in the plasma membrane is shown in Figure 2. Cardiolipin is a phospholipid that is exclusively limited to the mitochondrial membrane and is essential for proper assembly and functioning of the mitochondrial respiratory chain and oxidative phosphorylation (30).

The classic fluid mosaic model of the cellular membrane was modified recently to accommodate the
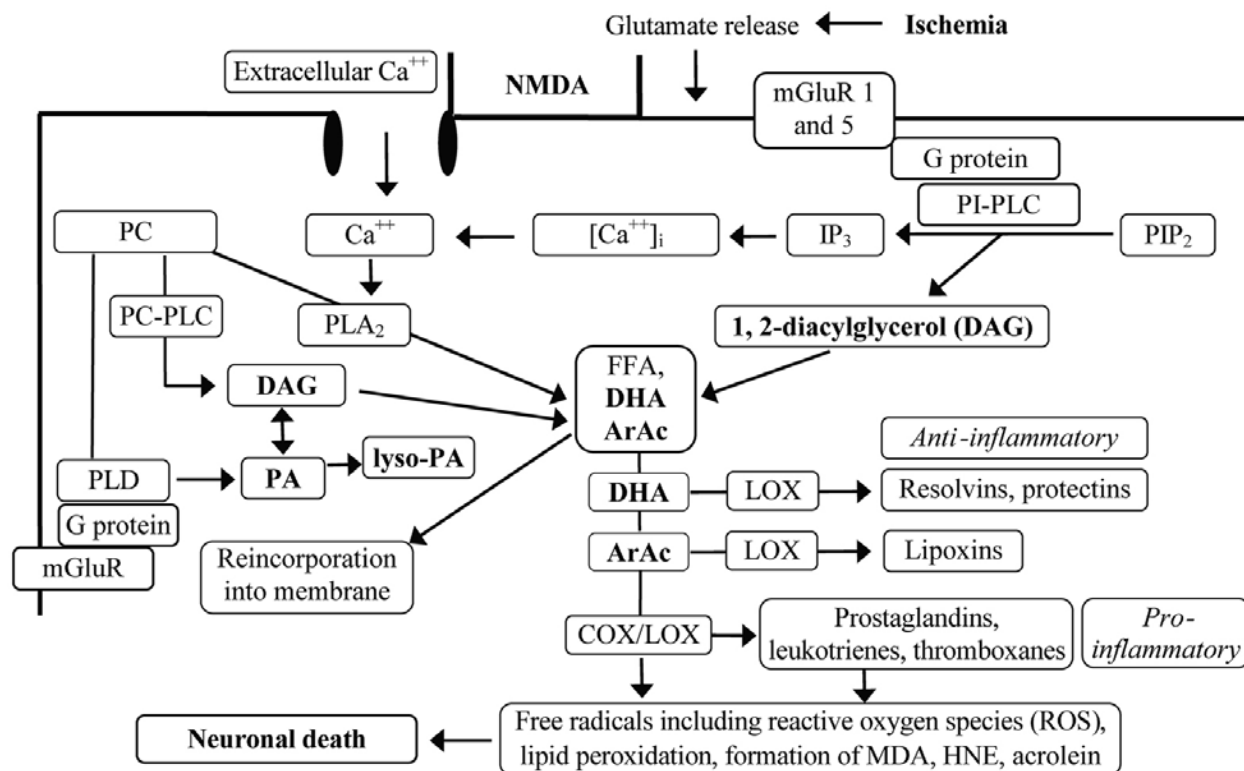
Figure 1. Lipid metabolism in ischemic neuronal death. Activation of phospholipases (PLA_2, PC-PLC, PI-PLC, PLD) sphingomyelinases (N-SMase and A-SMase) following cerebral ischemia results in release of lipid second messengers DAG, phosphatidic acid (PA), docosahexaenoic acid (DHA), ArAc and ceramide. PI-PLC releases inositol-trisphosphate (IP_3) in addition to DAG; IP_3 stimulates intracellular Ca^{++} release. PA and DAG can be readily inter-converted by phosphatases and DAG-kinases. ArAc undergoes further metabolism by LOX to generate important vasoactive eicosanoids and ROS. Recent studies suggest that COX does not directly produce ROS during ArAc oxidative metabolism, but does form free radicals (i.e., carbon-centered radicals on ArAc) (148, 200). DHA (204) is metabolized to 10,17S-docosatriene (Neuroprotectin D1), an endogenous neuroprotectant. DAG and ArAc stimulate A-SMase and N-SMase, respectively, to generate pro-apoptotic ceramide (148).

concept of distinct microdomains that serve signaling functions (31). The tight interaction of sphingolipids with each other and with cholesterol segregates these lipids into discreet membrane structures characterized by a liquid-ordered or gel-like phase, in contrast to the glycerophospholipids in the bulk cell membrane that reside in a more fluid liquid-disordered phase. These distinct sphingolipid- and cholesterol-enriched microdomains have been termed as "lipid rafts".

4. PHOSPHOLIPIDS: SYNTHESIS AND HYDROLYSIS

4.1. PC homeostasis

PC is the most abundant phospholipid in mammalian cells and constitutes ~50% of the total phospholipid content. PC homeostasis is regulated by a balance between hydrolysis and synthesis, which under normal conditions is tightly regulated to assure a constant amount of the phospholipid in the cell membrane (32, 33). Controlled hydrolysis of PC is required for signal transduction and efficient regulation of cellular functions (33). A limited amount of membrane damage can be handled by the cell provided the energy status (e.g. ATP

production and ion homeostasis) is not severely impaired. A major loss of membrane integrity is incompatible with cellular viability. Patho-physiological breakdown of membrane PC causes growth arrest (34) and even a 10% loss is sufficient to threaten cell viability (32). Excessive breakdown of PC by phospholipases not only changes membrane permeability, uncontrollably allowing small ions and metabolites to cross the cell membrane, but also releases second messengers such as phosphatidates, 1,2-diacylglycerol (DAG), and ArAc (20:4) and docosahexaenoic (22:6) acids, among others (26, 32). ArAc metabolites prostaglandins and leukotrienes are implicated in the development of inflammatory conditions.

4.2. Cytidine triphosphate:phosphocholine cytidylyltransferase (CCT) and PC synthesis

PC can be synthesized by methylation of PE by PE-N-methyltransferase (PEMT) or through the CDP-choline/Kennedy pathway. The PEMT pathway is significant only in the liver (35, 36) and PC synthesis in the brain is predominantly through the CDP-choline pathway (Figure 3), which is regulated by the rate-limiting enzyme CCT (32, 37). The majority of choline is derived from dietary sources (32). In the Kennedy pathway, choline is
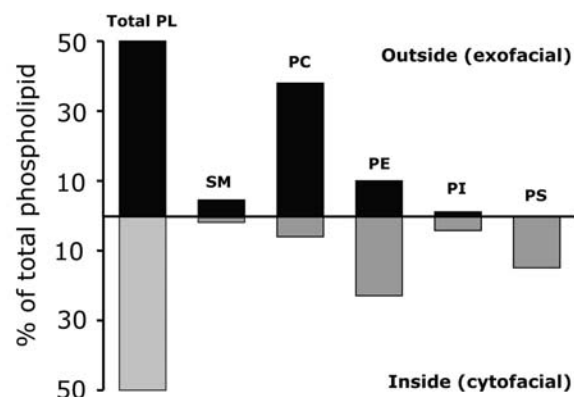
Figure 2. Asymmetric distribution of phospholipids (PL) in a plasma membrane.
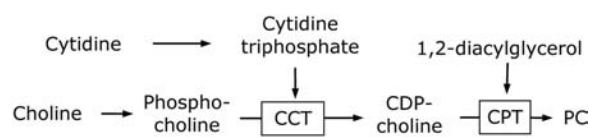

Figure 3. PC synthesis through CDP-choline/Kennedy pathway (148). CCT is down-regulated after stroke, impeding PC synthesis (41). CCT:cytidine triphosphate:phosphocholine cytidylyltransferase. CPT: CDP-choline:DAG cholinephosphotransferase.
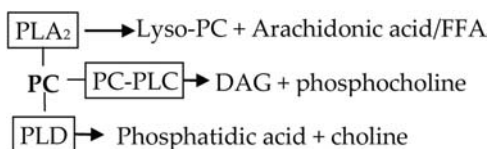

Figure 4. The immediate products formed from PC by each class of phospholipase.

first phosphorylated, and CCT (37) synthesizes CDP-choline from phosphocholine and cytidine triphosphate. PC is synthesized from CDP-choline and DAG by the action of CDP-choline:DAG phosphocholine transferase (CPT). Under certain conditions, the availability of DAG may become rate-limiting in PC synthesis (38). Several studies have shown that blocking PC synthesis causes cell death. A number of compounds (camptothecin, ceramide, chelerythrine, etoposide, farsensol, geranylgeraniol and hexadecylphosphocholine) that induce apoptosis also cause a build-up of CDP-choline (measured by ^{31}P nuclear magnetic resonance) (39). The accumulation of CDP-choline implies that a block of CPT is a common event in apoptosis. Inactivation of CCT also induces cell death (32).

