Evidence That Myo-Inositol Plus Ethanolamine Elevates Plasmalogen Levels And Lends Protection Against Oxidative Stress In Neuro-2A Cells

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EVIDENCE THAT MYO-INOSITOL PLUS ETHANOLAMINE ELEVATES PLASMALEGEN LEVELS AND LENDS PROTECTION AGAINST OXIDATIVE STRESS IN NEURO-2A CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
By

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I HEREBY RECOMMEND THAT THE DISSEPTION PREPARED UNDER MY SUPERVISION BY Isaie Sibomana ENTITLED Evidence that Myo-Inositol plus Ethanolamine Elevates Plasmalogen Levels and Lends Protection Against Oxidative Stress in Neuro-2A Cells BE ACCEPTED IN PARTIAL FULFILLMENT OF THEREQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy

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Abstract

Sibomana Isaie, Ph.D., Biomedical Sciences Ph.D. Program, Wright State University, 2016. Evidence that myo-inositol plus ethanolamine elevates plasmalogen levels and lends protection against oxidative stress in Neuro-2A cells

Plasmalogens are glycerophospholipids abundant in brain and heart tissues. Evidence suggests that they have antioxidant properties. Studies from our laboratory showed that rats treated with myo-inositol plus ethanolamine (ME) have elevated ethanolamine plasmalogens (PE-Pls) in brain and are protected against phosphine-induced oxidative stress. We hypothesized that ME elevates PE-Pls levels and protects against oxidative stress through oxidation of its vinyl ether bond. We tested this hypothesis in Neuro-2A cell culture and assessed the effects of treatments with myo-inositol (M), ethanolamine (Etn), or a combination (ME) on the: (1) effects on phospholipid (PL) classes, especially Etn PLs; (2) effects on cell viability in response to H₂O₂-induced oxidative stress; and (3) molecular species of Etn PLs preferentially affected by ME and H₂O₂ treatments, especially PE-Pls and their degradation byproducts – lyso-phosphatidylethanolamines (LPE). ³¹P NMR data show that treating the cells with equimolar amounts (500 μM) of M or Etn for 24 h did not influence PL levels, but ME yielded a 3-fold increase in both PE-Pls and PE (p<0.001). Cells exposed to 650 μM H₂O₂ for 24 h decreased cell viability to 53% ± 1.7. While pretreatment with M or ME significantly increased cell survival to 62% ± 1.2 or 80% ± 0.6, respectively (p<0.05), Etn alone had no effect. Mass spectrometry showed that ME preferentially elevated the
levels of PE-Pls species containing saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) by 60%, while PE-Pls containing polyunsaturated fatty acids (PUFA) increased by only 10%. H₂O₂ caused a significant decrease in PE-Pls (27%), producing a 39% increase in LPE and a 4-fold increase in glycerophosphoethanolamine (GPE), but had no impact on PE levels, suggesting that LPE and GPE were primarily byproducts of PE-Pls degradation. Surprisingly, all these effects were blocked by pre-treating cells with ME prior to H₂O₂ exposure. Taken together, these data suggest that a preferential increase in PE-Pls species containing SFA+MUFA in response to ME may protect cells from H₂O₂-induced oxidative stress. The mechanism for this effect is unclear, but further investigations to understand these processes may help to develop neuroprotective approaches to alleviate the progression of neurodegenerative diseases/disorders.
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LIST OF ABBREVIATIONS

AAPE: 1-Alkyl-2-Acyl-sn-glycero-3-phosphoethanolamine
AAPC: 1-Alkyl-2-Acyl-sn-glycero-3-phosphocholine
AAG3P-AT: Alkyl/Acyl-sn-glycero-3-phosphate acyltransferase
LPAAT: Alkylglycerophosphate acyltransferase
ANOVA: Analysis of variance
ARA: Arachidonic acid
BCA: Bicinchoninic acid
cALD: Cerebral adrenoleukodystrophy
CDP-Etn: Cytidinediphosphate-ethanolamine
CID: Collision-induced dissociation
CL: Cardiolipin
ddH$_2$O: Distilled and de-ionized water
DHA: Docosahexaenoic acid
DHAP: Dihydroxyacetonephosphate
DHAP-AP: Dihydroxyacetonephosphate acyltransferase
DHAP-S: Dihydroxyacetonephosphate synthase
AADHAP-R: Acyl/Alkyl dihydroxyacetonephosphate reductase
VLACS: Very long-chain acyl-CoA synthetase
ECT: CTP: phosphoethanolamine cytidylyltransferase
EDTA: Ethylenediaminetetraacetic acid
EK: Ethanolamine kinase
EMEM: Eagle’s Minimum Essential Medium
EPT: Ethanolamine phosphotransferase

