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Identification of disease markers in human cerebrospinal fluid using lipidomic and proteomic methods

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Abstract. Lipids comprise the bulk of the dry mass of the brain. In addition to providing structural integrity to membranes, insulation to cells and acting as a source of energy, lipids can be rapidly converted to mediators of inflammation or to signaling molecules that control molecular and cellular events in the brain. The advent of soft ionization procedures such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have made it possible for compositional studies of the diverse lipid structures that are present in brain. These include phospholipids, ceramides, sphingomyelin, cerebrosides, cholesterol and their oxidized derivatives. Lipid analyses have delineated metabolic defects in disease conditions including mental retardation, Parkinson's Disease (PD), schizophrenia, Alzheimer's Disease (AD), depression, brain development, and ischemic stroke. In this review, we examine the structure of the major lipid classes in the brain, describe methods used for their characterization, and evaluate their role in neurological diseases. The potential utility of characterizing lipid markers in the brain, with specific emphasis on disease mechanisms, will be discussed. Additionally, we describe several proteomic strategies for characterizing lipid-metabolizing proteins in human cerebrospinal fluid (CSF). These proteins may be potential therapeutic targets since they transport lipids required for neuronal growth or convert lipids into molecules that control brain physiology. Combining lipidomics and proteomics will enhance existing knowledge of disease pathology and increase the likelihood of discovering specific markers and biochemical mechanisms of brain diseases.

Keywords: Lipidomics, phospholipidomics, sphingolipidomics, cholesterol, proteomics, mass spectrometry, electrospray ionization, phospholipases, enzymes, lipoproteins, cytochrome P450, acetylhydrolases, fatty acids, eicosanoids, secretion, ion channels, receptors, inflammation, oxidation, cerebrospinal fluid, brain, neurological diseases

Abbreviations used:
AA, arachidonic acid
Apo, apolipoprotein
APCI, atmospheric pressure chemical ionization
BBB, Blood brain barrier
CDP, cystidine diphosphate
CSF, cerebrospinal fluid
COX, cyclooxygenase
CYP, cytochrome P
ESI, electrospray ionization
ESI EPA, eicosapentaenoic acid

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(EPA) DHA, docosahexaenoic acid
(DHA) HETEs
HDL, low density lipoprotein
IP, inositol phosphate
LC-MS^2, liquid chromatography tandem mass spectrometry
LDL, low density lipoprotein LO, lipoxygenase
LT, leukotriene
PA, phosphatidic acid
PAF, platelet-activating factor
PAFA, platelet-activating factor acetylhydrolase
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PhosGl, phosphatidylglycerol
PG, prostaglandin
PI, phosphatidylinositol
PIP, phosphatidylinositol phosphate
PL, phospholipase
PLA_2, phospholipase A_2
PLC, phospholipase C
PLD, phospholipase D
PS, phosphatidylserine
PUFA, polyunsaturated fatty acid
SRM, selected reaction monitoring
pAD, probable Alzheimer's disease
PD, Parkinson's disease
SRM, selected reaction monitoring

Glossary
Lipidome- All known lipids, includes phospholipids, fatty acids and cholesterol. The study of structure, biosynthesis and function of all lipids is *lipidomics*.
Phospholipidome- All known phospholipid classes, subclasses and molecular species. The study of structure, cellular distribution, biosynthesis and function of phospholipids is *phospholipidomics*.
Sphingolipidome- All known sphingolipid classes and molecular species. The study of structure, cellular distribution, biosynthesis and function of sphingolipids is *sphingolipidomics*.

1. What are lipids and why are they important in brain function?

Lipids are organic compounds with long chain hydrocarbon molecules that are soluble in organic solvents but not soluble in water. Lipids are derived from living organisms; some examples of lipids include long chain hydrocarbons, alcohols, aldehydes, fatty acids, their derivatives (glycerides, wax esters, phospholipids, glycolipids, sulfolipids, and fatty acid esters), fat soluble vitamins (A, D, E and K), carotenoids and sterols. Lipids are usually subdivided into neutral or polar lipids and are now classified into eight categories based on hydrophobic and hydrophilic composition [68]. About half of the dry weight of the brain is made of lipids. Lipids are important in many brain functions including membrane composition, signal transduction, and biological messenger functions [18,39,40,61,67,69,88, 206,221]. Thus, changes in the concentrations of brain lipids may reflect physiopathologic processes.

One class of lipids proposed to be important in brain function is the polyunsaturated fatty acids (PUFAs). PUFAs are released from phospholipids by lipases to carry a myriad of biological functions. For example, arachidonic acid (20:4, n-6) can be released and subsequently converted to eicosanoids by cycloxygenases (COX), epoxygenases, lipoxygenase (LO) in combination with prostaglandin or leukotriene synthases [6,160, 180,190,195]. Eicosanoids act on specific receptors or ion channels to influence physiological processes such as sleep and pain [94,95]. Likewise, several neurosteroids derived from cholesterol have been shown to have important physiologic functions in the brain [10, 99,116,170,213]. In addition to providing signaling molecules, lipids are the building blocks of cell membranes that confer structure and insulation to nerve cells and are a major reservoir of stored energy. With these important functions, changes in their amounts or defects in lipid metabolic pathways can have a significant impact on brain function. Therefore, an accurate measure of lipid concentrations in the central nervous system is needed for understanding their role in the pathology of diseases.

Lipids encompass a range of structurally dissimilar molecules consisting of several isomers that are difficult to isolate. Lipids do not easily ionize and upon collision-induced dissociation, fragment into ions that can not be useful fingerprints for distinguishing the thousands of molecular species found in cells. No single ionization method can be used for all lipid classes. Moreover, differently charged headgroups in lipids make some lipids easy to ionize in the positive mode while others are better measured in the negative mode. However, recent advances in mass spectrometry have made it possible to use electrospray ionization (ESI) [90,105,129,133] with negative or positive ions under atmospheric pressure conditions to measure several molecular species of lipids. Combined with liquid or gas chromatography, hundreds of lipid molecular species can now be identified.

2. Structures of lipids detected in human brain and CSF and their biosynthetic pathways

2.1. Phospholipidome

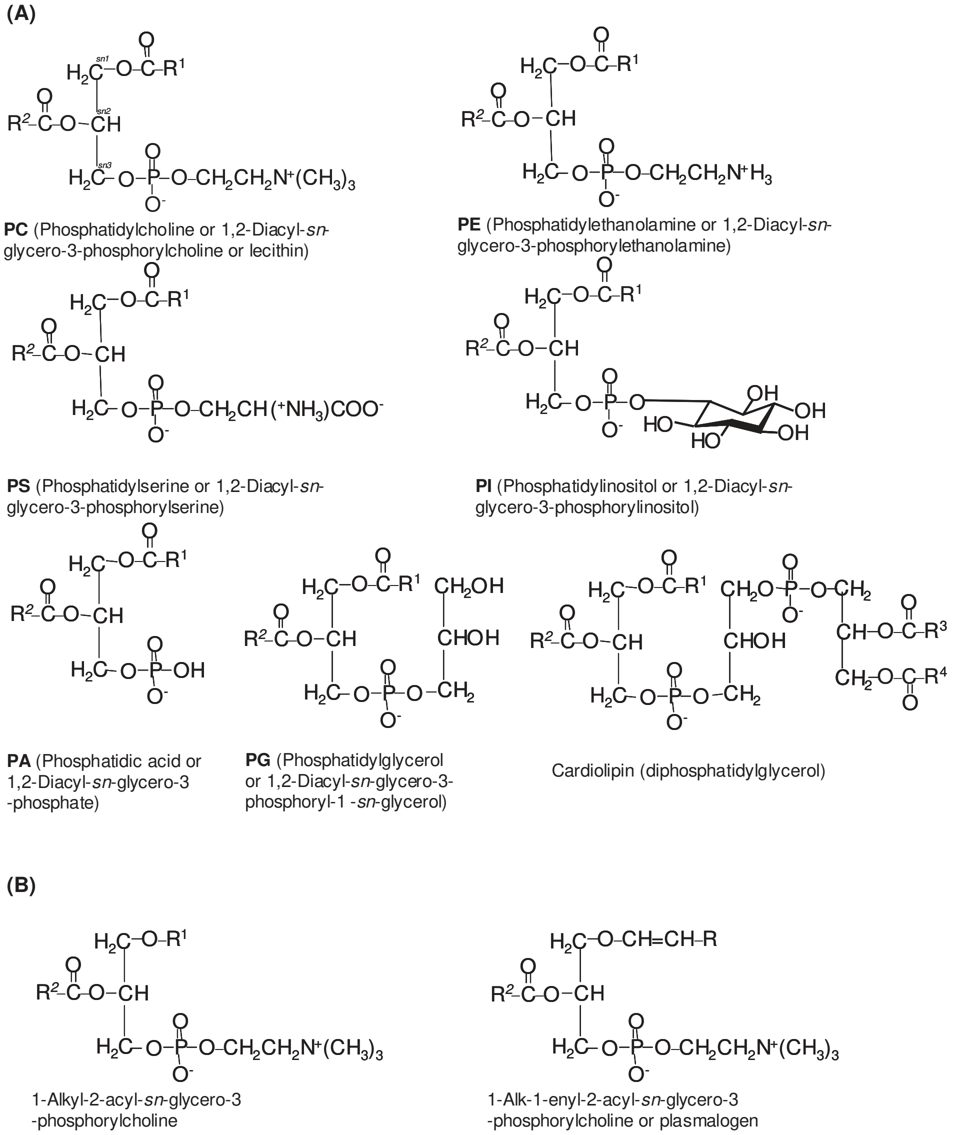
The phospholipidome consists of the major polar lipid class found in mammalian cells and the study of their structure, biosynthesis and catabolism is henceforth referred to as phospholipidomics. Structurally, phospholipids are composed of a glycerol backbone to which is esterified a fatty acid at the *sn-1* and *sn-2* carbon and a phosphor-headgroup moiety at the *sn-3* position. When the headgroup is choline, ethanolamine or serine, the phospholipids are known as phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylserine, respectively (Fig. 1A). Phosphatidylinositol (PI) and phosphatidylglycerol (PhosGl) are formed when inositol and glycerol are the headgroups, respectively (Fig. 1A). Phosphatidic acid (PA) and diphosphatidylglycerol (cardiolipin) are other important phospholipids classes with structures depicted on Fig. 1.