CCT activity decreased in transient forebrain (40) and focal (41) ischemia, which may have resulted from induction of TNF-alpha, since TNF-alpha inhibits CCT (42) (see section 6.1.7.3.). We have also shown up-regulation of phospholipases and down-regulation of CCT that collectively contributed to loss of PC following tMCAO (41). Our recent studies also indicate that loss of PC is a cause rather than a result of cell death (43). Thus

preserving membrane PC may offer benefit after stroke (41).

4.3. PC hydrolysis by phospholipases

Phospholipases have received wide attention for their role in progression of stroke injury (25). PC is hydrolyzed by phospholipase A_2 (PLA_2), PC-phospholipase C (PC-PLC), and phospholipase D (PLD) to release lyso-PC, DAG, phosphatidic acid (PA), and FFA, especially ArAc. The immediate products formed from PC by each class of phospholipase are given in Figure 4.

4.3.1. PLA_2

PLA_2 isozymes occur in multiple forms (25, 44) in the mammalian cell and are classified as calcium independent ($iPLA_2$, 84 kDa), and the calcium-dependent cytosolic ($cPLA_2$, 85-110 kDa) and secretory ($sPLA_2$ 14-18 kDa) forms. $cPLA_2$ preferentially cleaves ArAc at the *sn*-2 position of PC and forms lyso-PC, whereas $sPLA_2$s show no fatty acid preference and act on a broader range of phospholipids (45, 46). $iPLA_2$s also are not selective for ArAc and are considered to be involved primarily in membrane remodeling (47, 48). Full activation of $cPLA_2$ requires binding of Ca^{2+} for translocation from cytosol to the phospholipid membrane and phosphorylation of specific serine residues (49). Activation of PLA_2 is harmful to neurons in several ways: 1) membrane phospholipid degradation, 2) increased Ca^{2+} influx, 3) formation of lyso-PC that inhibits CCT (50), 4) ArAc release and metabolism by cyclooxygenases/lipoxygenases (COX/LOX) (Figure 1). Mitochondrial PLA_2 is a $sPLA_2$ that acts on PC, PE, and cardiolipin (51, 52). Induction of PLA_2 has been shown to result in damage to the mitochondrial respiratory chain (53).

4.3.2. PC-PLC

The mammalian enzyme has a strong preference for PC and hydrolyzes it to form phosphocholine and DAG. The primary intracellular target for DAG is activation of protein kinase C (PKC) which stimulates cell proliferation (54). In addition to acting as a second messenger, hydrolysis of DAG by DAG lipases releases FFA including ArAc. Our studies show that the PC-PLC inhibitor (55) tricyclodecan-9-yl potassium xanthate (D609) attenuated infarction after tMCAO, evidence for a role of PC-PLC in stroke injury (Figure 5). Our results demonstrated that PC-PLC activity increased after focal cerebral ischemia; D609 blocked the activity in the assay confirming this as PC-PLC activity (41). However non-specific actions of D609 should be taken into consideration while interpreting data (43, 203).

4.3.3. PLD

PLD catalyzes the hydrolysis of PC to choline and PA. PA may act directly as a signaling molecule and can be further converted to other messenger molecules such as DAG and lyso-PA. PLD catalyzes a unique transphosphatidylation reaction in the presence of a primary alcohol to form a phosphatidylalcohol instead of PA (56). This reaction has been used to measure PLD activity and assess the role of PA in cell signaling. Two mammalian forms (56) have been identified, PLD1 (~125
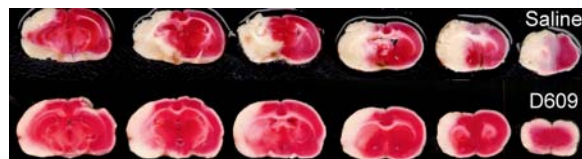
Figure 5. D609 (50 mg/kg in saline; i.p at the onset of reperfusion) attenuated ischemic injury volume (infarction) by $35 \pm 5\%$ after 1 hr MCAO/24 hr reperfusion (saline 270 ± 38 mm^3).
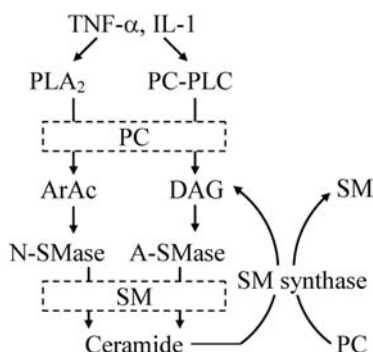

Figure. 6. Cytokine mediated generation of lipid mediators DAG, ArAc and ceramide. This scheme also shows the relationship between glycerophospholipids and sphingolipids (148). TNF-alpha and IL-1 activate N-SMase and A-SMase through stimulation of PLA$_2$ and PC-PLC and release of ArAc and DAG, respectively. N-SMase and A-SMase hydrolyze SM to liberate ceramide. SM synthase transfers the phosphocholine head group of PC to ceramide to form SM and DAG.

kDa) and PLD2 (~105 kDa). PLD1 is expressed in neurons and glial cells (56) and PLD2 is expressed in astrocytes (56). Expression of PLD in forebrain ischemia (56) and other CNS injuries and disorders (57) has been demonstrated. Interestingly there are no reports examining the role of PLD in stroke (focal cerebral ischemia) injury, which may be due to lack of specific inhibitors (primary alcohols block PA formation but should not be considered as PLD inhibitors). Our data showed increased protein expression of PLD2 after 1 hr MCAO and reperfusion over 7 day (41).

4.4. SM, SM synthases and SMases

PC also serves as the phosphocholine donor in SM synthesis, another phospholipid important for maintaining the membrane integrity. SM synthase (SMS) transfers the phosphocholine group from PC to ceramide to generate SM and DAG. SMS serves as a bridge between metabolism of glycerolipids (PC and DAG) and sphingolipids (SM and ceramide) since it regulates not only cellular SM and ceramide levels but also contributes to formation of DAG (Figure 6). Recent literature suggests there are three forms of SMS: SMS1 and SMS2. SMS1 is predominantly in Golgi while SMS2 is localized to plasma membrane (197-199). Another alternative SM synthesis pathway has been proposed in which ceramide is converted to ethanolamine phosphorylceramide (EPC) *via* transfer of

the head group from PE, a third SMS-related enzyme (SMSr). EPC later converted to SM by methylation in a reaction one similar to the S-adenosyl methionine (AdoMet or SAM) mediated conversion of PE to PC (197). SMS is closely related to PC-PLC in that both enzymes use PC as a substrate and generate DAG as a product. There has been some debate actually whether PC-PLC and SM synthase are distinct enzymes or one and the same (58). Mammalian PC-PLC has not yet been cloned and characterized. Commercial antibodies of this enzyme are presently unavailable.

SMases are C-type phospholipases with specificity for SM. SMase hydrolyzes SM to form ceramide, which inhibits cell proliferation (59) and induces apoptosis (60). The role of ceramide in cell signaling has been studied extensively (61). Two distinct SMases have been described: both acidic SMase (A-SMase) and neutral SMase (N-SMase) are expressed in brain tissue. Expression of A-SMase has been shown in astrocytes (62), but the cellular localization (neurons, astrocytes, etc.) of these two forms is not well characterized. These SMases exhibit distinct sub-cellular distribution: A-SMase is lysosomal while N-SMase is localized to the outer leaflet of the plasma membrane (63, 64). Since ceramide released by these enzymes is hydrophobic and does not diffuse from its site of release, A-SMase and N-SMase have different signaling functions. A-SMase deficient mice showed reduction in infarct volume compared to wild type (65). However one should bear in mind that these mice have Niemann-Pick related disease symptoms (148). Niemann-Pick disease is a lysosomal storage disorder that lacks A-SMase activity leads to lysosomal accumulation of SM.