ESI-MS/MS: Electrospray ionization tandem quadruple mass spectrometry

Etn: Ethanolamine

eV: Electron-volts

FAR1: Fatty Acyl-CoA Reductase 1

FAR2: Fatty Acyl-CoA Reductase 2

FBS: Fetal bovine serum

Formyl-GPE: 1-Formyl-2-acyl-sn-glycerophosphoethanolamine

GPA: Glycerophosphatidic acid

GPCho: Glycerophosphocholine

GPE: Glycerophosphoethanolamine

H$_2$O$_2$: Hydrogen peroxide

HILIC: Hydrophilic interaction liquid chromatography

LP: Lysoplasmalogenase

LPC: Lysoglycerophosphocholine

LPE: Lysoglycerophosphoethanolamine

LPEAT: Lyso-PE acyltransferase

LPI: Lysoglycerophosphoinositol

M: Myo-inositol

ME: Myo-inositol + Ethanolamine

MUFA: Monounsaturated fatty acid

N2A: Neuro-2A cells

NIPIS: Negative ion – precursor ion scan
NMR: Nuclear Magnetic Resonance
OPLS-DA: Orthogonal Projections onto Latent Structures - Discriminant Analysis
PA: Phosphatidic acid
PBS: Phosphate buffered saline
PUFA: Polyunsaturated fatty acid
PC: Phosphatidylcholine
PC-Pls: Phosphatidylcholine plasmalogens (Choline plasmalogens)
PCA: Principal Component Analysis
PE: Phosphatidylethanolamine
PE-Pls: Phosphatidylethanolamine plasmalogens (ethanolamine plasmalogens)
PG: Phosphatidylglycerol
PI: Phosphatidylinositol
PINLS: Positive ion–mode neutral loss scan
PL: Phospholipids
PLA1: Phospholipase A1
PLA2: Phospholipase A2
PLC: Phospholipase C
PLD: Phospholipase D
PS: Phosphatidylserine
ROS: Reactive oxygen species
SFA: Saturated fatty acid
SM: Sphingomyelin
S.E: Standard error of the mean
STDEV: Standard deviation
TSP: Trimethylsilyl propionate
U: Unidentified
UPLC: Ultra performance liquid chromatography
CHAPTER 1: INTRODUCTION AND SPECIFIC AIMS

1.1 Plasmalogens: Structure and Cellular Role

Plasmalogens belong to the glycerophospholipid group. They possess a vinyl ether bond at the \( sn-1 \) position of their glycerol backbone (Fig. 1). This bond is a unique physical and chemical characteristic feature that distinguishes them from other phospholipids. The bond links the fatty acyl chain at this position to the glycerol backbone. The \( sn-1 \) position of plasmalogens usually contains a fatty acid chain of C16 or C18 length and the vinyl ether double bond represents always the \textit{cis}-isomer (1). The \( sn-2 \) position of plasmalogens generally contains docosahexanoic acid (DHA) or arachidonic acid (ARA), which are the reservoirs for the second messengers (docosanoid and eicosanoid molecules) (2, 3).

Plasmalogens are found in mammalian tissues and are highly abundant in heart and brain (3). The predominant types of plasmalogens are choline plasmalogens (PC-Pls) and ethanolamine plasmalogens (PE-Pls), which contain choline or ethanolamine as the polar phospho-base headgroup at the \( sn-3 \) glycerol position. PE-Pls constitutes nearly 90 mol\% of ethanolamine glycerophospholipid pool (4) and 22\% of the phospholipids in human brain (5). Plasmalogens are not only the reservoirs for second messengers and structural components of the cell membrane (6), they are also involved many cellular processes such as in ion transport (3), cholesterol efflux (7, 8), membrane fusion (9), as
well as suppressing neuro-inflammation (10-12) and starvation-induced cell apoptosis (13, 14). Experimental evidences suggest that they have antioxidant properties (15-20). Studies have shown that oxidative stress plays a significant role in the pathogenesis of neurodegenerative diseases/disorders (21, 22). An association between a decrease in PE-Pls and the severity of these diseases/disorders has also been documented (23, 24). A systemic administration of PE-Pls can abolish lipopolysaccharide-induced neuroinflammation and β-amyloid protein accumulation in the mouse brain (10, 11). Additionally, rats treated with myo-inositol plus ethanolamine (ME) yield an increase in PE-Pls levels in brain and these rats are protected against phosphine-induced oxidative stress (25, 26). However, how ME and oxidative stress-inducing agents affect the molecular species of ethanolamine phospholipids, especially PE-Pls in neural cells remains unknown. This information can lead to a neuroprotective approach to a wide spectrum of stressors on brain and cognitive function, enhancing brain resiliency. The present study was designed to address this question using a neuronal cell (Neuro-2A cell line) culture system.

1.1 SPECIFIC AIMS

**Aim 1.** Measure the phospholipid contents of Neuro-2A cells, with specific interest in PE-Pls. This aim is designed to assess if neuro-2A cells synthesize PE-Pls.

**Aim 2.** Examine the effects of myo-inositol alone, ethanolamine alone and myo-inositol plus ethanolamine treatments on the level of myo-inositol, phosphoethanolamine and different phospholipid classes with special interest in PE-Pls in Neuro-2A cells. The
The purpose of this aim is to assess if these treatments induce an increase in myo-inositol, phosphoethanolamine and phospholipids with a special interest in PE-Pls in the cells.

**Aim 3.** *Examine the effects of myo-inositol alone, ethanolamine alone and myo-inositol plus ethanolamine treatments on Neuro-2A cell protection against hydrogen peroxide-induced oxidative stress.* This aim is intended to assess if cells that contain elevated levels of PE-Pls also show protection against oxidative stress as it has been reported in a rat model *in vivo* (26).

**Aim 4.** *Examine the molecular species of ethanolamine phospholipids that are preferentially affected by myo-inositol plus ethanolamine and H₂O₂ treatments, with a special interest in PE-Pls species and their degradation by-products (lysglycerophosphoethanolamines and glycerophosphoethanolamines).* This aim is designed to determine the molecular species of ethanolamine phospholipids that are preferentially affected by ME treatment and H₂O₂-induced oxidative stress.
CHAPTER 2: BACKGROUND