Phospholipids are further divided into subclasses based on the type of linkage of fatty acids at the *sn-1* position. In ester lipids, fatty acids are attached via 1-acyl bonds while for ether lipids or plasmalogens, fatty acids at the *sn-1* position are linked via 1-alkyl- or 1-alk-1-enyl- bonds (Fig. 1B). 1-Acyl-, 1-alkyl or 1-alk-1-enyl- subclasses are predominant in PC and PE while diacyl-linked subclasses comprise most of PI, PS and PG found in cells. Ethanolamine plasmalogens are abundant in myelin sheath and changes in their composition are proposed for several diseases including AD. Choline plasmalogen is a precursor of the potent biological mediator known as platelet-activating factor (PAF) or 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. PAF is a mediator of hypersensitivity, acute inflammation, anaphylactic shock, platelet aggregation and serotonin release [31,37,149,152]. Bazan et al. have proposed that PAF is important in brain plasticity [12].

In most phospholipids, the fatty acid at the *sn-1* position is palmitic (16:0), stearic (18:0) or oleic acid (18:1) while either a saturated, unsaturated or a polyunsaturated fatty acids (PUFAs) can be found at the *sn-2* position of the glycerol backbone. In certain classes and subclasses found in cells or tissues, PUFAs are the major fatty acids at the *sn-2* position. This distribution gives rise to subclasses of lipids that are targeted for the release of PUFAs and the generation of specific lipid mediators and signaling molecules. An example is 1-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, the precursor of platelet activating factor and leukotrienes [77,122]. The incorporation of PUFAs into phospholipid subclasses is highly choreographed such that most PUFAs are initially incorporated into 1,2-diacyl phospholipids subclasses before they are remodeled into the ether-linked phospholipids classes by coenzyme A (CoA)-dependent or CoA-independent transacylase activities [73,84,126, 165,167].

Phospholipids are not only variable in their head groups, fatty acids and bond profiles on the glycerol backbone, but they are asymmetrically distributed within lipid bilayers of cells. This molecular diversity results in hundreds of molecular species in a given cell and theoretically thousands of species in mammals. Even more intriguing is the fact that different organelles may be enriched with specific lipid classes or subclasses. These distributions are unique in establishing the functions of different organelles by influencing membrane fluidity or generating signaling molecules in specific sites when cells are stimulated.

Phospholipids are synthesized when cystidine diphosphate (CDP)-activated polar headgroups are attached to PA (1,2-diacyl-sn-glycerol-3-phosphate) or when CDP-activated diacylglycerol (DAG) is attached to polar headgroups. For example, PC or lecithin is synthesized when choline is phosphorylated by cholinephosphotransferase [106] and then coupled to CDP prior to attachment to PA. Cholinephosphotransferase catalyzes the final step in the synthesis of PC via the Kennedy pathway by transferring phosphocholine from CDP-choline to DAG [98]. For PE biosynthesis, ethanolaminephosphotransferase catalyzes a similar transfer of ethanolamine with CDP-ethanolamine as the intermediate. PC can also be ob-



PC (Phosphatidylcholine or 1,2-Diacyl-*sn*-glycero-3-phosphorylcholine or lecithin)

PE (Phosphatidylethanolamine or 1,2-Diacyl-*sn*-glycero-3-phosphorylethanolamine)

PS (Phosphatidylserine or 1,2-Diacyl-*sn*-glycero-3-phosphorylserine)

PI (Phosphatidylinositol or 1,2-Diacyl-*sn*-glycero-3-phosphorylinositol)

PA (Phosphatidic acid or 1,2-Diacyl-*sn*-glycero-3-phosphate)

PG (Phosphatidylglycerol or 1,2-Diacyl-*sn*-glycero-3-phosphoryl-1 -*sn*-glycerol)

Cardiolipin (diphosphatidylglycerol)

1-Alkyl-2-acyl-*sn*-glycero-3-phosphorylcholine

1-Alk-1-enyl-2-acyl-*sn*-glycero-3-phosphorylcholine or plasmalogen

Fig. 1. Structure of phospholipids- Ester-linked phospholipids (A) and ether-linked phospholipids (B). Stereospecific numbering (*sn*) system of nomenclature for the glycerol backbone is indicated while R^1 and R^2 denote fatty acyl moieties. The classification of phospholipids into subclasses (1-acyl-, 1-alkyl- or 1-alk-1-enyl-) is shown by the fatty acyl-bond at the *sn-1* position of glycerol.

tained when PS is decarboxylated to PE followed by N-methylation of PE by S-adenosylmethionine-dependent methyl transferase [216]. Base exchange reactions when ethanolamine is exchanged for serine in PE results in PS biosynthesis. Similar to PC biosynthesis, PI is formed when 1,2 DAG is activated by CDP followed by condensation with myo-inositol. PI can undergo a series of phosphorylations catalyzed by various PI-kinases to form phosphopolyinositides. PIP_2 is an example of phosphorylated PI that has been well characterized and been shown to be a signal for cell growth, differentiation and synaptic vesicle formation [59,177].

The phospholipidome undergoes dynamic remodeling of fatty acids and phospho-headgroups under resting conditions and this remodeling process is enhanced when cells are stimulated and decreased when cells are undergoing apoptosis [79,84]. Examples of enzymes that catabolize phospholipids and the products they generate are listed on Table 1. The major enzymes that modify phospholipids include phospholipases (PLA_1, PLA_2, PLC, PLD), acyl transferases and PI specific kinases. Phospholipases hydrolyze ester

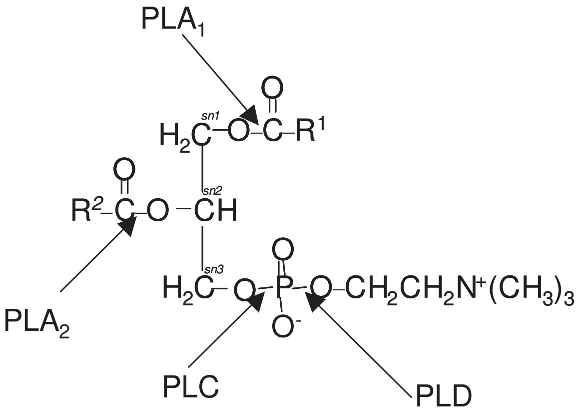


Fig. 2. Sites of action of phospholipases on phosphatidylcholine- The major groups of phospholipases include PLA$_1$, PLA$_2$, PLC and PLD. Products of these enzyme activities and their relevance to the brain are shown on Table 1.

bonds shown on Fig. 2. Many isoforms of PLA$_2$ that differ in structure, substrate specificity, requirements for calcium ions, mode of activation and cellular localization have been cloned and described in various mammalian cells [123]. Several PLA$_2$ isoforms have been characterized in the brain [73,204]. One PLA$_2$ isoform (cPLA$_2$) co-localizes with reactive astrocytes and glial cells and is involved in neurodegeneration [47]. Various PLC isoforms are involved in the generation of DAG and IP$_3$ in the rat and human brain [183,192]. Two isoforms of PLD (PLD$_1$ and PLD$_2$) generate signaling molecules in the brain and involved in the generation of choline required for acetylcholine biosynthesis [230]. PLD isoforms are implicated in neural outgrowth and hormonal/stress signaling [219,220,236]. Lysophospholipids generated by the action of PLA$_2$ can accept acyl groups from other phospholipids in a reaction catalyzed by lysolecithin-lecithin-acyltransferase (LLAT) [203]. Other CoA-dependent and -independent remodeling of PUFAs are responsible for the buildup of PUFAs in ether-linked phospholipids [82,121,165]. Lecithin cholesterol acyltransferase (LCAT), a protein with both lipase and transferase activity transfers fatty acids from lecithin to cholesterol [1,110]. LCAT is associated with lipoproteins that are involved in the transport of lipids from the liver to organs and vice versa. Although not recognized as the major pathway by which cholesterol is excreted from the brain, lipoprotein-mediated transport is important in neural outgrowth and may be the major transcellular transport mechanism within the brain.

2.2. *Sphingolipidome*

The sphingolipidome is a subset of the phospholipidome consisting of sphingomyelin and glycosphingolipids (cerebrosides, sulfatides, globosides and gangliosides). Sphingolipids are composed of a polar headgroup and two non-polar tails (Fig. 3). A long chain amino alcohol known as sphingosine is linked via an amine and a long chain fatty acid is attached to carbon-2 to yield ceramide. Sphingolipids are components of membranes found mainly in myelin sheaths. The major route of sphingolipid formation is the transfer of phosphorylcholine from PC to ceramide by sphingomyelin synthase (Fig. 3). Sphingomyelins are important in nerve cell membranes where very long chain saturated and monounsaturated fatty acids are the main N-acylated molecules at carbon-2 of sphingosine [96, 142,188].

The action of sphingomyelinase on sphingomyelin forms choline and ceramide. Ceramide can be further broken-down to sphingosine by ceramidase or can be converted to glucosylcerebroside by glycosyl ceramide synthase [179,184] (Fig. 3). Sphingosine phosphate and related molecules have recently been shown to regulate apoptosis [128,153]. Although these novel mediators have not been characterized in the brain, their presence may account for brain diseases that are known to result from defects in sphingolipid biosynthesis. Thus, one may postulate that changes in sphingosine phosphate levels in the neurodegenerative brain may be early indicators/biomarkers of brain atrophy.

In summary, the phospholipidome provides structure, is the precursor of signaling molecules and plays an important role in the formation of vesicles required for neurotransmitter release and the transport of metabolites. These important functions make it necessary to study the composition of brain phospholipidome because early changes are possible events in brain diseases.