The externalization of PS (an exclusive inner plasma membrane anionic phospholipid) to outer plasma membrane initiates an apoptotic death signal. The PS relocation also causes flipping of SM from outer to inner leaflet and hydrolysis by a cytosolic SMase (66-68) to generate ceramide with subsequent activation of death cascade mechanisms.

5. CYTOKINES

5.1. Cytokines are pleiotropic, multifunctional, and redundant with dual nature (pro- and anti-inflammatory functions)

Inflammation is the first response of the immune system to infection or irritation and may be referred to as the innate cascade (69, 70). Inflammation is characterized by the following quintet: redness (rubor), heat (calor), swelling (tumor), pain (dolor) and dysfunction of the organs involved (*functio laesa*). The first four characteristics were defined by Celsus around AD 40 (71); *functio laesa* was added to the definition of inflammation by Rudolf Virchow in 1858. Cytokines are low molecular weight, soluble proteins that are produced in response to an antigen and were originally described as mediators for regulating the innate and adaptive immune systems. Cytokines thus serve as chemical messengers and include tumor necrosis factors, interleukins, interferons, chemokines, and growth factors. One cytokine can act on a number of different cell types rather than one type

(*pleiotropic*); the same cytokine regulates a number of different functions (*multifunctional*); and a number of different cytokines can carry out the same function (*redundant*) (69, 70). The redundancy is due to the utilization of shared key components of intracellular signaling pathways.

A regulated inflammatory response is characterized by an interplay between pro-inflammatory (go signals) and anti-inflammatory effects (stop signals) mediated by various cytokines. Cytokines are commonly classified as either pro-inflammatory or anti-inflammatory: TNF-alpha, IL-1, interferon-gamma (IFN-gamma), IL-12, IL-18 and granulocyte-macrophage colony stimulating factor (GM-CSF) are well characterized as pro-inflammatory cytokines whereas IL-4, IL-10, IL-13, IFN-alpha and transforming growth factor-beta (TGF-beta) are recognized as anti-inflammatory cytokines. TGF-beta neuroprotectant action in brain lesions in stroke are based on neutralization experiments (72). Application of soluble TGF beta type II receptor to rats in stroke aggravated the infarction volume (73). Realistically, classification of cytokines as either pro- or anti-inflammatory is too simplistic: some cytokines, for example IL-6, are considered to have both pro- and anti-inflammatory actions (74, 75). Furthermore, some cytokines may behave as pro- as well as anti-inflammatory agents depending on the amount of the cytokine, the target cell, the activating signal, the timing and sequence of action, and the experimental model (76).

5.2. Induction of cytokines after CNS injury is complementary and reciprocal

Interestingly, expression of different cytokines is often related. TNF-alpha induces expression of IL-1 and IL-6, while IL-1 can induce both TNF-alpha and IL-6. Specifically, IL-1beta is a significantly more potent stimulator of IL-6 release compared to IL-1alpha (77). Thus after a CNS insult, the initial upregulation of cytokines will lead to infiltration of other inflammatory mediators to the site of injury and secondary cytokine signaling is subsequently initiated (78). Cytokines can also act in concert in regulating various pathways: for example, TNF-alpha, IL-1alpha, and IFN-gamma were shown to act synergistically in induction of cell death in mouse primary cortical neuron/glia co-cultures (79).

5.3. TNF-alpha, IL-1 and IL-6 in the CNS

Cytokines affecting CNS: a) originate from peripheral immune organs, cross blood-brain barrier or initiate signaling by binding to receptors on the vascular endothelium and b) produced by glial cells (astrocytes and microglia). Within the CNS, cytokines increase in response to a variety of injuries including cerebral ischemia. The inflammatory responses often include early and significant glia activation, presumably with intent of suppressing or removing the pathogenic initiation to inhibit the injury process (80). However an unchecked inflammatory response may promote or aggravate the injury process. Among the cytokines produced in the CNS in response to injury, TNF-alpha and IL-1 are the most extensively studied (78, 81). Both cytokines are produced as precursor

proteins (pro-TNF-alpha and pro-IL-1) and must be cleaved to release the active forms. TNF-alpha is initially synthesized as a 26 kDa membrane bound protein which is cleaved by the Zn-metalloprotease TNF-alpha converting enzyme (TACE) to form 17 kDa mature, soluble TNF-alpha (82). IL-1 exists in alpha and beta forms, both having a molecular weight of 17 kDa. Both IL-1alpha and IL-1beta are synthesized as 32 kDa precursor proteins. Pro-IL-1alpha is biologically active and can be cleaved to the 17 kDa mature form by membrane-bound calpain. Pro-IL-1beta is inactive and requires cleavage by interleukin-1 converting enzyme (ICE, also known as caspase 1) (83, 84). The majority of IL-1alpha remains membrane bound, while IL-1beta is released from the cells and is the main form expressed in the brain (85, 86).

5.3.1. TNF-alpha and IL-1 are increased in cerebral ischemia

In normal brain, expression of most cytokines, including TNF-alpha and IL-1, is very low (85). TNF-alpha and IL-1, major pro-inflammatory cytokines, are rapidly up-regulated in brain after cerebral ischemia (84, 87-89). TNF-alpha levels increased in CSF and sera of patients and correlated with stroke severity (90-92). Some of the effects caused by TNF-alpha in stroke are due to inhibition of glutamate uptake by astrocytes; induction of ICAM-1, increased MMP expression that leads to disruption of blood-brain-barrier and invasion of inflammatory leukocytes (82). TNF-alpha mediates its effects by binding to two receptors (p55 and p75), both of which are up-regulated in the ischemic brain (84, 93). The majority of biological effects of TNF-alpha, including cytotoxicity, ArAc release, and inflammatory responses are mediated through the p55 receptor (84, 93) that contains the death domain. The function of p75 remains obscure, although studies have indicated it may participate in protective signaling following injury (94). TNF-alpha receptors are differentially up-regulated after cerebral ischemia: p55 (contains the death domain) is expressed within 6 hr, whereas p75 increases at 24 hr (93). Increased expression of TNF-alpha p75 receptor was shown following permanent MCAO; it was suggested that the expression of this receptor was predominantly localized to microglia and macrophages over 1-10 day period, reaching a peak at day-5 (95). Nevertheless no definitive conclusions regarding the role of this receptor were provided (95) and still the function of this receptor remains elusive.

5.3.2. TNF-alpha has a dual role in mediating both beneficial and detrimental effects in brain injury

Studies on TNF-alpha neurotoxicity are considerable but controversial (81, 96-99). A number of studies have supported the deleterious effects of TNF-alpha in experimental stroke studies. TNF-alpha binding protein is a soluble protein produced by cleavage of the TNF-alpha extracellular binding domain of its membrane-bound receptor and is an endogenously produced inhibitor of TNF-alpha signaling (100). The actions of TNF-alpha can be blocked using specific TNF-alpha neutralizing antibody (TNF-alpha *ab*) or TNF-alpha binding protein to bind TNF-alpha and prevent it from interacting with its receptors (96). Administration of TNF-alpha *ab* (101) or TNF-alpha

binding protein (81, 101, 102) that attenuates the acute phase of TNF-alpha following cerebral ischemia have consistently demonstrated beneficial effects (81, 84, 96). Transgenic mice deficient in TNF-alpha (99) showed dramatic reduction in infarction compared to wild-type mice, and infusion of TNF-alpha exacerbated infarct volume in focal cerebral ischemia (102). However, transgenic mice with targeted deletion of TNF-alpha receptors (p55 and p75) showed increased infarction after tMCAO (98), providing conflicting results obtained with TNF-alpha null mice. A follow-up study showed that mice lacking p55 receptors are more susceptible for ischemic injury compared to wild type or p75 receptor deficient mice (103). Possible explanations for this discrepancy are a compensatory increase in other cytokines such as IL-1 in TNF-alpha p55 receptor deficient mice, or, although speculative, that another as yet unidentified TNF-alpha receptor exists in the CNS (104).