2.1 Phospholipids in Mammalian Cells

The improvement of the analytical tools used for structural characterization of lipids and their biosynthetic enzymes in the cells has greatly advanced our understanding on the diversity of the cellular membrane lipids (27, 28). The membranes of mammalian cells are complex and highly structured. They consist of hundreds of different phospholipid species, many different proteins, a variety of glycolipid molecules and cholesterol (29). The most abundant lipids in the membranes belong to the phospholipid group (30). Phospholipids play a crucial role in membrane functions. These include giving the membrane unique characteristics for proper function such as membrane fluidity, permeability, local curvature, molecular packing or hydration, charge, and ability to regulate the activities of various membrane-bound enzymes and ion-channels (31, 32). Although all membranes do not contain the same amount of each phospholipid class, the ratios between phospholipid classes and their molecular species can serves as a signature for various organelles (33, 34).
The chemical structure of glycerophospholipids consists of the glycerol backbone, which contains fatty-acyl chains esterified at \textit{sn}-1 and \textit{sn}-2 positions and a phosphate ester group at the \textit{sn}-3 position attached to one of a variety of head groups (Fig. 2). A variety of chemical moieties can serve as head groups, thus leading to a number of different phospholipid classes (phosphatidyl lipids) such as phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI), and cardiolipins (CL) \citep{29,30,35,36}. Brain phospholipids are highly heterogeneous with a wide range of molecular species that result from the combination of various fatty acyl chains at the \textit{sn}-1 and \textit{sn}-2 positions of their glycerol backbone \citep{37}. For instance, subclasses for ethanolamine phospholipid class include 1-acyl-2-acyl-\textit{sn}-glycero-3-phosphoethanolamine (phosphatidylethanolamine; PE), 1-alkyl-2-acyl-\textit{sn}-glycero-3-phosphoethanolamine (plasmanylethanolamine; AAPE) and 1-alkenyl-2-acyl-\textit{sn}-glycero-3-phosphoethanolamine (plasmenylethanolamine or phosphatidylethanolamine plasmalogen; PE-Pls) (Fig. 3). The only feature that distinguishes these subclasses is the type of linkage that attaches the acyl chain to the glycerol backbone at the \textit{sn}-1 position. This linkage involves an ester, ether and a vinyl ether bond for PE, AAPE and PE-Pls respectively \citep{6} (Fig. 3). Any phospholipid that
bears a vinyl ether linkage at the sn-1 position of the glycerol backbone is termed “plasmalogen” (6, 38). In eukaryotes, the acyl chain in sn-2 position is always linked to the glycerol moiety via an ester linkage (36, 38). Each of the PL subclasses is even further subdivided into various molecular species based on the characteristics of the acyl chains attached to sn-1 and sn-2 positions of their glycerol backbone (species from the same class contain a similar head group but differs in respect to their acyl chains). In plasmalogens, the fatty acyl chain at the sn-1 position is typically C16:0, C18:0 or C18:1, while that in the sn-2 position is commonly polyunsaturated (PUFA) and is mostly occupied by docosahexaenoic acid (DHA) or arachidonic acid (ARA) (3, 5, 39-41).

2.2 Plasmalogens in Neural Membranes

Plasmalogens are very unique types of phospholipids due to the vinyl ether bond linking the fatty acyl chain to the glycerol backbone at the sn-1 position (6, 38). At this position, they display a cis double bond on the fatty acyl chain, just adjacent to the ether linkage (6, 38). This bond is believed to confer special physical and chemical properties to plasmalogens, and to the cell membranes that harbor them (42). In the membrane, plasmalogens have a tendency of packing more closely with one another, thus reducing the membrane fluidity and passive ion permeability (43). It has also been reported that plasmalogens possess a property of forming an inverse hexagonal phase, a property that is important during membrane fusion processes (44).

Plasmalogens were initially discovered by Feulgen and Voit in 1924 and described as fuchsine-sulfurous acid staining compounds derived from tissue sections treated with acid or mercuric chloride as a part of a method routinely used to stain the
nucleus (6). The breakdown of plasmalogens through the oxidation of their vinyl ether linkage yielded aldehydes that reacted with fuchsine-sulfurous acid stain, giving rise to colored compounds inside (cytoplasm) of the cells. The term “plasmal” was then used to describe the aldehydes present in the plasma (cytoplasm) of the cells and the compounds that gave rise to these aldehydes were termed “plasmalogens” (6).

In mammals, the abundance of plasmalogens varies depending on the type of cells and tissues. They account for 4-32% of the total phospholipid mass (45). They are widely distributed throughout human and animal tissues, averaging about 18% of the total phospholipid contents in human body and 3.5% in human serum (46). They are mostly abundant in circulating immune cells, heart, lung, brain and neural tissues (3, 45). The main types of plasmalogens are choline plasmalogens (PC-Pls) and ethanolamine plasmalogens (PE-Pls) (47). PE-Pls constitute nearly 90 mol percent of myelin ethanolamine phospholipids (4) and over 50 mol percent of these phospholipids in neurons (4). The predominant fatty acyl chains attached to sn-1 position of plasmalogens in mouse and human brain are usually C16:0, C18:0 and C18:1, accounting for 98-99% of the acyl chains at this position (48-50). Phospholipids are asymmetrically distributed in neural membranes across the plane of the plasma membrane (51). Ethanolamine phospholipids and phosphatidylserine are localized in the inner leaflet of the membrane, whereas choline phospholipids and sphingomyelin are more concentrated in the outer leaflet of the membrane bilayer (32, 52). This distribution appears to be stable, as it has been shown that when ethanolamine phospholipids and phosphatidylcholine are placed in the outer leaflet of the membrane, the neural aminophospholipid translocase (flippase) is able to restore their normal distribution (53, 54). Phospholipid constituents of the neural
membranes are rich in PUFA (51, 55). The number of double bonds in the aliphatic groups at the sn-1 and sn-2 positions of these phospholipids is a crucial factor in dictating the phase transition temperature of membranes and their lateral diffusion velocity, thus playing a major role in cellular processes such as endocytosis and exocytosis, bilayer thickness, sorting of lipids and membrane fusion (51, 55).