2.3. *Cholesterol and hormones*

The brain is a very rich source of cholesterol and is estimated to represent 25% of total cholesterol in humans [60]. Although it is known that a major portion of this cholesterol is localized in myelin sheaths, little is known about the metabolic pathways that control this vast reservoir of cholesterol. Brain cholesterol is mainly produced by *de novo* synthesis (Fig. 4) and is segregated from plasma cholesterol by the blood brain barrier (BBB). Homeostatic control of brain cholesterol

Table 1
Some enzymes that modify phospholipids, their products and functions in CNS

Enzymes	Lipid substrates and products	Importance of products
PLA_2	Hydrolyzes the ester bond at the *sn-2* position of phospholipids (Fig. 2) to release free fatty acids (FFA) and lysophospholipids. For example, PC is hydrolyzed to LPC and FFA or PA hydrolyzed to LPA and FFA.	If the FFA is a PUFA, it may be a substrate of COX, CYP or LO. PLA_2 activity is implicated in endocytosis, fusion and membrane structure and asymmetry, neurodegenerative diseases and autistic disorders [20,73,204,208].
PLC	Hydrolyses phospholipids to DAG and phospho-headgroup moiety, e.g. $PI(4,5)P_2$ to DAG and $I(1,4,5)P_3$ [21]	Important in vesicle priming and synaptic vesicle docking [52,59]. DAG activates PKC and/or PKA while IP3 induces intracellular calcium release [22].
PLD	Releases PA and free headgroup from phospholipids (Fig. 2). An example is the release of choline from PC.	Implicated in synaptic vesicle fusion. PA promotes exocytosis, phagocytosis, membrane trafficking and cytoskeletal structure [48,51,62,147,219,220]. Headgroup such as choline can be used for neurotransmitter biosynthesis.
Lysophospholipase	Glycerophosphate and free fatty acids [218]. Implicated in ether lipid formation by brain microsomes [229] and anandamide biosynthesis [205]	Important in signal transduction [174,210,218].
PI kinase or PIP kinases and PITP	Phosphorylate PI to form PI(4)P and PI(4)P to $PI(4,5)P_2$. PITP is involved in membrane remodeling.	Required for synaptic vesicle formation, fusion, trafficking and exocytosis [49,50]

involves biosynthesis and cytochrome P450 (CYP)-mediated excretion with 24S-hydroxycholestrol as the major product [24,28]. Cholesterol serves several important functions in the brain. First, cholesterol helps maintain brain structure and is important in controlling lipid fluidity and the transport or permeability of ions and metabolites. Second, cholesterol provides the necessary insulation to neurons that allows efficient propagation of an action potential. Third, cholesterol is needed for the growth and development of neurons. Fourth, cholesterol is the precursor for the synthesis of steroid hormones that are important in controlling physiologic processes such as stress, plasticity, and depression [11]. Fifth, the cholesterol biosynthetic pathway generates molecules (isoprenenyl-pyrophosphate, geranyl-pyrophosphate, farnesyl-pyrophosphate) used for the modification of proteins and RNA and for the biosynthesis of ubiquinone, dolichol and Heme A [65, 66,196]. Modification of G-proteins is critical for their function. Thus cholesterol biosynthesis may indirectly influence receptor mediated physiologic processes controlled by G-protein coupled receptors. Finally, the cholesterol composition affects the activity of transmembrane proteins and receptors. For example, gamma amino butyric acid (GABA) transport requires cholesterol and lipid rafts/coated pits that are implicated in endocytosis and protein remodeling are all eriched with cholesterol [138,193,200,209,227]. These functions of cholesterol underscore its importance in brain function. For example, 24S-hydroxycholesterol, the major excretion product and has been shown to increase in CSF of AD subjects [24,60]. Moreover, CYP enzymes that oxidize cholesterol are differentially expressed in brain cells and around amyloid plaques in the AD brain [33]. Several other brain diseases are associated with metabolism, transport, recycling, excretion and degradation of cholesterol [2,24,64,120,135, 143,161,163,178,202,227].

Levels of cholesterol in the brain are controlled by the rate of biosynthesis, catabolism, transport and excretion. Two major pathways for cholesterol biosynthesis are known in mammals (Fig. 4). Both pathways require acetyl-CoA derived from glucose oxidation or from fatty acid metabolism (Fig. 4) [85,87,112, 113]. Conversion of HMG-CoA to mevolonic acid by HMG-CoA reductase is the rate-limiting step for both pathways in many cells including glial cells. In one pathway, 7-dehydrocholesterol is an intermediate while the alternate route involves 7-dehydrodesmosterol as an intermediate (Fig. 4). Several enzymes involved in cholesterol synthesis have been characterized in various brain cells. The differential expression of these enzymes in brain cells suggests that there is transcellular metabolism or highly specialized functions of cholesterol products by brain cells or specific brain regions. For example, astrocytes synthesize two to three times more cholesterol than neurons and fibroblasts [169]. Oligodendrocytes that are normally involved in myelination have higher capacity to synthesize cholesterol, underscoring the need and importance of cholesterol for myelination [212].

Cholesterol synthesis and distribution may play a critical role in the onset and progression of degenerative brain disorders. A study showing that cholesterol synthesis is higher in developing neurons but is decreased with aging has several implications for aging and neurodegenerative diseases [227]. While total cholesterol may not change, the distribution of choles-

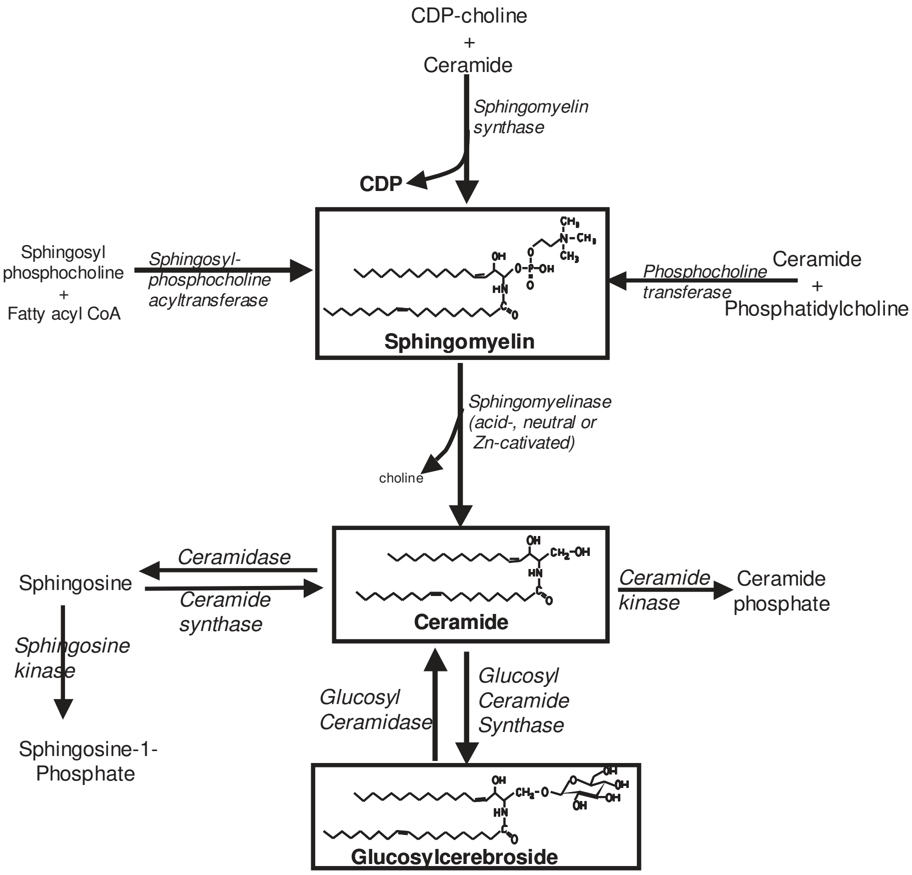


Fig. 3. Metabolism of sphingomyelin and ceramide.

terol favors amyloid peptide accumulation upon aging. In terms of biomarker discovery, it may be important to examine the biosynthetic pathway in individuals with a neurodegenerative disease to determine whether cholesterol biosynthesis is slower than in normal subjects. Alternatively, a compromised BBB may disrupt the tight control of brain cholesterol levels. Finally, enhanced catabolism via CYP-dependent mechanisms or by auto-oxidation may generate oxidative products that cross the BBB easily and are excreted from the brain. Any decrease in cholesterol level may result in enhanced proteolytic digestion of membrane bound proteins by exposing them to proteases or secretases in the case of amyloidosis (AD) [27,60]. Other ramifications may include enhanced oxidation or increased inflammation, leading to neuronal cell death.

Since cholesterol is independently controlled in the brain, it is unlikely to be strongly influenced by inhibitors of biosynthesis if these do not cross the BBB or by dietary manipulation, since the plasma pool is not interchangeable with the brain pool. Given an estimated half-life of 5 years in the brain, enhanced degradation or CYP-mediated catabolism of cholesterol will significantly alter its levels [28,33,136,224].

Lipoprotein-mediated uptake and reverse transport is proposed to play a small role in the removal of cholesterol from the brain. However, several groups have characterized many cholesterol-binding proteins in the CSF and brain [2,53,131,217,226]. If not directly involved in cholesterol transport out of the brain, these proteins may be crucial in transporting cholesterol within the brain from sites of synthesis to sites needed for neuronal growth or for the synthesis of neurohormones. There may also be cross-talk between 24S-hydroxycholesterol and lipoprotein-dependent transport since 24S-hydroxycholesterol has been shown to enhance ApoA1 dependent efflux of cholesterol from cultured cells. Several lipoproteins synthesized in the brain have been detected in CSF by 2D gels [80]. These include ApoA1, ApoD, ApoJ, and ApoE. Expression of one ApoE allele (ApoE4) increases the risk of late onset AD [32,41,124,127,134,238]. ApoE pro-