Ischemic preconditioning is a phenomenon wherein an ischemic insult of short, sub-lethal duration confers protection against a subsequent severe ischemic injury, which has been attributed to altered gene expression by the initial ischemic event. Pre-treatment of mice with TNF-alpha 48 hr before experimental stroke conferred neuroprotection (105) while another study demonstrated that TNF-alpha pretreatment 24 hr before MCAO aggravated the infarction (102). These studies suggest that exposure to TNF-alpha in the absence of injury (pretreatment) alters gene expression or similar effects that confers protection against subsequent stroke and that this phenomenon requires 48 hr. *In vitro* studies have also shown that pretreatment with TNF-alpha attenuated neuronal damage induced by oxygen-glucose deprivation (OGD) in organotypic hippocampal cultures, whereas TNF-alpha exacerbated neuronal damage when added after the injury (97). Thus the actions of TNF-alpha are dependent on whether it is present before or after an ischemic event. One school of thought is that TNF-alpha stimulates generation of reactive oxygen species (ROS), activation of the transcription factor NFkappaB, and up-regulation of manganese superoxide dismutase (Mn-SOD), which would provide defense against subsequent ROS generation. In cells stressed by an injury, addition of TNF-alpha during the injury stimulates ROS generation and aggravates the damage. The actions of TNF-alpha may be dependent on concentration and time of treatment (97), vary with the cell type, expression of its receptors, and the specific injury.

The over-expression of TNF-alpha in the early stage after brain injury may be deleterious, whereas lower levels in later phases may facilitate recovery (84, 96). In support of this proof-of-concept are studies showing that TNF-alpha null mice subjected to controlled cortical impact injury showed fewer neurological deficits compared to wild-type mice at 7 d post-injury. However, wild type mice had better recovery from brain injury by 2-3 weeks post-injury, whereas TNF-alpha null mice had poor recovery and exhibited greater neurological deficits compared to wild type mice at 4 weeks post-injury (106). This phenomenon apparently has not been investigated in stroke models. TNF-alpha null mice had smaller infarcts after tMCAO and 1 day reperfusion compared to wild type mice, evidence that elevated levels of TNF-alpha during the early reperfusion time are deleterious. However, studies have not examined longer reperfusion times (beyond day 1) to determine if TNF-alpha expression is needed during the later, recovery phase after tMCAO.

5.3.3. IL-1 and stroke

IL-1 is present in two forms in the brain, IL-1alpha and beta, which interact with two IL-1 receptors (107). IL-1 signaling is mediated by IL-1 receptor type I, while receptor type II is believed to be a non-signaling or "decoy" receptor (85). IL-1beta is the primary form expressed in the brain and up-regulated in response to stroke (85). Since both forms of IL-1 interact with the same receptor, they are believed to mediate much of the same signaling; however their functions may not completely overlap (77). A third member of the interleukin family is IL-1 receptor antagonist (IL-1ra), an endogenous protein that binds to the type I receptor without agonistic signaling and thus blocks effects of IL-1alpha/beta. IL-1ra knockout worsened the infarction after tMCAO and also exacerbated NMDA or AMPA induced neuronal death in mixed glial-neuronal co-cultures (108). Also administration of neutralizing antibody specific for IL-1ra to rats undergoing MCAO increased the neuronal injury. Expression of endogenous IL-1ra thus limits infarction after tMCAO and this neuroprotection is mediated through glia (108, 109). Treatment with exogenous IL-1ra reduced neuronal death in all forms of experimental cerebral ischemia (permanent and transient focal, global, and hypoxic) in a variety of species (rat, mouse, and gerbil) (110, 111). Individual knockout of IL-1alpha or IL-1beta did not significantly alter ischemic damage after tMCAO, which may have been due to compensatory responses. However transgenic mice deficient in both IL-1alpha and beta showed dramatic reduction in infarcts compared to wild-type (112).

Enzymatic cleavage of the extracellular binding domains of IL-1 type I and type II receptors produces soluble IL-1 receptors. Soluble type II IL-1 receptors bind IL-1beta with much higher affinity than IL-1ra and may function as inhibitors of IL-1 *in vivo*. In contrast, soluble type I IL-1 receptors bind IL-1ra selectively and thus may block or neutralize the natural anti-inflammatory actions of IL-lra (113).

Similar to TNF-alpha, the evidence that IL-1 is neurotoxic is also considerable but controversial. A few studies have indicated a neuroprotective role of IL-1 (114, 115). Pretreatment of cultured mouse primary cortical neurons with IL-1alpha or IL-1beta attenuated neurotoxicity induced by NMDA. IL-1 mediated neuroprotection was inhibited by addition of a neutralizing antibody to neuronal growth factor (NGF) (114). In the other study (115), treatment of cultured rat primary cortical neurons with IL-1beta attenuated neuronal death induced by exposure to excitotoxic amino acids (glutamate, NMDA, AMPA, and kainate). The neuroprotective effects of IL-1beta appeared to be mediated, at least in part, by induction of NGF. In these studies (115), IL-1beta was added to culture media both before (pretreatment) and after (post-
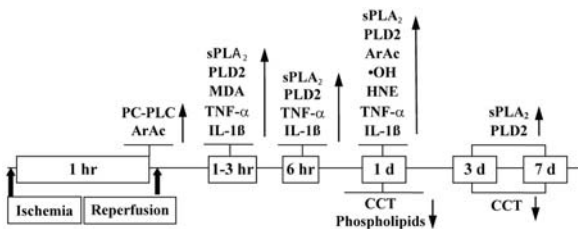
Figure 7. Time course of changes related to cytokines, lipid metabolism and oxidative stress after stroke (148). ↑=increase, ↓=decrease, compared to control. HNE: 4-hydroxynonenal; MDA: malondialdehyde; •OH: hydroxyl radical. PLA$_2$ enzyme activity, sPLA$_2$ mRNA and protein expression, PC-PLC activity and PLD2 protein expression were increased after stroke. CCT catalyzes the rate-limiting step in the biosynthesis of PC. CCT activity and protein expression decreased following stroke. Activation of phospholipases and loss of CCT collectively resulted in loss of PC (41).

Table 1. TNF-alpha and IL-1beta levels after 1 hr tMCAO and 1 hr reperfusion in rat, pg/mg protein

		Cortex		Striatum	
	Treatment	Contra-	Ipsi-	Contra-	Ipsi-
TNF-α	Saline	5 ± 2	25 ± 5[1]	6 ± 3	27 ± 5[1]
	CDP-choline	4 ± 2	15 ± 2[2]	5 ± 2	17 ± 5[2]
IL-1 β	Saline	3 ± 2	17 ± 2[1]	5 ± 3	14 ± 1[1]
	CDP-choline	4 ± 3	9 ± 2[2]	5 ± 2	10 ± 2[2]

Quantification of TNF-alpha and IL-1beta were done by using R&D Systems Quantikine M ELISA kits. [1] $p < 0.05$ ipsi- *vs* contra-, [2] $p < 0.05$ CDP-choline *vs* saline (n=5).

treatment) exposure to excitatory amino acids. It was not determined whether pretreatment alone would have conferred greater neuroprotection nor if post-treatment alone would have exacerbated neurotoxicity. The common factor in both studies (114, 115) appears to be that the neuroprotection afforded by IL-1 depends on exposure of cultures to IL-1 prior to injury (pretreatment).

5.3.4. IL-6: A complex cytokine and does it play a role in maintaining body temperature?

Unlike TNF-alpha and IL-1, IL-6 does not up-regulate major inflammatory mediators such as prostaglandins, nitric oxide, MMPs and synthesis of intercellular adhesion molecule ICAM-1 (74). Several reports have indicated that IL-6 induces PLA$_2$ (a pro-inflammatory response) while other studies revealed that IL-6 may inhibit PLA$_2$ induction. IL-6 exhibits several anti-inflammatory characteristics: it inhibits synthesis of TNF-alpha and IL-1 and promotes synthesis of TNF-alpha binding protein and IL-1 receptor antagonist (IL-1ra), which further inhibit TNF-alpha and IL-1 signaling (74).

IL-6 (89) is up-regulated following cerebral ischemia. Elevated levels of IL-6 in serum and CSF of stroke patients correlated with infarct volume and early worsening of neurological score (116, 117). IL-6 deficient mice showed either no reduction in infarction (118) or increased infarction (119) after MCAO compared to wild-type mice. Cytokines can affect body temperature through activation of the hypothalamic-pituitary-adrenal axis (120, 121). IL-6 deficient mice undergo severe prolonged hypothermia (26.7°C) with a survival rate of 90% when body temperature is unregulated following MCAO (119), which by itself attenuates infarction (122). A recent review on hypothermic neuroprotection discussed the role of cytokines and inflammation in stroke (123). When normal body temperature was maintained, IL-6 deficient mice showed increased infarction and very high mortality (20% of mice die within the first hour; ~90% die within 10 hr) after MCAO. These studies are difficult to interpret since both permanent (up to 24 hr) and transient (1 or 2 hr occlusion) MCAO models were used (119), and it was unclear in one study whether body temperature was monitored during reperfusion (118).