### 2.3 Biosynthesis of Ethanolamine Plasmalogens in Brain

The enzymes involved in plasmalogen biosynthesis in brain tissues have not yet been purified and characterized. This is due to the heterogeneity and complexity in organization of brain tissue, low activity of these enzymes as well as tedious and time consuming assays required for characterization of their activities (55). However, it has been reported that the pathway leading to the biosynthesis of plasmalogens in brain tissues is similar to that previously described from other tissues (56). Several literature reports describing this pathway in non-neural tissues are now available (5, 6, 39, 40, 57). All the steps involved in plasmalogen biosynthesis are shown in Fig. 4 and the topology of the enzymes that carries out each step is shown in Fig. 5. The first three enzymes that initiate

![Figure 4. Schematic representation of the pathway for plasmalogen biosynthesis. See text for nomenclature.](image-url)
this process are located in the peroxisome, whereas enzymes that complete the process are located on the endoplasmic reticulum (ER) (Fig. 5) (5, 40, 55). The first step is carried out by dihydroxyacetone phosphate acyltransferase (DHAP-AT; EC 2.3.1.42; (58)) and involves esterification of dihydroxyacetone phosphate (DHAP) at the sn-1 position with a long chain (> C10) acyl-CoA ester to form 1-acyl-DHAP (5, 6, 39, 57). The source of DHAP in peroxisomes is unknown. However, DHAP can either be generated inside the peroxisomes from glycerol-3-phosphate via the activity of glycerol-3-phosphate dehydrogenase or transported from the cytosol (59, 60). Characterization and activity of DHAP-AT have been reviewed (6). In the next step, Alkyl-DHAP synthase (ADHAP-S; EC 2.5.1.26; (61, 62)) introduces an ether bond at the sn-1 position by replacing the sn-1 fatty acid with a long chain fatty alcohol and yielding 1-alkyl-DHAP (5, 6, 39). The source of fatty alcohol for this reaction includes dietary intake (39) and an action of fatty acyl CoA reductase 1 and 2 (FAR 1/FAR 2) catalyzing a reduction of the long chain acyl-CoAs (63, 64). Honsho et al. (63) have demonstrated that FAR 1 enzyme catalyzes the rate limiting step in the biosynthesis of plasmalogens. Cheng and Russell (64) have shown that the preferred substrates for FAR1 are saturated and unsaturated fatty acids of 16 or 18 carbons, while FAR2 only prefers reducing the saturated fatty acids of 16 or 18 carbons. The third step in plasmalogen biosynthesis is catalyzed by acyl/alkyl-DHAP reductase (AADHAP-R; EC 1.1.1.101; (65, 66)) enzyme and involves a reduction of the ketone group at the sn-2 position of 1-alkyl-DHAP to form 1-alkyl-2-lyso-sn-glycero-3-phosphate (1-alkyl-sn-G3P) (5, 6, 39). DHAP-AT and ADHAP-S enzymes are exclusively intraperoxisomal enzymes facing the peroxisomal lumen (39, 62, 67, 68), while the fatty acyl CoA reductase and AADHAP-R enzymes are bound to
the membrane of peroxisomes on the side facing the cytosol (39) (Fig. 5). The implication of this enzyme setting in peroxisomes is that the supply of the acyl CoA and DHAP to DHAP-AT must either be generated inside the peroxisomes or transported from the cytosol into peroxisomes (39). For DHAP-AT to re-use the fatty acid released by ADHAP-S reaction, it must be re-activated to its CoA-ester form. It has been shown that peroxisomes possess acyl CoA synthetase also known as very long-chain acyl-CoA synthetase (VLACS), which has the ability to activate a wide range of fatty acids, including those released by ADHAP-S (69, 70). However, it has been reported that AADAHP-R has a dual distribution, in peroxisomes and ER (65, 71, 72). However, the localization of AADAHP-R seems to have less impact on its activity since in human and rodent cells defective in peroxisome assembly, this enzyme displays a normal level of activity (73-75).

Further steps in plasmalogen biosynthesis take place in ER. First, an acyl chain is esterified at the sn-2 position of 1-alkyl-sn-G3P by an alkyl/acyl-glycero-3-phosphate acyltransferase (AAG3P-AT; EC 2.3.1.51; (76-78)), yielding 1-alkyl-2-acyl-sn-glycero-3-phosphate (5, 39). It has been demonstrated that this enzyme and the one

**Figure 5.** Topology of the peroxisomal enzymes involved in the biosynthesis of plasmalogens. (1) DHAP-AT; (2) ADHAP-S; (3) acyl CoA reductase (long-chain alcohol-forming); (4) glycerol-3-phosphate dehydrogenase; (5) AADAHP-R; (6) peroxisomal membrane protein; (7) VLACS [39]. See text for nomenclature and abbreviations.
that attaches an acyl chain at the sn-2 position of 1-acyl-glycero-3-phosphate are different (79, 80). Substrate specificity studies carried out to assess the selectivity of AAG3P-AT also known as alkylglycerophosphate acyltransferase (LPAAT) on different acyl-coA species (16:0, 18:0, 18:2, 20:4, 22:4, 22:6) have shown that its specificity for acyl chains depends on the concentration of 1-alkyl-sn-glycero-3-phosphate (its substrate) (79). This enzyme prefers using polyunsaturated acyl-CoA over saturated species when 1-alkyl-sn-glycero-3-phosphate is available at low concentrations (79). The removal of the phosphate group is catalyzed by phosphatidic acid phosphohydrolase (PH; EC 3.1.3.4) and forms 1-alkyl-2-acyl-sn-glycerol (5, 39). Subsequently, ethanolamine phosphotransferase (EPT) (EC 2.7.8.1) uses cytidinediphosphate-ethanolamine (CDP-ethanolamine) and 1-alkyl-2-acyl-sn-glycerol as substrates in the presence of magnesium to synthesize 1-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine (1-alkyl-acyl-GPE) (39, 81). The last step in PE-Pls synthesis involves the dehydrogenation at the C1 and C2 position of the alkyl group in 1-alkyl-acyl-GPE and this process is catalyzed by a cytochrome b5-dependent microsomal electron transport system and a plasmenylethanolamine desaturase (Δ1’-desaturase; EC 1.14.99.19) enzyme (5, 6, 39).

There is no plasmenylicholine desaturase enzyme for PC-Pls synthesis (82, 83). PC-Pls is synthesized via from PE-Pls by either a base exchange or by a removal of ethanolamine phosphate by phospholipase C (PLC) and by choline phosphotransferase using CDP-choline (82, 84, 85).