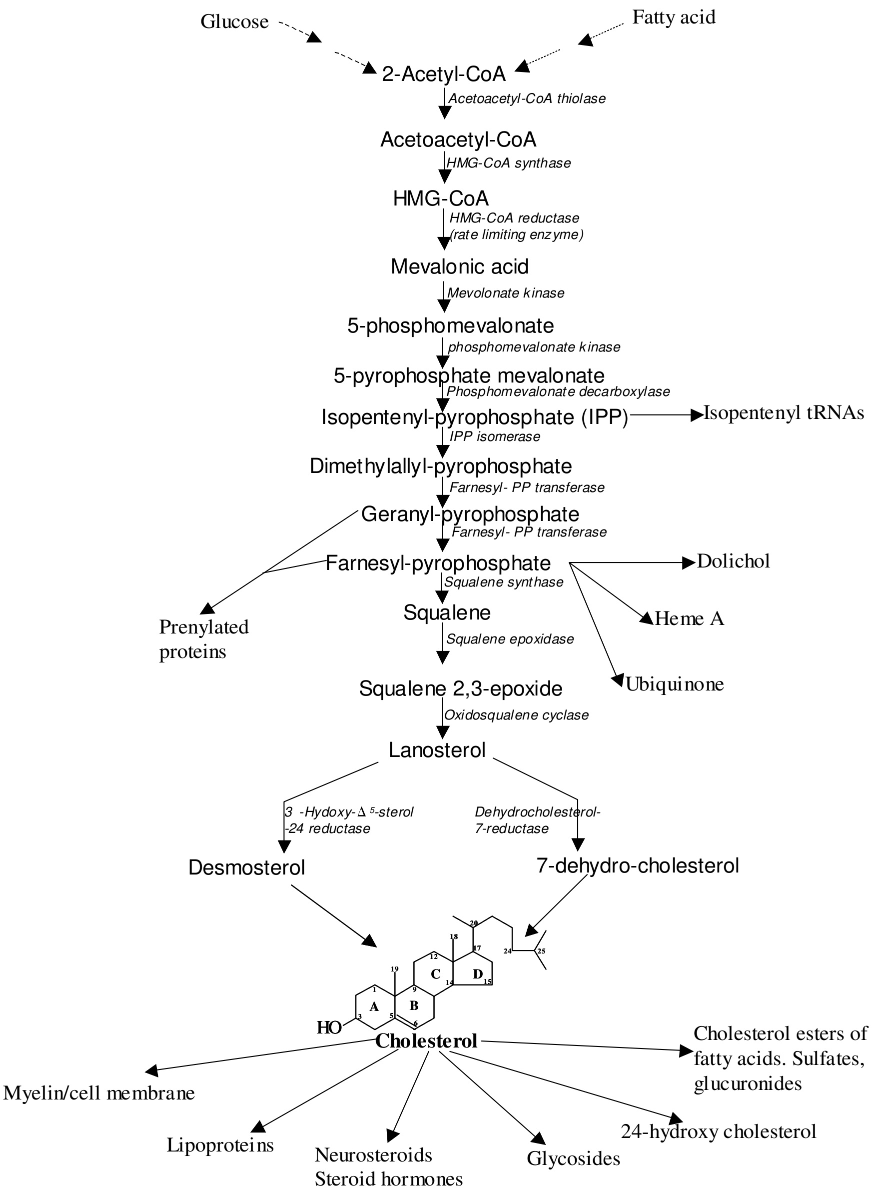


Fig. 4. Metabolism of cholesterol- Cholesterol is synthesized by a multi-enzyme pathway starting with 2 molecules of acetyl-CoA. Hydroxyl-methylglutaryl-CoA reductase is the rate limiting enzyme of this pathway. Several intermediates of cholesterol biosynthesis are substrates for molecules such as dolichol, Heme A and ubiquinone. Some intermediates are also used for post-translational modification of proteins. Once formed in the brain, cholesterol is used for nerve cell growth, for the formation of steroids and glucosides. CYP activity converts cholesterol to hydroxycholesterol that easily crosses the BBB for excretion. Cholesterol is also converted to sulfate or glucuronides dereivatives.

duced in high amounts by astrocytes is important in cholesterol transport in the brain and is a major component of cholesterol-containing lipoproteins found in CSF. *In vitro* studies show that ApoE3 is more efficient in cholesterol transport and delivery to neurons than ApoE4. In addition, ApoE3 from astrocytes stimulates neuronal outgrowth more than ApoE4 expressing cells [156]. Lipoproteins are integral parts of HDL or LDL particles that are the suggested route of cholesterol transport from the liver to organs and vise versa. Scavenger receptors (SR-B1) or other lipoprotein receptors may be responsible for cholesterol removal from the brain. LDL receptor (LDLR) is expressed in the brain [17]. Seven members of the LDLR family have been characterized. Typical features of the LDLR include a ligand binding domain, an EGF repeat, a trans-

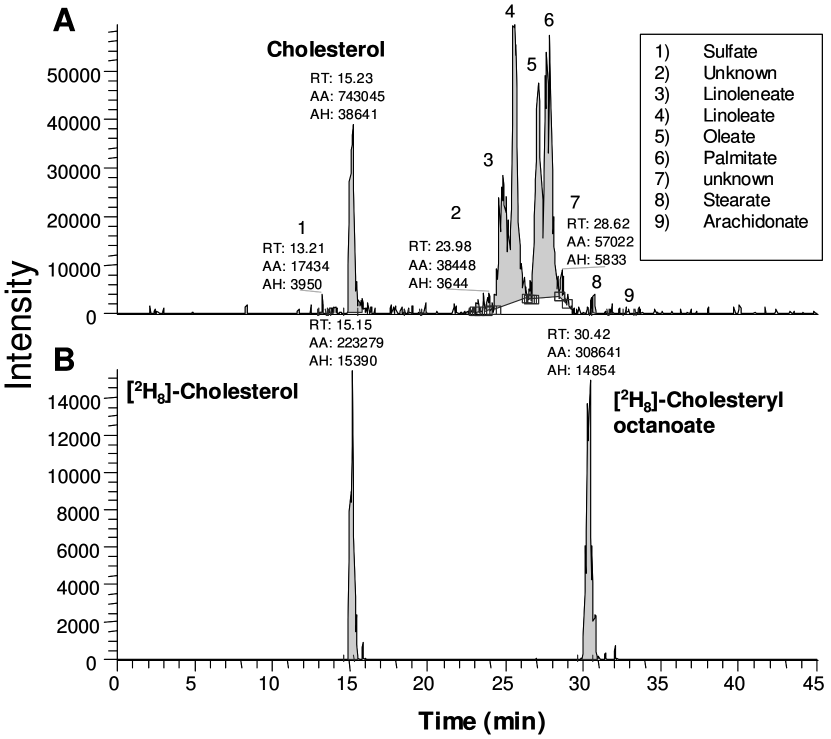
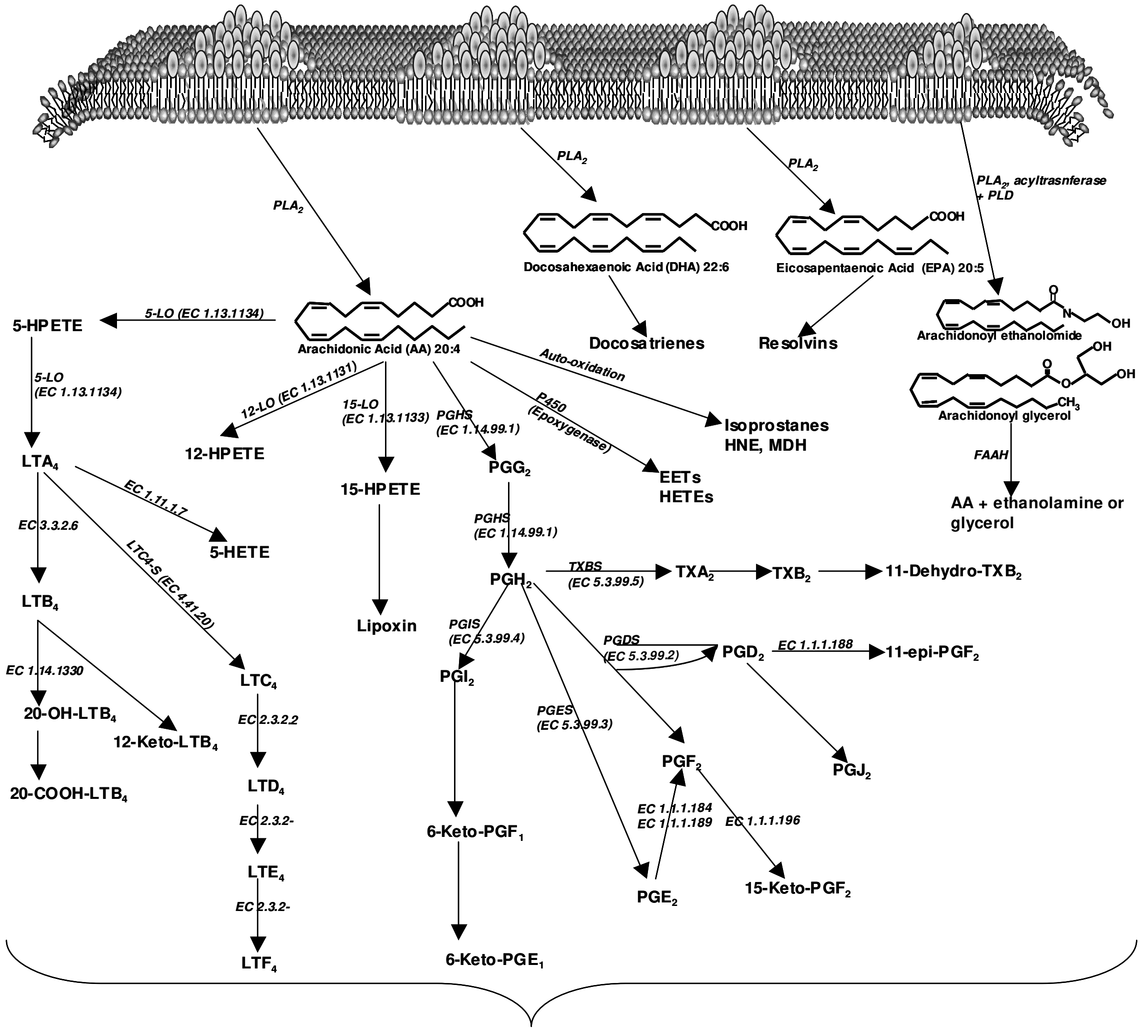


Fig. 5. LC-APCI tandem MS of cholesterol in CSF- Cholesterol and esters in CSF from 79 year old female- Positive ion mass spectra obtained by APCI/MS^2 for cholesterol and its derivatives in CSF (A) and SRM of internal standards (B). Peaks for cholesterol sulfate, free cholesterol and fatty acid esters of cholesterol are numbered from 1–9 (A). Deuterated internal standards are shown on Fig. 5(B).

membrane segment and a cytosplasmic tail containing a NPxY motif that controls endocytosis and interaction with the phosphotyrosine binding-containing proteins [17,158,166,185]. ApoE is a common ligand of the LDLR family. ApoE binds to LDLR and initiates a signaling pathway that promotes cell survival (Akt).