These studies suggest that hypothermia in IL-6-null mice compensated for the detrimental effects of IL-6 deficiency, that at least two pathways are involved in the overall outcome and that endogenous IL-6 in wild-type mice limits infarction after tMCAO. This also highlights the need for careful monitoring of physiological parameters, particularly body temperature, to rule out a contribution of hypothermic effects to infarct reduction in transgenic animals or with drug interventions after cerebral ischemia (122). In contrast to the results from IL-6 null mice, other studies demonstrated that mice over-expressing IL-6 in astrocytes developed severe neurological deficits, suggesting that IL-6 could have a pathogenic role in CNS injury (124). These contrasting studies may highlight the differential effects resulting from the extent and duration of cytokine expression.

6. CYTOKINES AND LIPID METABOLISM IN STROKE

The time course of changes related to cytokines, lipid metabolism and oxidative stress after transient focal cerebral ischemia are shown in Figure 7.

6.1. Cytokine up-regulation stimulates PC and SM hydrolysis, inhibits PC and SM synthesis and disrupts membrane integrity in stroke

Our studies have shown increases in TNF-alpha and IL-1beta in the ipsilateral cortex after tMCAO (Table 1), consistent with other studies in the literature (84, 87-89). A number of studies suggest that up-regulation of these cytokines alter phospholipid metabolism after stroke. CDP-choline, an intermediate in PC synthesis, significantly attenuated TNF-alpha and IL-1beta levels and may differentially affect phospholipases and phospholipid synthesis in restoring the lost phospholipid levels after stroke.

6.1.1. Do TNF-alpha and IL-1 up-regulate cPLA$_2$ in stroke?

Increased cPLA$_2$ *m*RNA (125) and immunoreactivity (126, 127) have been demonstrated after both focal and global cerebral ischemia. cPLA$_2$ immunoreactivity was present in reactive glia cells where neuron loss occurred, but was absent where neurons are

protected or did not degenerate (126-128). Human brain also expressed $cPLA_2$ immunoreactivity that was associated with astrocytes (128). These studies demonstrate that $cPLA_2$ is up-regulated after stroke and expression is largely confined to astrocytes. However, other studies did not observe any obvious change in the $cPLA_2$ mRNA expression in the penumbral area of the ischemic cortex at 3 days after tMCAO (129), which may be due to the selection of time points. To assess the role of $cPLA_2$ in stroke, transgenic 129/Sv mice (derived from C57BL/6) deficient in $cPLA_2$ were developed. $cPLA_2$ null mice showed smaller infarcts after tMCAO compared to wild-type counterparts (130), demonstrating the role of $cPLA_2$ in brain injury after stroke. It should be noted that 129/Sv are naturally deficient in $sPLA_2$ IIA due to a point mutation in the gene (131, 132) while other mouse strains such as BALB/c and DBA/1 have normal $sPLA_2$ II genotype (131, 132). Inhibition of PLA_2 attenuated TNF-alpha cytotoxicity (133, 134), indicating that induction of PLA_2 is one of the major pathways mediating TNF-alpha cytotoxic signaling.

In vitro studies have demonstrated that exposure of normal mouse primary astrocyte cultures to IL-1beta resulted in 4-fold increase in $cPLA_2$ protein expression as demonstrated by Western blotting (135). Simultaneous exposure of primary rat cultured astrocytes to TNF-alpha, IL-1beta, and IFN-gamma resulted in increased phosphorylation of $cPLA_2$ without a net change in overall $cPLA_2$ protein levels (136), however, the effects of individual cytokines on $cPLA_2$ were not shown. In our tMCAO studies in spontaneously hypertensive rat (SHR) we did not observe any significant change in $cPLA_2$ protein expression over 24 hr reperfusion. However the phosphorylated *vs* non-phosphorylated forms of $cPLA_2$ might not have been resolved to observe the changes (41). In an astrocytoma cell line, TNF-alpha stimulated phosphorylation of $cPLA_2$ which was preceded by activation of c-Jun N-terminal kinase (JNK) and p38 MAP kinase (137). Since $cPLA_2$ has been shown to be phosphorylated by MAP kinase (138), TNF-alpha likely stimulates $cPLA_2$ phosphorylation through activation of the MAP kinase pathway. TNF-alpha also activated the transcription factor NF-kappaB and up-regulated COX-2 mRNA and protein expression (137). Thus activation of $cPLA_2$ by MAP kinases and NF-kappaB-dependent COX-2 induction are major steps in the ArAc cascade regulated by TNF-alpha in astrocytoma cells.

These studies strongly suggest that the increases in TNF-alpha and IL-1 initiate the ArAc cascade through activation of $cPLA_2$ and COX-2 in astrocytes after stroke, although this connection has not yet been demonstrated *in vivo*.

6.1.2. TNF-alpha and IL-1 up-regulate $sPLA_2$ IIA in stroke

To-date most of the studies in ischemia focused on $cPLA_2$. Recent studies showed that $sPLA_2$ IIA was up-regulated after tMCAO and (129). $sPLA_2$ mRNA expression was maximum between 12 hr to 72 hr; *in situ* hybridization showed that $sPLA_2$ IIA mRNA was expressed mainly in the penumbral area of the ischemic cortex. Immunohistochemical studies showed that $sPLA_2$

IIA is localized to GFAP positive astrocytes. Our data also showed that $sPLA_2$ IIA mRNA and protein levels were elevated after 1 hr tMCAO (41). Indoxam (139), a putative $sPLA_2$ IIA inhibitor, reduced infarction in MCAO. However, one should be aware of non-specific actions of indoxam: it not only inhibits the enzymatic activity of $sPLA_2$-IB, -IIA and -V, but also blocks the binding of $sPLA_2$-IB to its receptor (140) (see next section on PLA_2 receptors 6.1.3.). Transient forebrain ischemia for 20 min in rat also showed an increase in $sPLA_2$ expression localized to hippocampal CA_1, CA_3, dentate gyrus and neocortex during 7 and 20 day reperfusion demonstrated by *in situ* hybridization (141). Pancreatic $sPLA_2$ (group IB) was not expressed in ischemic brain. A large number of $sPLA_2$ IIA inhibitors have been developed as anti-inflammatory agents (142-144) but to the best of our knowledge they have not been tested in stroke models.

In unstimulated cultured rat astrocytes, basal PLA_2 activity and expression of $sPLA_2$ II mRNA and protein is very low. Exposure of astrocyte cultures to TNF-alpha or IL-1beta resulted in a substantial increase in $sPLA_2$ II mRNA and protein expression and increase in PLA_2 activity in the culture medium (145). TNF-alpha stimulated PLA_2 II expression was enhanced by forskolin, an agent commonly used to stimulate cellular levels of cyclic AMP. Forskolin alone did not directly activate PLA_2 II gene expression in astrocytes, but up-regulated stimulation by TNF-alpha, suggesting the involvement of protein kinase A (PKA) (145). These studies also demonstrated that lipopolysaccharide (LPS) stimulated release of PLA_2 activity from astrocytes, which was attenuated by H-7, a protein kinase C (PKC) inhibitor. H-7 had relatively little effect on TNF-alpha stimulated release of PLA_2 activity, indicating that in astrocytes, PLA_2 II expression is regulated by two distinct protein kinases, PKA and PKC, depending on the extracellular stimulus (145). It all likelihood the PLA_2 form II mentioned in these studies is now referred to as type IIA since only IIA had been identified and cloned at that time (146). Later studies also showed that TNF-alpha in addition to inducing $sPLA_2$ IIA also induced $sPLA_2$ V in cultured rat astrocytes (147).