CDP-ethanolamine is one of the substrates in the Kennedy pathway leading to the synthesis of phosphatidylethanolamine (PE) (86, 87) (Fig. 4). Ethanolamine kinase (EK) catalyzes the initial reaction which involves the ATP-dependent phosphorylation of
ethanolamine to form phosphoethanolamine and ADP as products (88). The second step, which is a rate-limiting step in this pathway requires the CTP:phosphoethanolamine cytidylyltransferase (ECT) to combine phosphoethanolamine and CTP to form a high-energy donor CDP-ethanolamine and produces pyrophosphate as a byproduct of the reaction. In the last step, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT) combines CDP-ethanolamine with diacylglycerol to generate PE and releases cytidinemonophosphate-ethanolamine (CMP) as a byproduct (88). Both PE and PE-Pls species requires the availability of CDP-ethanolamine moiety and EPT enzyme for their de novo synthesis.

2.4 Phospholipid Homeostasis in Neural Membranes

Brain tissues actively and constantly catabolize phospholipids (51). Maintaining a phospholipid homeostasis in mammalian cells is a gigantic task because of the great number of different molecular species of phospholipids in cell membranes and various biological processes such as biosynthesis, degradation, remodeling and interorganelle trafficking of these molecules (29). Establishing homeostasis requires a balance between phospholipid catabolism, re-synthesis via re-acylation, and de novo synthesis pathways (52). In all these processes, the brain spends about 20% of its entire ATP pool (89). Various forms of phospholipase and lysophospholipase enzymes have been purified and characterized from brain tissues (51, 90). These include phospholipases A₁, A₂, C, D and lysophospholipase A 1 / 2 (90-99). These enzymes are grouped in two categories. Those that hydrolyze the acyl groups (hydrolases) and those that cleave the phosphodiester bond (phosphodiesterases). The hydrolases include phospholipase A₁ (PLA₁), phospholipase A₂ (PLA₂), phospholipase B (PLB), and lysophospholipase A 1 / 2 (Lyso PLA 1 / 2) (99).
Phosphodiesterases consist of phospholipase C (PLC) and phospholipase D (PLD) (99). PLA₁ (EC 3.1.1.32) catalyzes exclusively the hydrolysis of the ester bond at the sn-1 position, thus yielding a free fatty acid and a 2-acyl lysophospholipid (51, 99). PLA₂ (EC 3.1.1.4) catalyzes the hydrolysis of the acyl ester bond at the sn-2 position, thus forming a free fatty acid and a 1-acyl lysophospholipid (51, 99), which can be re-acylated by acyl-CoA in the presence of acyltransferase enzyme (51). Lysophospholipids can also be further hydrolyzed by lysophospholipases to generate free fatty acids and glycerophosphobases (EC 3.1.1.5) (96, 99-101). PLC (EC 3.1.4.11) catalyzes the hydrolysis reaction of the phosphodiester bond at the sn-3 position that yields the 1,2-diacylglycerol and a phosphobase, whereas PLD (EC 3.1.4.4) removes a head group from a glycerophospholipid to form a phosphatidic acid and a free base (51, 99).

Phospholipids in neural membranes undergo a rapid de-acylation–re-acylation process that involves activities of phospholipases and acyltransferases (51, 102, 103). The de-acylation–re-acylation cycle continuously shuttles fatty acyl chains between molecular species within different phospholipid subclasses, thus introducing PUFAs into these subclasses (51). For instance, when a PLA₂ hydrolyzes a phospholipid, a resulting lysophospholipid molecule is rapidly re-acylated with a different fatty acid (51, 102, 103). In fact, this cycle is an important mechanism for neural cells to control the saturated and PUFAs esterified to phospholipids in their membranes (104).

2.5 Role of Plasmalogens in Neurological Diseases/Disorders

In addition to being the primary constituents of cellular membranes and acting as the binding sites for a variety of intracellular and intercellular proteins, a number of
phospholipid molecules also serve as the precursors of the second messengers in eukaryotic cells (105). Several investigators have reported that the sn-2 position of plasmalogens acts as a reservoir of neuroprotective DHA (5, 39, 106) or ARA, the storage depots for second messengers such as docosanoid and eicosanoid molecules (2, 3). Alterations in cellular levels of plasmalogens have been associated with various pathologies such as chronic obstruction pulmonary disease (107), rhizomelic chondrodysplasia punctate (108, 109), asthma (19), bronchopulmonary dysplasia (110), and neurodegenerative diseases/disorders (111-118).

Since brain tissues are rich in plasmalogens, it is not surprising that a deficiency in brain plasmalogens can be linked to various types of neurodegenerative diseases/disorders (5). Although the physiological role of plasmalogens is not fully understood, they are required for normal development of the central nervous system (CNS) (51, 55). The significance of plasmalogens in neuropathologies is highlighted by the findings that neurodegenerative disorders/diseases are associated with a decrease in PE-Pls levels (23, 24, 119). These include cerebro-hepato-renal (Zellweger) syndrome where a lack of peroxisomes (cellular organelles involved in plasmalogen biosynthesis) leads to impaired cerebellar development and neuronal cell death (119, 120), impaired neurotransmission (23), aggravation of brain injury (121), and progression of diseases such as Alzheimer disease (111), and chronic bronchopulmonary dysplasia (110), Parkinson’s disease (112), experimental autoimmune encephalomyelitis (113), and Niemann Pick type C disease (122). However, it is not yet known if the cellular deficiency in plasmalogen levels is a direct cause or a downstream effect of pathology (5). There is evidence that supports both relationships. For instance, Goodenowe and
colleagues (111) investigated the relationship between serum PE-Pi levels, dementia severity and pathology of Alzheimer’s disease. Their findings indicate a correlation between PE-Pi levels and severity of Alzheimer’s disease. It has also been shown that the affected brain regions of Alzheimer’s disease patients are deficient in PC-Pi and PE-Pi contents (115, 116). Moreover, there is evidence to suggest that the severity of the disease correlates with the magnitude of the deficiency (115, 116). Cerebral adrenoleukodystrophy (cALD), the most common peroxisomal disorder that severely affects the myelin in the CNS (114) has also been found to correlate with a decrease in PE-Pi (123).