In addition to lipoproteins, several ATP-binding cassette (ABC) transporters assist in the shuttling of cholesterol from glial cells to neurons [217]. Preliminary results from our laboratory combining 2D-LC with a linear ion trap mass spectrometer have revealed several isoforms of ABC proteins in CSF (unpublished data). Studies to determine whether their expression or isoform profiles change in brain diseases or whether their expression is linked to cholesterol are underway in our laboratory.

Cholesterol metabolism is associated with several diseases including neurodegeneration, hypercholesterolemia, AD, multiple sclerosis, and Niemann Pick disease type C. CNS cholesterol is mainly unmodified; it is not conjugated to fatty acids, or modified by sulfates and glucuronides. Our studies show several cholesterol molecular species in CSF including fatty acid esters and sulfates (Fig. 5). CSF may be a medium of transport of cholesterol from areas of synthesis to other parts of the brain where it may be needed for hormone synthesis or neuronal growth. Alternatively, cholesterol and its esters may exist in lipoprotein bound particles that are needed for LDLR binding. Studies showing LCAT, cholesterol esters and various constituents of lipoproteins in CSF suggest that this mode of cholesterol transport may be important within the brain for neuronal function. These studies underscore the importance of cholesterol in growth and neurodevelopment. Considerable levels of esterified cholesterol in human CSF may reflect a preferred mode of extracellular transport since most cholesterol within brain cells is free. It remains to be determined whether levels of free or esterified cholesterol are altered in CSF from subjects with neurological diseases compared to normal subjects. Moreover, it would be of interest to determine whether profiles of cholesterol, CYP, lipoproteins, receptors and ATP-binding cassette proteins can form a multiplex biomarker panel for the detection of neurological pathologies linked to cholesterol metabolism in the brain.



Products of PUFA oxygenation and endocannabinoids act on specific receptors (G-protein coupled) to control physiologic processes such as sleep, pain, secretion or inflammation.

Fig. 6. Oxygenation of PUFAs and endocannabinoid biosynthesis- PUFAs are released from the lipid bilayer by the action of PLA_2 enzymes. Free PUFAs such as AA, DHA or EPA are metabolized by major oxygenases pathways numbered 1–4. 1) Lipoxygenases (5-LO, 12-LO or 15-LO) generate hydroxyperoxyeicosatetraenoic acid (HPETE) that are subsequently converted to leukotrienes (5-LO) or lipoxins (15-LO). 2) PUFAs can also be metabolized via the Prostaglandin H synthase (PGHS) pathway to form PGH_2. Terminal synthases convert PGH_2 to prostanoids. PGIS, PGES, PGDS and TXBS are the major terminal synthases responsible for PGI_2, PGE_2, PGD_2 and TXB_2 biosynthesis. 3) Cytochrome P450 monooxygenases (P450) convert PUFAs by hydroxylation to hydroxyeicosatetraenoic acids (HETEs), by allylic oxidation to generate isomers of HETEs and by olefin bond epoxidation to generate regioisomers of epoxyeicosatetraenoic acids (EETs). 4) Auto-oxidation of PUFAs can generate isoprostanes or neuroprostanes and oxidized lipid products such as malonaldeylde and 4-hydoxynonenal (HNE). 5) DHA and EPA can serve as substrates of these oxygenases to for docosatrienes and resolvins, respectively. 6) A combination of acyltransferase activity in concert with PLD is proposed for the biosynthesis of the endocannabinoid, arachidonoyl ethanolamide (AEA). AEA levels in the brain may be controlled in part by fatty acid amide hydrolase (FAAH) activity. Overall, products from these pathways are implicated in processes such as sleep, pain, secretion and inflammation.

2.4. Fatty acids, eicosanoids and endocannabinoids

Compared to other tissues, the brain is highly enriched with PUFAs . The major classes of PUFAs in the human brain belong to the $n - 6$ or $n - 3$ classes where n denotes the total number of carbon atoms and the numbers denote the presence of a double bond 6 or 3 carbon atoms from the terminal omega (ω) carbon atom. The major PUFA species are arachidonic acid (AA, 20:4, n-6), eicosapentaenoic acid (EPA, 20:5, n-

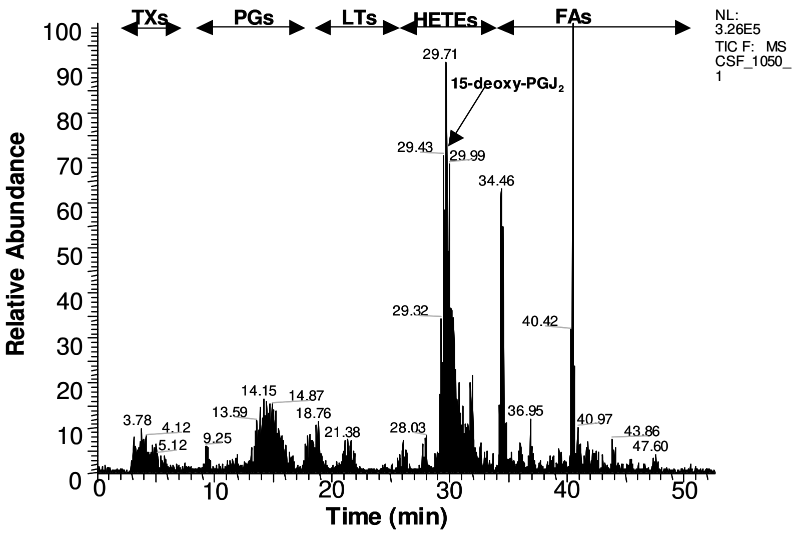


Fig. 7. LC-ESI tandem MS of eicosanoids, isoprostanes and fatty acids in CSF- After the addition of 2 ng deuterated internal standards to 400 ul CSF, eicosanoids and free fatty acids were extracted using ethyl acetate. Samples were reconstituted in water/methanol (70:30) containing 0.01% acetic acid. LC-negative ion ESI-MS^2 was performed with SRM of parent and product ions for thromboxanes (TX), prostanoids (PTs), leukotrienes (LT), isoprostanes and fatty acids (PUFAs).

3) and docosahexaenoic acid (DHA, 22:6, n-3) [146]. Mammals cannot synthesize AA, EPA and DHA because they lack the desaturase enzyme required to introduce a double bond at the n-6 and n-3 position [125]. Therefore, the major precursors of PUFAs must be provided by the diet and are thus referred to as essential fatty acids (EFAs). Linoleic acid (18:2, n-6) and α-linolenic acid (18:3, n-3) are the major plant oil-derived EFAs. EFAs are important in brain development and function [54,125,186,214]. Once ingested, EFAs are subjected to elongation and desaturation to form several PUFAs including AA and DHA [199]. Considerable evidence suggest that PUFAs diffuse through the lipid bilayer and FA transporters have also been implicated in their uptake [173,176]. Upon internalization, PUFAs are converted to CoA-derivatives by acyl-CoA synthetases (ACS), which require ATP. PUFA-CoAs are utilized for the synthesis of phospholipids, triacylglycerides or can be utilized for energy generation via mitochondrial β-oxidation [38,130,145,157]. PUFA-CoAs also function as signaling molecules [38, 93]. PUFA levels and metabolism have been implicated in several neurological processes. These include neural outgrowth, neurodegeneration, depression, membrane activity of receptors and the sodium pump, synaptic lipid signaling and plasticity [29,86,115,140,142,194, 214,232]. Although PUFAs are thought to be involved in diseases ranging from AD, PD, stroke, and anxiety, the mechanisms that would account for their roles still awaits discovery [115,140]. PUFA levels may be influenced by uptake from the diet, biosynthesis and release/catabolism within the brain.

Phospholipases release PUFAs from membrane phospholipids. Released PUFAs are converted to bioactive lipids or other signaling molecules (Fig. 6). Bazan has recently reviewed the significance of PUFAs in synaptic lipid signaling [14]. Likewise, the importance of phospholipases in releasing PUFAs from phospholipids and their potential role in brain function has been reviewed [73]. However, the isoforms of phospholipases and the source and mechanism by which PUFAs may be mobilized for eicosanoid formation are not yet defined in CSF. Given the presence of several oxygenases and terminal synthases that can convert PUFAs into eicosanoids, resolvins and docosanoids/neuroprostanes [139,186] (Fig. 6), it is important to determine the PUFA composition of brain lipids and the ancillary pathways that control their availability to neurons. Using LC-negative ion ESI tandem MS, we have detected many eicosanoids, isoprostanes and fatty acids in human CSF (Fig. 7). Changes in the amounts of these molecules in CSF may not only be an indication of physiologic process in the brain, but could suggest pathologic conditions.

In addition to eicosanoids, another AA-derived class of molecules implicated in pain reduction, motor regulation, learning, memory, reward and appetite has been recently described [57,100,215]. These molecules are