These studies suggested that increased TNF-alpha and/or IL-1 levels after cerebral ischemia could be responsible for the up-regulation of $sPLA_2$ IIA. To determine this aspect, rats were injected with TNF-alpha antibody (TNF-alpha *ab*, 0.36 mg/kg i.p.) or IL-1ra (20 micrograms by intracerebroventricular injection) at the onset of reperfusion after 1 hr MCAO. After 24 hr reperfusion, Western blots demonstrated a 3.7-fold increase in $sPLA_2$ IIA protein expression in the ipsilateral cortex (IC) compared to the contralateral cortex (CC) (Figure 8). Administration of TNF-alpha *ab* or IL-1ra attenuated the ischemia-induced increase in $sPLA_2$ IIA in the ipsi-cortex to contralateral levels, indicating the role of TNF-alpha and IL-1 in $sPLA_2$ IIA induction after stroke. Administration of CDP-choline (500 mg/kg i.p.), the rate-limiting intermediate in PC synthesis, attenuated the increases in TNF-alpha and IL-1beta (Table 1), $sPLA_2$ IIA protein expression (Figure 8) and PLA_2 activity (Table 2). These data suggested that anti-inflammatory effects of CDP-
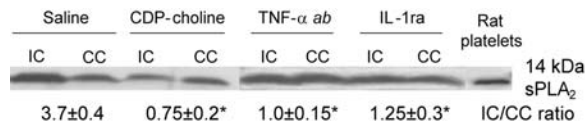
Figure. 8. $sPLA_2$ protein expression increased in ipsi. cortex (IC) vs contra. cortex (CC); treatment with CDP-choline, TNF-alpha antibody (TNF-alpha *ab*), or IL-1 receptor antagonist (IL-1ra) attenuated this increase to CC levels. Relative $sPLA_2$ protein expression is given as IC/CC ratio.

Table 2. Effect of CDP-choline, TNF-alpha antibody (TNF-alpha *ab)* or IL-1 receptor antagonist (IL-1ra) on PLA_2 activity (pmol/min/mg protein) after tMCAO

Reperfusion	Contra./Saline	Ipsi./saline	Ipsi./CDP	Ipsi/TNF-α *ab*	Ipsi/IL-1ra
3 h	38 ± 7	65 ± 5[1]	47 ± 7[3]	45 ± 5[3]	48 ± 3[3]
6 h	28 ± 8	56 ± 8[1]	42 ± 5[3]	40 ± 5[3]	39 ± 5[3]
1 d	40 ± 8	92 ± 4[2]	50 ± 6[4]	50± 8[4]	54 ± 8[4]

[1] $p<0.05$ and [2] $p<0.01$ compared to contra; [3] $p<0.05$ and [4] $p<0.01$ compared to ipsi/saline (n=4). Neither CDP-choline, TNF-alpha *ab* nor IL-1ra had any significant effect on contralateral PLA_2 activity. PLA_2 activity was determined as previously described (46).

choline may be mediated through attenuation of cytokine expression and $sPLA_2$ IIA activation (148).

6.1.3. $sPLA_2$ receptors

In addition to their catalytic activity as enzymes, $sPLA_2$s also know to function as ligands by binding to specific receptors (149). Two types of $sPLA_2$ receptors have been identified: muscular (M-type) and neuronal (N-type) (146, 150). The M-type receptor is mainly expressed in skeletal muscle tissue and was originally characterized for its high affinity binding of snake venom $sPLA_2$s. M-type receptors are also expressed in lung, liver, heart, and kidney, and have endocytic properties, rapidly internalizing $sPLA_2$ (146). N-type receptors are present at high levels in rat brain, are structurally and pharmacologically distinct from M-type receptors and exhibit a different spectrum of affinities for various $sPLA_2$s. High affinity "N-type-like" receptors, similar but not identical to brain N-type receptors, are present in other tissues such as lung, liver, heart, and kidney (146). Although ten different mammalian $sPLA_2$s have been identified, binding studies to M-type and N-type receptors have been conducted only for group IB (pancreatic) and IIA (synovial or non-pancreatic) $sPLA_2$. Both $sPLA_2$ IB and IIA bind to M-type receptors but do not have high affinity for N-type receptors (151, 152). Surprisingly, human $sPLA_2$ IB and IIA do not bind to either M-type or N-type human receptors (150). Possible mechanisms of actions of $sPLA_2$s through binding to these membrane receptors have been hypothesized, but none have been clearly demonstrated (150). Elucidating the physiological roles of these $sPLA_2$ receptors is hampered by the lack of specific antagonists that block receptor function without inhibiting $sPLA_2$ enzymatic activity, allowing these two functions of $sPLA_2$ to be differentiated.

6.1.4. Crosstalk between $iPLA_2$, $cPLA_2$ and $sPLA_2$

Incubation of astrocytoma cells with $sPLA_2$ IIA was shown to activate MAP kinases and phosphorylation of $cPLA_2$ (153). Treatments with $sPLA_2$ inhibitors did not prevent the effect of $sPLA_2$ on $cPLA_2$ activation, indicating that this is independent of its catalytic activity. In contrast, pre-incubation with an antagonist to the $sPLA_2$ receptor

prevented activation of $cPLA_2$ by $sPLA_2$. However, since the astrocytoma cell line in these studies is of human origin, the involvement of M-type or N-type $sPLA_2$ receptors is not supported since these do not bind $sPLA_2$ IIA. It was suggested that the action of $sPLA_2$ involves interaction with a membrane receptor similar to the macrophage mannose receptor or engagement of heparan sulfate proteoglycans (153). Other studies showed that $sPLA_2$ is an up-stream regulator of $cPLA_2$ *via* 5-LOX metabolites in HaCaT cells (154). Functional coupling between $cPLA_2$ and $sPLA_2$ through COX-2 has been demonstrated in macrophages (155). In contrast, other studies have shown that products generated by the $cPLA_2$-12/15-LOX pathway served as regulators of $sPLA_2$ IIA expression in rat fibroblastic cells. These studies demonstrate important coupling between $sPLA_2$ and $cPLA_2$ expression, although the pathways linking them may be dependent on cell type. A novel role for $iPLA_2$ in up-regulating IL-1beta that may drive the subsequent induction of $sPLA_2$ (IIA and V) and $cPLA_2$ (IV) during inflammation has been reported recently (156).

6.1.5. PLA_2 metabolites in inflammatory resolution

Activation of PLA_2s release ArAc, eicosapentaenoic acid, and DHA. Metabolism of ArAc to prostaglandins and leukotrienes is a major pathway in induction of inflammation. PLA_2s also play an important role in the later stage of inflammation by releasing ArAc, eicosapentaenoic acid, and DHA for the formation of lipoxins, resolvins, and protectins that are important in resolution of inflammation (157, 204). Specialized chemical mediators such as aspirin can acetylate COX-2; prostaglandin synthesis is inhibited when COX-2 is acetylated and metabolism is shifted to generate pro-resolution lipoxins.

The DHA metabolite 10,17S-docosatriene (neuroprotectin D1, NPD1) was recently identified in cerebral ischemia/reperfusion in mouse. NPD1 was found to serve an endogenous neuroprotective role by inhibiting apoptotic DNA damage, up-regulating anti-apoptotic proteins Bcl-2 and BclxL, and down-regulating pro-

apoptotic Bax and Bad expression. NPD1 also inhibited oxidative stress-induced caspase-3 activation and IL-1beta-stimulated COX-2 expression (158, 159). Administration of albumin causes systemic mobilization of n-3 polyunsaturated fatty acids (PUFA) including DHA, and provides substantial neuroprotection in models of brain ischemia and trauma. It was demonstrated that after DHA-albumin administration there was an increase in NPD1 in the ipsilateral brain after tMCAO in rat (160).

6.1.6. TNF-alpha and IL-1 activate PLD

A few studies have shown activation of PLD by TNF-alpha (161-163) or IL-1 (164). In some studies (161-163), PLD activation was determined as increased activity measured by its transphosphatidylation reaction and thus did not differentiate whether PLD1, PLD2, or both were induced. However, not all cell lines respond to TNF-alpha stimulation by up-regulating PLD (162). There are conflicting data as to whether activation of PLD mediates (161, 162) or protects against (163) TNF-alpha cytotoxicity. The role of PLD in cell survival *vs* death may depend on the specific cell line, such as tissue origin, normal *vs* neoplastic cells. Another factor could be whether cells have been sensitized to TNF-alpha cytotoxicity by inhibition of protein synthesis (163). Apoptotic signaling does not require protein synthesis and is potentiated by inhibition of protein synthesis; protein synthesis-dependent feedback processes may antagonize or modulate apoptotic signaling (81). The physiological role of PLD is not well understood at this time and whether activation of PLD is a cellular defense mechanism or part of the pathological cascade towards cell death remains an unresolved issue (56). *n*-Butanol, which blocks PA formation (transphosphatidylation reaction of PLD) (165) but does not as such inhibit PLD activity, increased cell death during hypoxia or oxygen-glucose deprivation (OGD) suggesting that PLD activation has beneficial effects (43, 166). In this context it is worth noting that beneficial effects of TNF-alpha in stroke may be partly mediated through PLD, but this needs further investigation.