It is probable that a decrease in plasmalogen contents in the brain is related to their degradation by reactive oxygen species (ROS) (5). Khan and colleagues (123) demonstrated that reduced levels of PE-Pi lead to an accumulation of ROS in human cALD brain tissues and a transformation of a metabolic disorder into a fatal neuroinflammatory disease. Additionally, it has been shown that oxidative stress plays a significant role in the pathogenesis of Parkinson’s disease, a neurogenerative disorder of the CNS (21, 22, 124). The mechanisms underlying Parkinson’s disease involve activation of microglial cells which produce nitric oxide and superoxide during neuroinflammatory responses (125, 126). Gaucher’s disease reflects another type of pathological conditions characteristic of the reduction in plasmalogen levels (117, 118). Moraitou and colleague’s study (117) demonstrated that the red blood cell membranes from Gaucher’s disease patients exhibit significant reduction in plasmalogen contents for both C16:0 and C18:0 fatty acyl chains at the sn-1 position. This reduction strongly
correlates with the disease severity. All these neurogenerative diseases have in common a reduction in cellular plasmalogen contents probably due to oxidative stress.

2.6 Antioxidant Properties of Plasmalogens

Cells generate a small amount of free radicals such as superoxide (O$_2^-$) or hydrogen peroxide (H$_2$O$_2$) during their normal biological processes (57, 127-129). The production of free radicals can be initiated extracellularly and intracellularly (57). The presence of these free radicals in the cells together with trace amounts of transition metals such as Fe$^{2+}$ and Cu$^{2+}$ can lead to production of hydroxyl radicals (HO$^-$), a highly reactive radical species (130, 131). A single cell can produce about 50 hydroxyl radicals every second, thus generating 4 million of this species a day (132). Hydroxyl radicals can attack biomolecules located in less than a few nanometers from their site of production (133). Phospholipids in the cell membrane bilayer are the main targets of these free radicals (57) (Fig. 6). Oxidized lipids can also exist in radical forms, thus attacking other molecules in a step known as propagation of chain reactions which can proceed through tens to thousands of molecular intermediates (57, 129). The propagation phase is terminated by the action of antioxidants forming non-radical products (129, 134). The impact of lipid

![Figure 6](image-url)
oxidation on cell membranes and physiological processes of cells have been extensively described in several reviews (135-138).

Of all brain cells, neuronal cells are particularly vulnerable to oxidative injury because they contain high levels of PUFAs (51) and don’t possess sufficient reduced glutathione (139). Under normal conditions, ROS are involved in maintaining cellular “redox homeostasis” aimed at protecting cells against excessive oxidative stress (128). However, elevated levels of ROS inflict damage to neural membranes, changing not only their fluidity (140), but also decreasing activities of other membrane actors such as membrane-bound enzymes, ion channels, and receptors (141). It has also been reported that they are capable of inducing alteration in cell signaling (142, 143) and surface charge (51).

There is a growing support for the hypothesis that plasmalogens are natural antioxidants that act as scavengers of free radicals in tissue and plasma (15-20). The
The protective role of PE-PLs serving as antioxidant against free ROS has been documented (16, 144). Studies have demonstrated the sensitivity of plasmalogen’s vinyl ether bond to a variety of oxidative agents (39, 40, 145, 146). The vinyl ether linkage is more susceptible to oxidation than the corresponding ester bond linking the phospholipid moieties to their glycerol backbone (3, 5, 39-41). This bond is more preferentially affected by reactive chlorinating species as compared to the olefinic residues in sn-2 position of diacyl-phospholipids that are embedded in the hydrophobic domain of cell membranes (19, 147, 148). This bond may be serving as a sacrificial trap for ROS that would otherwise cause oxidative damage to membrane lipids (15, 17) (Fig. 7). Another explanation for the susceptibility of the vinyl ether bond to oxidative attack is inherent to its location in the cell membrane. The enol ether double bond is exposed to the hydrophilic domain of the membrane and its sensitivity to attack by oxygen radicals is associated with an enhanced electron density donated by oxygen to the vinyl ether bond (148). Additionally, the hydrogen atoms attached to the carbon atom adjacent to the vinyl ether substituent exhibit a relatively low bond dissociation energy, which makes the vinyl ether moiety a suited target for ROS (57).

The sensitivity of a vinyl–ether bond to oxidative attack (15-20, 145, 146) has reinforced the hypothesis that plasmalogens are natural antioxidants. It has been suggested that plasmalogens may be acting on a wide range of free radicals such as ROS (149) and iron-induced peroxidation (150). The peroxidized phospholipids in neural membranes can result in a membrane-packing defect, thus, facilitating PLA2 access to the sn-2 ester bond for hydrolysis (51). In fact, it has been shown that peroxidized phospholipids are the better substrates for PLA2 compared to native phospholipids (151).
This has led to a suggestion that cytosolic PLA$_2$ (cPLA$_2$) is involved in repairing and restoring the physiological physicochemical state of neural membranes (51). Other studies have demonstrated that cPLA$_2$ preferentially hydrolyzes unoxidized over oxidized arachidonic acid (152), suggesting that cPLA$_2$ is not involved in cell membrane repair function (153). Besides sacrificing their vinyl ether bonds during free radical attack, it has also been shown that plasmalogens can block the iron-mediated peroxidation of PUFAs (150) and copper-induced oxidation of low-density lipoproteins (144, 154).

Luoma et al. (12) have shown that plasmalogens protect internodal myelin of mice against oxidative damage. The findings that an increase in PE-Pls level in rat brain is protective against phosphine-induced oxidative damage (26) suggest that PE-Pls may be a useful target in addressing health issues that are associated with plasmalogen deficiency. Mechanisms for enhancing the baseline level of PE-Pls in the cells and restoration to their normal level are needed.