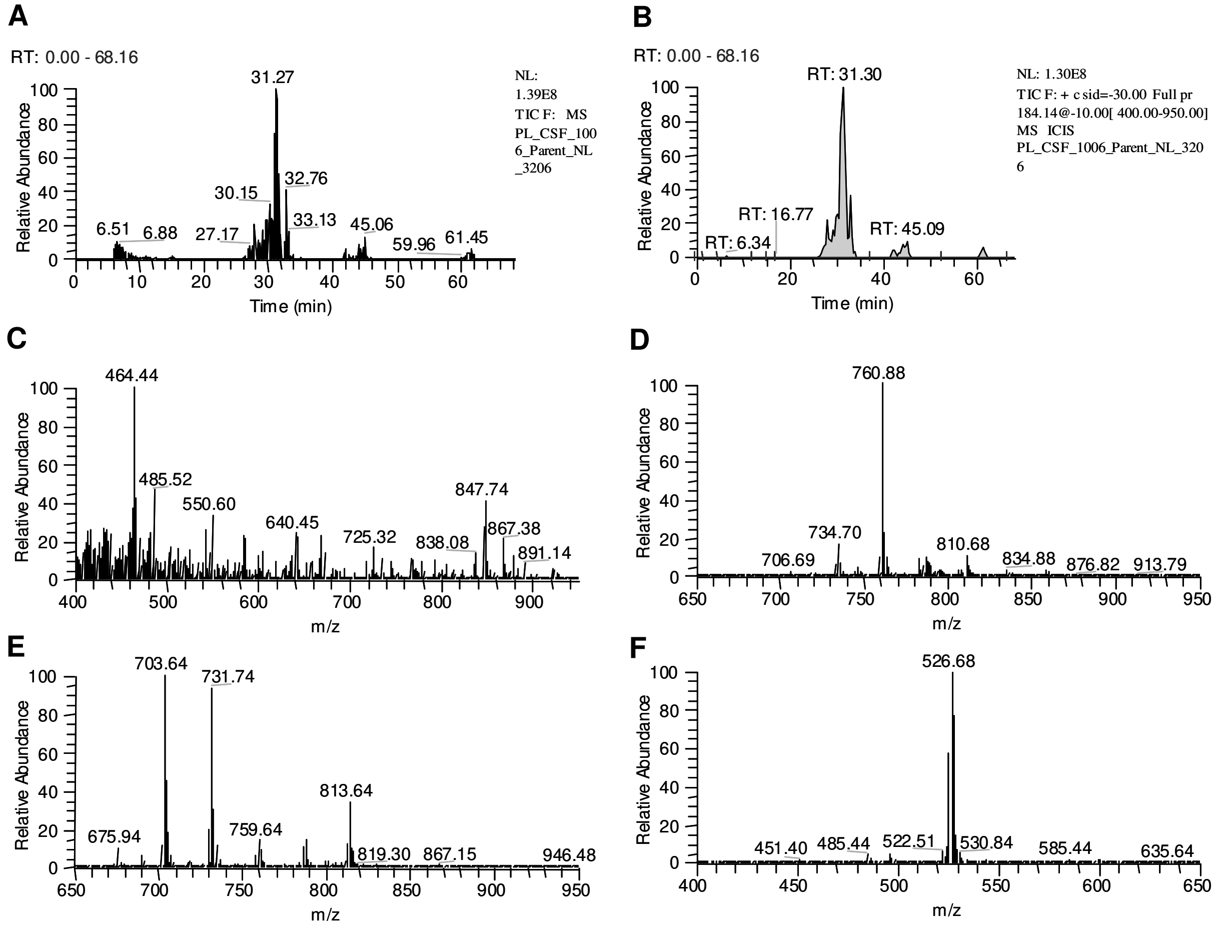


Fig. 8. LC-ESI tandem MS of phospholipids in human CSF- 10 ng phospholipids standards were added to 200 μl CSF and total lipids were extracted using chloroform and methanol. Samples were reconstituted in chloroform/methanol (2:1) and LC-positive ion ESI-MS^2 was performed with parent ion monitoring for choline containing phospholipids or neutral ion loss for PE, PI, PG and PS. Figure 8(A) shows the TIC obtained for all phospholipids classes while Fig. 8(B) shows a chromatograph of choline-containing molecular species. Figure 8(C) shows spectra of sphingosylphosphocholine/phosphocholine species eluting at 1–20 min, PC at 20–35 min (Fig. 8D), sphingomyelin at 35–45 min (Fig. 8E) and lysophosphatidylcholine/PAF at 45–65 min (Fig. 8F). Over 450 choline-containing molecular species are identified in CSF.

known as endogenous cannabinoid ligands or endocannabinoids. The first endocannabinoid was initially identified as an ethanol amide derivative of AA and termed arachidonamide or anandamide (Fig. 6). A second endocannabinoid also contains AA that is attached to glycerol (2-arachidonoyl glyceryl ester or 2AG). Endocannabinoids are synthesized and released from neurons upon stimulation [215]. Inactivation of anandamide and 2-AG is accomplished by rapid uptake via a membrane transporter (AMT) followed by intracellular enzymatic degradation by fatty acid amide hydrolase (FAAH) [100,215]. Endocannabinoids bind to the CB1 receptor. Similar to the CB1 receptor, the distribution of AMT and FAAH are high in the hippocampus, cerebellum and cerebral cortex. Recent studies have clarified endocannabinoid signaling. Upon release by postsynaptic neurons, endocannabinoids diffuse back to the presynapatic neurons where they act on CB1 receptors to inhibit release of neurotransmitters such as GABA and glutamate [3]. Interestingly, recent studies show that CB1 receptors are coupled to another lipid second messenger, ceramide [3]. Also a proposed pathway for endocannabinoid-induced apoptosis involves ceramide. Structural similarities between 2-AG and lysophosphatidic acid (LPA) also suggest possible cross-talk between these lipid-signaling ligands and their receptors. Measurement of these signaling molecules in CSF and correlation of any changes in their levels with enzyme activity or protein levels will represent disease markers and provide tangible pathways that can be influenced by therapy.

3. Methods for measuring lipids in CSF

Early methods for analyzing the lipidome depended on organic extraction coupled with thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). Further structural identification of lipid classes and molecular species were possible only after extensive digestion of lipids, derivatization and further chromatography (Table 2). This laborious strategy employed over many decades revealed several metabolic pathways. However, low sensitivity and the labor-intensive process limited extensive profiling of lipid molecular species. Advances in mass spectrometry have made it possible to detect thousands of lipid molecular species. Initial advances in lipid analyses were made by Murphy and colleagues using fast atomic bombardment [44,117]. The advent of soft ionization processes such as ESI-MS^2 [108,171,175] or APCI-MS^2 [35] have revolutionized metabolic profiling of lipids. Not only are these modern instruments sensitive, but enzyme digestion and derivatization procedures are not needed for most lipids. By combining LC with MS, many more molecular species of lipids can be measured. For example, Fig. 8(A) shows the total ion current (TIC) obtained from CSF phospholipids monitored using LC tandem MS with parent ion monitoring of PC or neutral ion loss for PE, PI, PS and PhosGl. Figure 8(B) shows the TIC of choline-containing phospholipids species. Spectra of choline-containing molecular species corresponding to sphingosylphosphocholine/phosphocholine, PC, sphingomyelin and PAF/LPC are shown in Figs 8(C), 8(D), 8(E) and 8(F), respectively. Over 450 different molecular species were identified in human CSF. Similar to PC, hundreds of PE, PI, PS and PhosGl molecular species were identified in CSF (data not shown). Other studies use 2D mass spectrometry with either parent ion monitoring, neutral ion loss of specific lipid fragments or multiple reaction monitoring (MRM) to identify hundreds of lipid species [102,207] (Table 2). Information from these studies is important in obtaining structure, composition of lipids in cells, turnover of lipids and characterization of lipid synthesis/transport and degradation pathways.

4. Proteomic strategies for the identification of lipid-metabolizing proteins in CSF

While lipidomic approaches reveal lipid composition, protein expression and the putative biosynthetic pathways in CSF that account for specific changes in lipid levels or distribution can best be understood if the levels and activity of metabolizing enzymes and transport proteins are determined. For example, if the level of PAF is found to change in CSF for a specific disease, various possibilities may account for this change. An increase in PLA_2 activity resulting in the generation of lysophosphatidylcholine (LPC) and subsequent acetylation of LPC by acetyltransferase can result in an increase in PAF. Likewise, a decrease in PAF acetylhydrolase activity may result in a build up in PAF levels. Alternatively, an increase in PAF acetylhydrolase activity will decrease PAF levels. Similar arguments can be applied to most lipid signaling molecules (eicosanoids, DAG, IP_3 etc). One way of understanding why there is a change in the lipidome is to examine all the proteins in the biosynthetic and catabolic pathways associated with specific lipid molecules as outlined on Fig. 9. In addition to the use of systematic animal knockout models, enzyme analyses or use of proteins chips, 2D gel electrophoresis and shotgun sequencing of CSF proteins are the most valuable approaches for interrogating changes in lipid-metabolizing proteins. In 2D gel electrophoresis, CSF proteins are identified by their isolelectric point (pI) and by their size. A 2D map is then obtained by staining gels with fluorescent dyes or with silver stains [92,101]. Densitometric profiles and normalized total densities are obtained for relative quantification. Proteins on 2D gels are identified by extraction and MS sequencing, immunoblotting, or by reference to published 2D database (SWISS-2DPAGE, http://www.expasy.org/ch2d/). While very laborious, 2D gels are useful because they resolve posttranslationally modified isoforms of proteins [101].

In shotgun sequencing, CSF proteins are reduced, denatured and alkylated prior to enzyme digestion, often with trypsin. Peptide fragments are subsequently resolved using capillary columns and detected using an ion trap mass spectrometer. Sequences are obtained using several software packages such as Sequest/Bioworks and multidimensional protein identification technology [225]. Improvements in mass spectrometric methods and the availability of human sequence databases makes it possible to analyze complex mixtures of peptides in CSF by liquid chromatography coupled to tandem mass spectrometry. The handling of

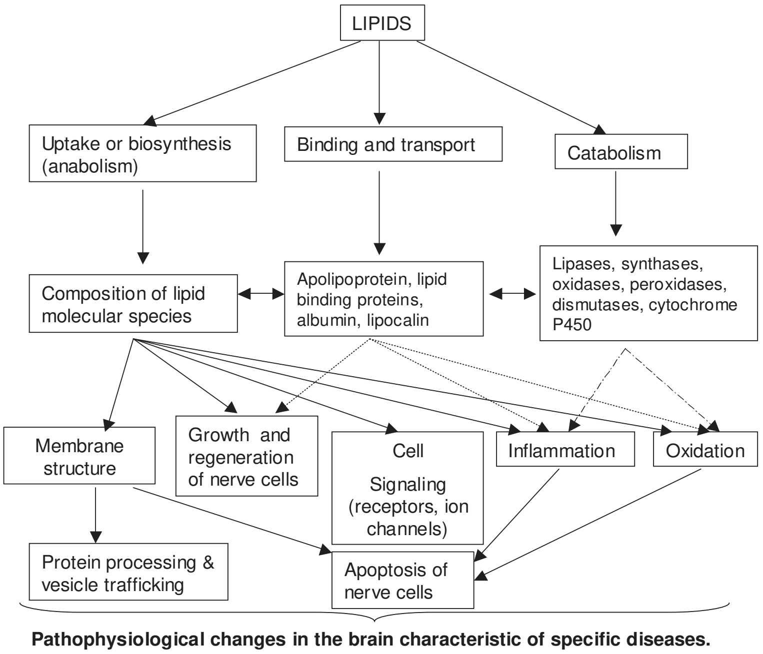


Fig. 9. An overview of lipid metabolism and its pathological significance- A comprehensive analysis of CSF lipids composition by LC tandem MS will reveal how lipids get into the brain, their usage once in the brain and physiologic processes that they influence. Lipid compositional data are complemented by measures of proteins that metabolize lipids in the CSF. This approach will result in a better understanding of the pathophysiology of brain diseases.

data using automated data processing with improved algorithms has increased confidence in the identification of proteins [42,63].