6.1.7. Cytokine involvement in PC, SM homeostasis: Role of PC-PLC, SMases, CCT and SM synthase

CD95 (APO-1, Fas) receptor is one member of the TNF receptor superfamily that is involved in triggering apoptosis. CD95 and its endogenous agonist CD95 ligand (CD95L) are up-regulated in cerebral ischemia (99). Transgenic mice lacking functional CD95L showed significant reduction in infarct volume after tMCAO, demonstrating its contribution to ischemic brain injury (99). It was also demonstrated that mice lacking both TNF-alpha and functional CD95L showed a dramatic reduction in infarction, indicating that TNF-alpha and CD95 act synergistically in stroke injury (99). It has been shown that CD95 activates PC-PLC, which appeared to be a requirement for subsequent activation of A-SMase, since the PC-PLC inhibitor D609 blocked CD95-induced A-SMase activation (167). CD95 also induced PLA2 and N-SMase, which was independent of PC-PLC/A-SMase pathway since N-SMase activation was unaffected by D609 (167). Both TNF-alpha and IL-1 have been shown to activate PC-PLC and A-SMase (168). Activation of A-

SMase is mediated by formation of the PC-PLC product DAG (168). Besides TNF-alpha and IL-1, PC-PLC has been shown to be involved in signaling pathways of a number of cytokines such as IL-3, colony-stimulating factor 1, platelet-derived growth factor, epidermal growth factor, and interferons (168). Independently of PC-PLC activation, TNF-alpha and IL-1 also stimulate N-SMase (168). Because of the rapid up-regulation of N-SMase by TNF-alpha, it is believed that activation of N-SMase couples directly to the p55 TNF-alpha receptor (169). Alternatively, ArAc was shown to be a potent stimulator of N-SMase but not A-SMase in a cell-free system (170), suggesting that TNF-alpha and IL-1 can stimulate N-SMase through activation of cPLA2 or sPLA2 and release of ArAc.

6.1.7.1. SM hydrolysis by SMases generates pro-apoptotic and anti-proliferative ceramide

Due to their distinct subcellular distribution: A-SMase is lysosomal while N-SMase is localized to the plasma membrane (63, 64), ceramide generated by these two enzymes mediates different cellular signaling pathways. Ceramide produced by N-SMase triggers the MAP kinase cascade *via* ceramide activated protein kinase, eventually resulting in phosphorylation of cPLA2 (169). On the other hand, A-SMase appears to be imbedded in the pathway from PC-PLC to activation of the transcription factor NF-kappaB, whereas ceramide generated by N-SMase is apparently excluded from NF-kappaB activation pathway (169). In A-SMase null mice subjected to tMCAO, ceramide levels showed little or no increase in ischemic cortical tissue compared to a 3- to 5-fold increase in wild type mice (65). Levels of mRNAs encoding for TNF-alpha, IL-1alpha, IL-1beta, granulocyte-colony stimulating factor, and IL-2 were significantly increased in wild type mice after tMCAO, whereas there was no detectable increase in mRNAs for these cytokines in A-SMase null mice (65). These studies indicated that A-SMase is primarily responsible for increased ceramide levels after tMCAO and that ceramide released by A-SMase regulates expression of several cytokines, which likely involves activation of NF-kappaB. Inhibition of PC-PLC with D609 significantly reduced infarction after tMCAO, which was ascribed to inhibition of PC-PLC/DAG/A-SMase/ceramide pathway. D609 largely prevented the increases in ceramide levels and mRNA expression for cytokines, suggesting that activation of A-SMase was attenuated (65). D609 was also shown to block pro-inflammatory actions of TNF-alpha, which was attributed to PC-PLC inhibition (171). We have also shown that D609 significantly attenuated infarction by 35±5% after 1 hr MCAO and 24 hr reperfusion in spontaneously hypertensive rat (Figure 5).

6.1.7.2. PC-PLC inhibitor D609 inhibited cell proliferation (5'-bromo-2-deoxyuridine (BrdU) incorporation)

The PC-PLC product DAG is necessary for protein kinase C activation and cell proliferation (54). D609 (100 µM, IC$_{50}$=94 µM) (43) inhibited proliferation of cultured neuronal progenitor cells. After 3 days in culture with D609, neurospheres were significantly smaller (Figure 9B) compared to controls (Figure 9A). The number of BrdU-positive cells was reduced by 75±5% (Figure 9D)
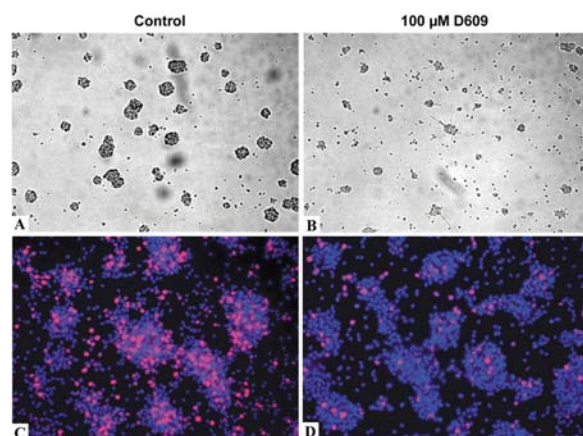
Figure 9. D609 inhibited proliferation (75 ± 5%) of neurospheres. (A, B) bright field images; (C, D) BrdU incorporation (red); Hoechst nuclear stain (blue). (A, C) controls; (B, D) 3 days after exposure to 100 µM D609.
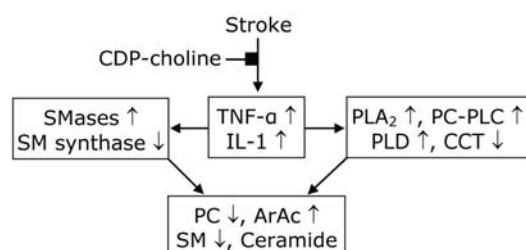

Figure. 10. Integration of cytokine response and lipid metabolism after stroke (148). Up-regulation of TNF-alpha and IL-1 differentially affects phospholipases, SMases; CCT and SM synthase in collectively causing loss of PC and SM and release of ArAc and ceramide.

compared to controls (Figure 9C), indicating that D609 inhibited proliferation of the neurospheres. D609 also inhibited proliferation of primary astrocytes (59) as well as neural stem cells (172). This could also suggest that D609 beneficial effects could arise from inhibiting proliferating microglia/macrophages that are the primary source for TNF-alpha and IL-1 and other pro-inflammatory cytokines in stroke (173), however this needs further investigation.

6.1.7.3. TNF-alpha and IL-1 inhibit PC and SM synthesis by affecting CCT and SM synthase respectively

PC homeostasis is regulated by the opposing actions of hydrolysis by phospholipases (174) and synthesis through CCT (Figure 10). TNF-alpha can activate caspases (175) which in turn can down-regulate CCT (176) and SM synthase (177, 178) through proteolytic cleavage during apoptosis. Other studies have shown that TNF-alpha can stimulate degradation of CCT through the ubiquitin-proteasome and calpain-mediated proteolytic pathways (42). TNF-alpha and IL-1 may inhibit PC and SM synthesis through activation of SMases and release of ceramide. Ceramide and/or its metabolites such as sphingosine inhibit both CCT and SM synthase, and thus inhibit synthesis of both PC and SM (179). In conjunction with activation of

phospholipases and SMases, this results in further loss of PC and SM, disruption of the phospholipid membrane, and contributes to cell death. We have shown CDP-choline counteracted these actions of cytokines on phospholipid metabolism (Tables 1, 2 and Figure 8) in restoring PC levels and provided benefit after stroke (41).

6.2. Arachidonic acid (ArAc): regulation of cytokines expression

Energy failure and increases in intracellular Ca^{++} during cerebral ischemia result in activation of phospholipases and large increases in FFA including ArAc (25, 180). There is a surge of ArAc release during ischemia (23, 25, 27, 181, 182) which may stimulate expression of TNF-alpha and IL-1beta (183). Thus the release of ArAc during ischemia (25, 182) may be one of the initial events that up-regulate cytokine expression. Activation of phospholipases by TNF-alpha and IL-1 may reinforce the feedback regulation, suggesting a potential self-re-enforcing cycle which could be interrupted by blocking either phospholipases or cytokines.