2.7 Goal of the Present Study

The goal of this study was to (1) assess if ME treatment increases ethanolamine plasmalogens in N2A cells and provides protection against H$_2$O$_2$-induced oxidative stress, and (2) determine the molecular species of ethanolamine phospholipids (PE-Pls, PE and LPE) that are preferentially affected by these treatments. It has been shown that rats treated with myo-inositol plus ethanolamine yield an increase in PE-Pls level in the brain (25, 26, 155) and these rats are protected against phosphine-induced oxidative stress (26). However, the relationship between the level of PE-Pls and cell protection against oxidative stress is not known. It is likely that PE-Pls exerts a control on oxidative stress
through the oxidation of vinyl ether linkage. We used a N2A cell culture system to investigate the impact of ME ethanolamine phospholipid species, with a special interest in PE-Pls species and how they are affected by H₂O₂-induced oxidative damage. These studies will enhance our understanding on the relationship between ethanolamine phospholipid species, PE-Pls species in particular, and cell’s response to H₂O₂-induced oxidative stress. This research may lead to a neuroprotective approach to a wide spectrum of stressors on brain and cognitive function, enhancing brain resiliency.

2.8 Hypothesis and Strategy

It has been shown that the vinyl-ether bond of plasmalogens is highly susceptible to oxidation and can serve as a sacrificial trap for reactive oxygen species (15-20). These findings have led to a suggestion that plasmalogens possess antioxidant properties. The findings reported by Kuczynski and Reo (26) raise the possibility that the vinyl-ether linkage of PE-Pls is involved in rat brain tissue protection against phosphine-induced oxidative damage. I, therefore, hypothesized that myo-inositol plus ethanolamine enhances ethanolamine plasmalogen (PE-Pls) synthesis which in turn, protects the cells against oxidative stress through oxidation of its vinyl ether bond, thus serving as a sacrificial trap for oxidants that could otherwise damage cells.

My strategy was to test this hypothesis using a Neuro-2A cell (murine neuroblastoma cell line) culture system. These cells have been used extensively in screening novel compounds for neurotoxic properties and associated mechanisms (156). They have also been used as a model system in mechanistic studies of plasmalogen-induced neuronal protection against apoptotic stimuli (13). Hossain et al. (13) have
demonstrated that treating N2A cells with a mixture of plasmalogens (consisting of 96.5% ethanolamine plasmalogens and 2.5% choline plasmalogens) at concentrations of 5 μg/ml and 20 μg/ml abolishes serum starvation-induced cell death, enhances phosphorylation of AKT and ERK 1/2, which are known to inhibit the cleavage of pro-apoptotic caspase-9 and caspase-3 (157-159) and blocks the activity of these caspases. N2A cells were also used to study the cell viability to ascorbic acid and H2O2 (160). The estimated LC50 for H2O2 was above 400 μM (160). This cell culture system was, therefore, a suitable in vitro system to use in testing my hypothesis. I also used H2O2 to induce oxidative stress in N2A cells. Similarly, Hardway et al. (160) and other investigators have used H2O2 in various studies to induce oxidative stress in cells (161-164). NMR spectroscopy and mass spectrometry analyses of cell extracts were performed to elucidate the effects of ME and H2O2 treatments on phospholipid profiles in general and more specifically on molecular species of PE-Pls, PE and LPE in N2A cells.
CHAPTER 3: METHODS

3.1 Nuclear Magnetic Resonance (NMR) Analyses

3.1.1 Cell line and culture conditions

Mouse neuroblastoma derived cells (Neuro-2A) were maintained in a growth medium consisting of Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). They were cultured in a humidified chamber provided with an atmosphere of 95/5% O\textsubscript{2}/CO\textsubscript{2} at 37 °C. Cells used in experiments were seeded in T-75 cm\textsuperscript{2} flasks (three flasks/group) at a starting density of 2 x10\textsuperscript{6} cells per flask in a total medium volume of 15 ml. After two days, the growth medium was discarded and cells were washed twice with 4 ml of PBS, and then supplied with 15 ml of fresh growth medium pre-warmed to 37 °C.

3.1.2 Neuro-2A cell treatment with myo-inositol +/- ethanolamine

At 80% confluency, cells were washed twice with 4 ml of PBS and supplied with either growth medium, medium supplemented with 500 μM myo-inositol (M), medium supplemented with 500 μM ethanolamine (Etn), or growth medium supplemented with 500 μM myo-inositol + 500 μM ethanolamine (ME) for 24 h. Lipids were extracted from cells as described below and an aliquot of the sample was taken for protein determination (see below). Working stocks of Etn, M and ME were always prepared fresh using growth medium.
3.1.3 Neuro-2A cell treatment with hydrogen peroxide

Dose-response assays were carried out to determine a concentration that results in approximately 50% cell death following a 24 h exposure to hydrogen peroxide (H$_2$O$_2$). Cells were grown in T-75 flasks (three flasks/group) as described above. Treatment was initiated after the cells reached 80% confluency. After washing cells twice with 4 ml of PBS, they were then supplied with either growth medium alone (control), or medium supplemented with various concentrations of H$_2$O$_2$. At 24 h, cell viability in each group was determined using the trypan blue staining method (see details below). A concentration that yields about 50% cell viability was chosen for subsequent H$_2$O$_2$ treatment experiments. A working stock solution of H$_2$O$_2$ was always prepared fresh using growth medium.