Once both the identities and the levels of CSF proteins have been determined, apparent differences in the proteomes of normal and diseased individuals can be used to explain the mechanisms by which changes in the lipidome occur in disease. Mechanistic changes could be reflected in observed differences in protein abundance (COX, 5-LO, PLA_2, etc.), or variance in the levels of protein activation (e.g., phosphorylation of $cPLA_2$). This combinatorial strategy will be useful in assessing confidence for novel lipid or protein biomarkers and allow for direct comparison between variable levels attributable to chance, diet, genetic variability and environment and changes related to the onset and progression of disease. Furthermore, this strategy will prove to be clinically useful in helping to develop intervention strategies involving drug inhibitor discovery as well as in the rational design of diet/lifestyle changes necessary for disease prevention.

4.1. *Some examples of lipid metabolizing proteins of interest in CSF*

Several 2D gel electrophoresis studies have revealed lipid-metabolizing proteins in human CSF. These proteins may be classified by their functions in biosynthesis, transport or catabolism/degradation of lipids. Our studies show nine different prostaglandin D synthase (PGDS) isoforms, 4 apolipoprotein (ApoA1) isoforms, and 9 apoliprotein J (Apo J) isoforms in CSF [80]. These initial studies are being expanded using 2D-capillary LC in combination with high resolution, high sensitivity linear ion trap mass spectrometry. With this approach, we have been able to increase the dynamic range more than 7 fold such that less abundant proteins not visualized on 2D gels are discovered with high statistical confidence [104].

Lipid metabolizing proteins that we have found in CSF include enzymes with a variety of functions such as: ligases, synthases, transferases, lipid binding proteins, ABC cassette proteins, all major lipoprotein isoforms, lipases, COX, LO, CYP450 enzymes, kinases for choline, ethanolamine, inositol and PI, and receptors for steroids and prostaglandins (unpublished data). Bazan and colleagues, and others have extensively reviewed roles of COX in brain physiology [13,16]. Other studies have shown close association of $cPLA_2$ to amyloid plaques in AD brain and the importance of phospholipases in the brain has been reviewed [47, 70,72,73,201]. The importance of apolipoproteins in lipid transport in the brain has been suggested based on

Table 2
Methods for profiling lipids classes

LIPIDS	Lipidomic procedures
Phospholipids	1) Normal phase HPLC or TLC followed by PLC digestion and derivatization of diglycerides. ([76] and references therein) 2) LC-ESI-MS^2 [102,207] 3) ESI-MS^2 [91,171,198,235] 4) NMR [25,26]
Ceramides, sphingomyelin.	1) LC-ESI-MS^2 [34] 2) ESI-MS^2 [91]
Cholesterol and steroid hormones	1) LC-ESI-MS^2 [91,132,172] 2) GC/MS [187]
Fatty acids and eicosanoids	1) Saponification, derivatization and GC or GC/MS [7,89] 2) LC-ESI-MS^2 [83] 3) LC-APCI-MS2 [83] 4) NMR [107,228]
Triacylglycerides	1) ESI-MS^2 [91] 2) APCI-MS [36]

cell studies showing increased secretion of ApoE and cholesterol by astrocytes over-expressing ApoE [156]. Close association of ApoE4 alleles to late onset AD and epidemiological studies showing a link between high cholesterol to AD has given more credence to a link between ApoE and cholesterol transport [137,150, 156]. However, the functions of other CSF lipoproteins are not known. Given that ApoA1 interacts with the ABC cassette transporter and ATP phospholipid binding proteins to transport cholesterol in cells [223, 237], it is likely that a similar mechanism takes place in the brain. While of great importance to brain lipid metabolism, these can not be fully addressed because of the limited knowledge in the living brain. We will concentrate on our discovery of groups of enzymes that exemplify synthesis and degradation of bioactive lipid molecules. Specifically, we will review platelet activating factor acetylhydrolase and CYP enzyme isoforms that are important in cholesterol, neurosteroids and PUFA metabolism.

4.2. *Platelet-activating factor acetylhydrolase* (*PAFA*)

At the site of inflammation, several cells are recruited to counteract tissue damage. Cells are recruited to these sites by mediators such as platelet-activating factor (PAF). PAF is a phospholipid that is a potent mediator of inflammation. PAF is formed by sequential action of PLA_2 on PC to generate LPC that is subsequently acetylated at the *sn-2* position by acetyltransferase. PAF-like molecules can also be formed by oxidative fragmentation of PUFAs at the *sn-2* position of PC [46,77,97]. PAF receptor antagonists, thromboxane B_2 (TxB_2) and leukotrienes C_4 (LTC_4) reduce PAF activity [69]. The biological properties of PAF are diminished by hydrolysis of the acetyl moiety or the short-chain oxidized esters.

PAFA hydrolysis of the *sn-2* ester bond of platelet-activating factor (PAF) and PAF-like oxidized phospholipid species release an acetate or oxidized moiety and lysophosphatidylcholine, thus attenuating the bioactivity of PAF by reducing its levels. Several PAFA isoforms (1 secreted and 4 intracellular) have been cloned or characterized based on substrate specificity, cellular localization and structure. Isoform1b forms a G-protein-like complex consisting of two catalytic subunits (a1 and a2) and a regulatory subunit (b). Another well-characterized isoform of PAFA consists of a single polypeptide and is homologous to plasma PAFA. Isoform II has anti-oxidant properties and a catalytic triad of amino acids characteristic of most esterases, including the G motif found in most serine esterases and lipases [4,114].

Deficiency in plasma PAFA is associated with stroke, asthma, myocardial infarction, brain hemorrhage and non-familial cardiomyopathy [4,114]. Animal studies and preclinical studies show that recombinant plasma PAFA can prevent inflammation and thus has the potential of controlling human inflammatory diseases [211, 222]. As much as two thirds of PAFA activity is associated with LDL and the remainder is found in HDL particles. Expression of PAFA is regulated by bacterial liposacharides (LPS), cytokines, PAF and the lyso-PAF concentration. PAFA levels are strongly correlated to LDL cholesterol and an increase in PAFA has been shown in essential hypertension, vascular disease, ischemic stroke, diabetes mellitus, rheumatoid and non-rheumatoid arthritis [8,159,211,231]. In other diseases such as asthma, Crohn's disease, sepsis, acute myocardial infarction, multiple organ failure, juvenile rheumatoid arthritis and systemic lupus erythematosis, PAFA has been shown to decrease. Given the proposed role of PAF in plasticity and the role of inflammation in brain diseases, PAFA levels may change with diseases and control of PAFA activity may influence neurological pathologies.

4.3. *CYP450 and brain function*

Cytochrome P450 (CYP) are phase 1 enzymes involved in oxidative activation /deactivation of compounds or toxins [233]. CYPs belong to four major families (CYP1, CYP2, CYP3 and CYP4) that are further subdivided into subfamily members. The liver is a very rich source of CYP [233]. However, whole brain CYP activity is approximately 1% activity of the liver. Specific brain regions or cell types may have higher expression of CYP than others. For example, the major CYP families are localized at the BBB, choroid plexus and posterior pituitary [148,168,197,233]. CYP1 family proteins are often expressed in basal ganglia and cerebellum of human brain. CYP2, especially the D6 subfamily has been mapped to the BBB of human brain, are expressed mainly in arachnoid, choroid plexus and vascular areas as well as in neuronal cells. CYP3 family enzymes are mainly localized in pituitary cells and are probably involved in the regulation of growth hormone.

Higher levels of CYP are associated with oxidative stress. Drugs, xenobiotics and endogenous compounds are known to be substrates of CYP. Endogenous substrates include neurotransmitters such as dopamine, neurosteroids and PUFAs. PUFAs are modified by CYP1A, 2A, 2C, 2D, 2E, 2J and 4A [23,103,131]. Arachidonic acid is converted to epoxygenase metabolites (14,15, 11,12, 8,9, 5,6-epoxyeicosatetraenoic acid, EETs) or ω-terminal hydroxylase metabolites (20, 19, 18, 17, 16 hydroxyeicosatetraenoic acids, HETES) and lipoxygenase-like metabolites (15-, 12-, 9-, 8-, 5 HETES) [45,131,234]. In the brain, EETs are produced by astrocytes close to cerebral microvesicles and are likely involved in control of cerebral blood flow. The pituitary and hypothalamus both produce HETEs that stimulate neuropeptide formation. HETEs are also potent vasoactive agents that modulate normal brain function and are altered in cerebrovascular pathologies.

CYPs are also involved in cholesterol and steroid biosynthesis. Steroid hormones influence brain growth and development [131,144,182,191]. Steroids produced by the adrenal glands and the gonads readily cross the BBB. In addition to steroids synthesized outside the CNS, the brain is the site of synthesis of several neurosteroids that are known to influence its function. Steroids are implicated in behavior, mental illness, activation of the immune system, fatigue, depression, some forms of epilepsy and dementia [131]. The role of CYP family enzymes in PUFA metabolism, steroid biosynthesis and neurotransmitter modification underlines their importance in brain function.

5. Lipid-related mechanisms in neurological diseases

The brain is composed of cells (neurons, astrocytes, glial cells, oligodendrocytes, etc) that communicate via chemical or electrical signals in response to stimulation. These cells are highly shielded to prevent fluctuations in ion or chemical concentrations and are separated from the rest of the body by a blood brain barrier. The cell membranes of cells are critical in maintaining ion and chemical balance in the brain. Therefore, drastic changes in lipid composition may have significant pathological ramifications (Fig. 9).