7. INFLAMMATION, CYTOKINES, LIPID METABOLISM, AND REACTIVE OXYGEN SPECIES (ROS)

Cessation of blood flow to the brain leads to energy loss and necrotic cell death. This initiates the immune response, activating inflammatory cells including microglia/macrophages and generating ROS (including hydroxyl radical, superoxide anion radical, and hydrogen peroxide). ROS can further stimulate the ischemic cells to release cytokines that cause up-regulation of adhesion molecules, mobilization and activation of leukocytes, platelets and endothelium that will result in additional ROS production (184). These activated inflammatory cells further release cytokines, MMPs, nitric oxide and more ROS in a feed-back fashion (80). ArAc metabolites, synthesized by and liberated from astrocytes, microglial cells and macrophages, are intimately involved in the inflammatory process by enhancing vascular permeability and modulating inflammatory cell activities and ROS generation.

The study of ROS and oxidative stress in inflammation is difficult due to the transient nature of ROS, the number and complexity of ongoing processes, the interactions between these processes due to direct and reverse relations, and the capacity of ROS to alter a large number of cellular components. ROS are produced by a number of cellular oxidative metabolic processes including oxidative phosphorylation by the mitochondrial respiratory chain, xanthine oxidase, NAD (P)H oxidases, metabolism of ArAc by LOX, and monoamine oxidases (25). However, recent studies suggest that COX does not directly produce ROS during ArAc oxidative metabolism, but does form free radicals (i.e., carbon-centered radicals on ArAc) (148, 200). ROS cause oxidative damage to nucleic acids, proteins, carbohydrates, and lipids. Beyond damage to membranes, lipid peroxides give rise to reactive alpha, beta-unsaturated aldehydes including malondialdehyde, 4-hydroxynonenal (HNE) and acrolein. These aldehydes covalently bind to proteins through reaction with thiol

groups and alter their function. Although there are intracellular defenses against ROS, increased production of ROS or loss of antioxidant defenses leads to progressive cell damage and decline in physiological function. "Oxidative stress" results when generation of ROS exceeds the cell's capacity to detoxify them. The brain is believed to be particularly vulnerable to oxidative stress as it contains high concentrations of PUFAs that are susceptible to lipid peroxidation, consumes relatively large amounts of oxygen for energy production, and has lower antioxidant defenses compared to other organs. The time-course of alterations in lipid metabolism and formation of lipid metabolites and lipid peroxidation products after transient cerebral ischemia is presented in Figure 7. Generation of HNE has been demonstrated by us and others in brain tissue after stroke (25). Recently, elevated levels of an acrolein-protein conjugate were demonstrated in plasma of stroke patients (185).

8. SUMMARY AND PERSPECTIVE

There are substantial data from both animal models and clinical studies that cytokines including TNF-alpha, IL-1 and IL-6 are up-regulated after stroke. Although the roles of cytokines in stroke pathology remain controversial, the majority of studies support their deleterious effects, at least in the early phase of stroke injury. Whether cytokines mediate pro-survival or pro-apoptotic signaling appears to depend on their concentration, the target cell, the activating signal, and the timing and sequence of action (76). Although a great deal of information has been published individually on cytokines as well as lipid metabolism, the integration of these two areas in stroke is less explored and certainly needs more attention. A number of *in vitro* studies have shown that TNF-alpha and IL-1 activate phospholipases. However, most of these studies were conducted in cell lines not derived from brain origin, and their applicability to stroke must be interpreted with caution since response to cytokines is cell-specific. The increases in TNF-alpha and IL-1 together with activation of phospholipases in animal models of stroke suggest that these events are inter-related, although *in vivo* experimental data is limited. This is supported by our studies demonstrating that neutralization of TNF-alpha or IL-1 in animal model of stroke attenuated sPLA$_2$ protein expression (Figure 8). It should be reminded that various sPLA$_2$ inhibitors have been developed but not many have been tested in stroke models (142-144).

A systemic inflammatory response involving up-regulation of TNF-alpha and IL-1 is believed to be instrumental in the formation and destabilization of plaques, one of the greatest risk factors for ischemic stroke (3, 4). sPLA$_2$ IIA (inflammatory PLA$_2$) has been related to various inflammatory diseases such as rheumatoid arthritis may contribute to development of atherosclerosis. A variety of cells relevant to atherosclerosis such as macrophages and neutrophils can release sPLA$_2$ in response to TNF-alpha and IL-1 (17). The inter-relationship of systemic inflammation- inflammatory PLA$_2$ with stroke pathology has not been well studied, even though considerable clinical data indicate that it is associated with unfavorable outcome in stroke patients (9).

9. FUTURE TRENDS: INFLAMMATION, LIPIDOMICS AND RNA SILENCING

The emerging field of lipidomics tries to define the crucial role of lipids in the cell; the research is aimed at mapping the entire lipid population in a biological system, describing the composition and biological function (186-188). The main progress that has spurred recent advances in lipid analysis was the development of new mass spectroscopic techniques, particularly the "soft ionization" techniques electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Lipidomics seeks to provide a molecular signature to a certain pathway or a disease condition. These approaches have provided crucial information that was previously unachievable (189). Lipidomic approaches also hold the promise of elucidating how various signaling pathways such as cytokines interact in determining the net cellular outcome.

RNA interference (RNAi) (190-192) takes advantage of a naturally occurring process to degrade RNA, the intermediary translator between the DNA of genes and the protein molecules they encode. RNAi takes advantage of a naturally occurring process to degrade RNA. By degrading RNA, genes can be selectively and temporarily "turned off.", RNAi provides a tool to analyze protein function and is a method of choice particularly for genes where transgenic knockouts are not possible due to lethality, for example CCT (36) and PLD (56). There have been few reports on the use of siRNA in animal models (193) and the development of appropriate vector systems is a prerequisite (194) before siRNA can be regarded as a suitable experimental tool for *in vivo* studies (195). Use of siRNA in cell culture has been achieved with striking potency at a relatively low concentration (190, 196, 199).

Lipidomic analyses together with RNAi may provide powerful tools to identify lipid mediators, characterize new lipid intermediates, and elucidate their roles in specific cell signaling pathways. In particular, by selectively silencing expression of specific cytokines and lipid metabolizing enzymes, lipidomics may elucidate the roles of cytokines during the acute (pro-inflammatory) and resolution (anti-inflammatory) phases of inflammation. The role of lipids in formation of cell membranes makes them both ligand and substrate for proteins, suggesting that advances in lipidomics could have far reaching implications in genomic, proteomic and metabolomic fields. Impressive achievements and advancements have surfaced during the last few years (157, 159). Lipidomics holds the promise of characterizing complex mixtures of lipids, identifying previously unknown changes in lipid metabolism (21). A deeper knowledge and understanding of the complexity of cytokine-lipid signaling will elevate our understanding of their role in stroke and various CNS disorders, opening new opportunities for drug development and therapies for neurological diseases.

10. ACKNOWLEDGEMENTS

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Abbreviations: ArAc: Arachidonic acid; CDP-choline: Cytidine-5'-diphosphocholine; CNS: Central nervous system; COX/LOX: Cyclooxygenase/lipoxygenase; D609: Tricyclodecan-9-yl potassium xanthate; DAG: 1,2-Diacylglycerol; DHA: Docosahexaenoic acid; FFA: Free fatty acid; HNE: 4-Hydroxynonenal; IL-1beta: Interleukin 1beta; IL-1ra: IL-1 receptor antagonist: Lp-PLA$_2$: Lipoprotein-PLA$_2$; MMP: Matrix metalloprotease; PA: Phosphatidic acid; PAF: Platelet activating factor; PC: Phosphatidylcholine; PI: Phosphatidylinositol; PLA$_2$: Phospholipase A$_2$; sPLA$_2$: Secretory PLA$_2$ or inflammatory PLA$_2$; PLC: Phospholipase C; PS: Phosphatidylserine; PUFA: Polyunsaturated fatty acid; ROS: Reactive oxygen species; SM: Sphingomyelin; SMase: Sphingomyelinase; SMS: SM Synthase; TNF-alpha: Tumor necrosis factor-alpha; TNF-alpha *ab* : TNF-alpha antibody

Key Words: Atherosclerosis; Arachidonic acid; Ceramide; CNS injury; Cytokines; Inflammation; Interleukin-1beta; Lipid metabolism; Lipoprotein-PLA$_2$; Phospholipases; Phosphatidylcholine; Sphingomyelin; Stroke; TNF-alpha; Review

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