5.1. *Structure and distribution*

Cell membranes are composed of complex lipids (diacyl glycerophospholipids, sphingomyelin, ether phospholipids, plasmalogens and cholesterol) and contain ion channels as well as several receptors for neurotransmitters, neuropeptides or neurohormones. The complexity of lipids is reflected in important ways. First, lipids are composed of different classes and subclasses (e.g., PC, PE and plasmalogen). Second, each class is further differentiated by the fatty acid content that make up thousands of molecular species. Third, some complex lipids such as glycerophosphatidylinositols are modified by phosphorylation of the inositol base at the 3, 4 or 5 position to generate PIP, PIP_2 or PIP_3 derivatives. Fourth, complex lipids are asymmetrically distributed within cell membranes and lipid composition varies within organelles of the same cell. For example, lipid rafts have been shown to be rich in PI-anchored proteins and cholesterol while PUFAs seem to initially accumulate in the nuclear membrane before being remodeled to organelles [155,227]. Fifth, the difference in distribution is not limited to specific cells but also to specific regions of the brain. Sixth, these complex lipids are subject to constant remodeling of fatty acyl groups and are rapidly degraded by phospholipases (A_2, C or D) to generate several lipid derivatives [14]. This complexity coupled with the fact that lipids interact with the internal and external environment of cells makes it critical that their composition be well controlled. An abnormal lipid composition or distribution within the cell bilayer is likely to influence important physiologic processes associated with ion channels or receptor function. Several brain diseases described in this review display changes in these physiologic processes.

5.2. *Lipids and secretion*

In response to stimulation, some cells secret neurotransmitters, hormones and enzymes to the extracellular space by a regulated process involving the fusion of cytoplasmic organelles to the plasma membrane. Under other conditions, there is a transfer of organelle membrane components to the cell membrane. The former process involving release of contents has recently been termed secretory exocytosis and the latter involving fusion to cell membrane is known as non-secretory exocytosis[43]. Fusion of organelles to plasma membrane may be important in the transfer of membrane proteins to the cell surface or transfer of specific lipid domains to the plasma membrane. Once transferred to the plasma membrane, these proteins serve as either receptors, transporter of ions and metabolites, or are enzymes generating signals that regulate cell growth and function [43,52,55,58,59,74,111].

Other particles variously called endosomes, vesicles or exosomes are also secreted into the extracellular space without releasing their contents [43]. These particles have been characterized in brain where they may be important in transporting metabolites, hormones or enzymes [56,75]. Lipid-rich particles conjugated with various proteins such as HDL and LDL are important in the transport of cholesterol and other lipids between cells and organs. A common feature of these particles, vesicles or exosomes is the involvement of lipids or lipid signaling molecules that are essential components of the particles or are signals that control the secretory process.

An important component in the secretory cascade is the involvement of proteins that generate specific phospholipid moieties needed for secretion. The major proteins involved in synaptic vesicle formation bind major lipids or have activities that modify the phospholipidome. Several classes of proteins known to play important roles in secretion have been characterized. PI kinases generate polyphosphoinositol moieties while lipases form several signaling molecules (Table 1). For example, PIP2 is formed from PI by the action of PI kinases. PIP2 has been shown to be required for synaptic vesicle formation [59]. Another example involves the signaling protein, phospholipase D1 (PLD1) that has been shown to control the secretion of tissue plasminogen activator and thus to facilitate neural outgrowth by generating PA [147]. PA can be further metabolized to lysophosphatidic acid by PLA_2 or to DAG by PLC [51, 236]. PA facilitates membrane fusion events and serves as an anchor for membrane proteins. Likewise, several PI species are important in membrane fusion and in the anchor of membrane bound proteins. Lipid-metabolizing enzymes also initiate signaling cascades within cells by forming co-activators of kinases such as PKC or PKA. Activation of PKC by DAG and the release of intracellular calcium mediated by inositol phosphates are events closely associated with secretion. During secretion, cells have to synthesize, and transport lipids to the plasma membrane. The major substrates of phospholipases are the phospholipid components of plasma membranes that are constantly remodeled by the action of lipases and acyltransferases [5,78,82,84]. The activities of all these enzymes replenish lipid components or aid in the transfer of the correct components to specific domains on cell membranes during the cytosis cascade.

5.3. *Lipids, ion channels and receptors*

In addition to anchoring membrane bound proteins, lipids are required for the functions of ion channels, ion pumps and receptors for several neurotransmitters [71]. Lipids not only provide the proper environment for the assembly of ion channel complexes and the barrier that maintains an ion gradient across the cell membrane, but they are also modulators of these same complexes. For example, AA has been shown to regulate the Na channel and DHA is required for the action of the Na^+ pump [29,118,164]. IP_3 and related molecules are involved in intracellular calcium mobilization. Byproducts of lipids such as eicosanoids induce signaling processes that eventually influence ion channels and ion pumps. Together, these data underscore the role of lipids and proteins that metabolize lipids in regulating important functions of the brain. The proteins that regulate lipid metabolism or the lipid products that they generate may be altered in various pathological conditions and may be useful indicators or biomarkers of diseases. A recent example is temporal lobe epilepsy (TLE), a common form of epilepsy affecting 1–2% of the population where expression of PLD1 has been implicated in tissue plasminogen activator release and neurite outgrowth [119,236].

5.4. Signaling molecules, mediators of inflammation and oxidation damage

Phospholipases and kinases generate several signaling molecules described above. In addition, several oxidized lipids are important in the pathophysiology of brain diseases [14,15,80–82]. Lipids are modified by specific enzymes or by non-specific auto-oxidative processes (Fig. 6). For example, stimulated brain cells release PUFAs such as AA, DHA or EPA that can be converted to eicosanoids or docosanoids by the action of enzymes (COX, LO) [9,14]. Under conditions of oxidative stress, PUFAs are oxidized by reactive oxygen species (ROS) alone or in combination with enzymes to form bioactive signaling molecules known as isoprostanes [30,141,151,154,162,189]. Free PUFA and the concentrations of several oxidized products are known to increase in the brain of AD subjects or subjects with ischemic stroke and PUFA supplementation is neuroprotective [19,109,115]. Phospholipids in lipid bilayers are also subject to enzymes and auto-oxidation. LDL-associated phospholipids are more readily oxidized to form lipids that induce inflammation or can induce apoptosis of cells. Several hydroxylated-derivatives of cholesterol are also formed by specific CYP450 enzymes [131,144]. These hydroxylated or oxidized derivatives of lipids are implicated in neurodegenerative diseases, making it important for the development of sensitive and specific methods to measure products.

Improvements in ionization technology, especially soft ionization methods typified by ESI or APCI have facilitated study of an expanding area of lipidomics dealing with oxidized products (Table 2). This exciting area of research is likely to reveal oxidized lipids that could be biomarkers of biochemical processes (inflammation, immune response, infection, environmental toxicity, etc.) in the CNS. For example, our preliminary studies show a significant increase in oxidized lipid derived products in urine from probable AD (pAD) and PD subjects compared to individuals with no classifiable neurologic disease (N) (Fig. 10). These data were obtained using the less specific thiobarbituric acid (TBAR) assay. Thus, it is not known whether these excreted products are brain-derived or represent a more systemic increase in oxidative stress. Furthermore, the mechanism by which these oxidized lipid products are formed and excreted is not understood.

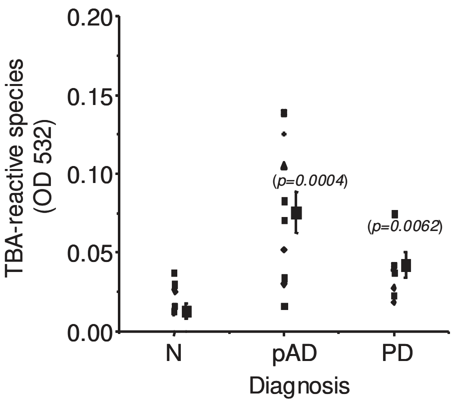


Fig. 10. Thiobarbituric acid-reactive products in urine from normal, AD and PD subjects- Urine samples from normal (N, $n = 9$), probable AD (pAD, $n = 19$) or from PD ($n = 5$) subjects were reacted with TBA. The OD at 532 nm was obtained. The mean OD for each group was obtained and comparison between groups was performed using non-parametric statistics for unpaired data. The p values for pAD versus N and PD versus N are indicated.

We have also used negative ion LC-ESI tandem MS with SRM to examine 40–75 oxidized lipid products (thromboxanes, prostaglandins, leukotrienes, HETES, isoprostanes) and their fatty acid precursors in human CSF. As shown by Fig. 7, several eicosanoids are present in CSF. Total ion monitoring in combination with chromatographic methods (chiral phase chromatography or GC) will likely reveal hundreds if not thousands of isomers derived from many PUFAs in CSF. Overall, our knowledge of lipidomics in combination with free radical chemistry or with proteomics should increase our understanding about the formation of oxidized lipids and their utility as potential disease markers.

6. An overview of brain pathology and lipids

In examining the structure, biosynthesis and importance of lipids in the CNS, several biochemical pathways and mechanisms were proposed for their role in pathophysiology. The major pathways involve lipid biosynthesis, oxidative and structural damage of lipids, lipid transport and formation of signaling molecules (Fig. 9). Lipids from the diet or obtained from *de novo* synthesis are transported and catabolized in the brain. Transport is affected by lipoproteins, lipoprotein receptors, albumin and binding proteins such as PGDS/lipocalin. Lipids are transported to sites where they are needed for nerve cell regeneration or for secretion of transmitters. When the brain is under stress due to infection, trauma or environmental factors, lipids are also metabolized by oxygenases. Simultaneously, lipids that control ion channels, induce inflammation, sleep or control pain may be formed. Lipids can be controlled at the various points of this metabolic chain. Impairment at any level may result in destabilization of metabolic processes manifested by pathologic processes linked to brain diseases. An accurate measure of lipids or proteins that bind/transport or metabolize lipids can help uncover what has precipitated the neurodegenerative processes in the brain.

7. Conclusions

PUFAs, cholesterol, phospholipids and enzymes that metabolize these lipids are important in brain function. Processes that control lipid metabolism are altered in several brain diseases. Measurement of lipid composition and examination of enzyme activity or protein levels may constitute a multiplex approach for discovering how defects in lipid metabolism affect brain physiology. Our studies involving a combination of lipidomics and proteomics of CSF have started to reveal how lipid-related proteins may be linked to disease pathology. Lipids, their oxidized products or signaling molecules are implicated in disease mechanisms (inflammation, oxidation, pain), and physiologic processes (sleep, release of neurotransmitters or induction of signaling cascades). Discovery of lipid pathways and elucidation of mechanisms will provide not only an indication of what may be wrong as a disease process is initiated, but preempt strategies to control, prevent or alleviate symptoms of diseases.

For example, dietary supplements using PUFA-enriched fish oils, antioxidants that prevent oxidation of PUFAs or specific inhibitors of enzymes may be indicated once defects in lipid specific pathways have been discovered.

Acknowledgment

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