3.1.4 Evaluation of cell viability

Cell viability was assessed using the trypan blue staining method. Briefly, after discarding the growth medium, cells were washed twice with 4 ml of PBS. A volume of 2 ml of trypsin-EDTA solution was placed in each T-75 cm$^2$ flask. The flask was subjected to a gentle rotation to ensure that the trypsin-EDTA solution covers the monolayer of cells. Cells were then incubated at 37° C for 3-5 minutes to allow cell detachment from the surface of the flask. Detached cells formed a sheet and the trypsin-EDTA became cloudy. Once about 50% of the cells had been detached and are floating in trypsin-EDTA solution, the flask was shaken gently to release any remaining attached cells from the flask surface. The cells were then re-suspended in 6 ml of fresh growth medium to inactivate trypsin. Clumps or sheets of cells were disaggregated by trituration with a 10
mL pipet. A volume of 10 μL of cells was mixed with 10 μL of 0.4% trypan blue dye (cell/trypan blue ratio of 1:1) in a 96-well plate. Then 10 μL of this mixture was pipetted into a counting chamber slide (Countess®; Invitrogen) to completely fill the chamber. The slide was inserted into the automated cell counting machine (Countess®) to determine the number of live cells and dead cells. The Countess® machine counts the cells that are stained blue as dead cells, while those without dye are counted as live cells. The output of Countess® includes a total cell count/ml, live cells/ml, dead cells/ml as well as the percentage of cell viability. The number of live and dead cells in each flask was determined by multiplying the Countess® outputs by the appropriate dilution factor (8x). The percentage of cell viability in a treated group was calculated using the following formula:

\[
\% \text{ of viable cells} = \frac{\text{Number of live cells in a treated group}}{\text{Number of live cells in a control group}} \times 100\%
\]

This method normalizes the cell viability to the control group (cells treated with growth medium only) set at 100%. We also assume that cell density after washing with PBS is similar in both treated and control groups if the treatment has no deleterious effects on cell survival. Therefore, any difference in the number of live cells was presumed to be a result of treatment effects on cell growth and/or viability.

3.1.5 Evaluation of apoptosis

Apoptosis was assessed using cell death detection ELISA\textsuperscript{PLUS}, 10x kit. N2A cells were prepared as described above in the section on “Evaluation of cell viability”. After assessing the total number of cells and the cell viability per treatment group, 200 μL of
the re-suspended control cells were transferred into a 1.5 ml Eppendorf tube and diluted with a fresh growth medium to obtain a concentration of $1 \times 10^5$ cells/ml (100,000 cells/ml). The dilution factor in the control group was calculated based on the number of live cells determined by the cell viability assays. This dilution factor was used to dilute the cells in treated groups. A volume of 200 µL of treated cells were transferred into a 1.5 ml Eppendorf tube and diluted to $1 \times 10^5$ cells/ml (100,000 cells/ml using the dilution factor calculated for the control group). The cells were centrifuged 200 x g for 10 minutes. The supernatant was carefully removed by pipetting and the pellet re-suspended in 200 µL of lysis buffer (provided in the kit) and incubated at 15 °C to 25 °C for 30 minutes. The lysate was centrifuged at 200 x g for 10 minutes. A volume of 20 µL of the lysate were carefully transferred into streptavidin-coated microplate well. Each sample was run in triplicate. Three microplate wells served as background control (blank). Three additional wells were also reserved for the positive control (DNA-Histone complex). A volume of 80 µL of immunoreagent solution composed of 72 µL of incubation buffer, 4 µL of anti-histone-biotin and 4 µL of anti-DNA-peroxidase was added to each well containing the sample, positive control and blank. The streptavidin-coated microplate was covered with an adhesive cover foil and incubated on a microplate shaker at 300 rpm for 2 h at 25 °C. The solution was removed thoroughly by pipetting and the wells were rinsed carefully 3x with 300 µL of incubation buffer. A volume of 100 µL of ABTS substrate solution were added to each well. A volume of 5 ml of ABTS substrate solution was prepared by dissolving 1 ABTS substrate tablet into 5 ml of substrate buffer and allowing the solution to come to 25 °C in the dark before use. The microplate then was incubated on a microplate shaker at 300 rpm for 10 – 20 minutes (until the color development is
sufficient for a photometric analysis). A volume of 100 µL of ABTS stop solution was added to each well and the absorbance was measured using a microplate reader (FlexStation® 3 reader, Molecular Devices, LLC., CA, USA) set at 405 nm for samples and positive control and 490 nm for blank. The values from each triplicate were averaged and the background value (blank) was subtracted from it. Apoptosis for treated groups was calculated using the following equation:

\[
\text{Apoptosis} = \frac{\text{Absorbance in treated group}}{\text{Absorbance in control group}}
\]

### 3.1.6 Lipid and aqueous phase extractions

Lipids were extracted from N2A cells grown in T-75 flasks (three flasks/group) according to a dual phase extraction method as described by Tyagi, et al (165), but with a slight modification to accommodate the protein assay requirements. Briefly, the cells in each flask were washed twice with 4 ml of PBS solution at room temperature. Then 4 ml of ice-cold methanol were added to each T-75 cm² flask and the cells kept on ice for 5-10 min. Cells from each flask were scrapped with a rubber policeman and transferred into a 50 ml glass centrifuge tube kept on ice. The flask was rinsed with 4 ml of ice-cold distilled-deionized water (ddH₂O) and the rinse solution added to the centrifuge tube containing the cells suspended in methanol. Since cells in each treatment group were grown in three flasks, the vial for each group at this point contained cells suspended in 24 ml (8 ml x 3 flasks) of methanol and ddH₂O. After sonicating the mixture, a 0.5 ml aliquot was taken from the 24 ml for protein assay. Next, a volume of 11.75 ml [(24 ml – 0.5 ml) /2] of chloroform (keeping the methanol-chloroform-water ratio at 1:1:1, v/v/v)
was added to the mixture in the 50 ml glass centrifuge tube using a glass pipette. The sample was vigorously mixed before being transferred into a separatory funnel. The sample was left to stand at 4°C for 17 – 24 h to allow a complete separation of lipid and aqueous phases. The upper methanol-water phase containing the water-soluble metabolites and the lower chloroform phase containing the lipids were carefully collected separately. The lipid phase (chloroform phase) was collected into a dark amber vial and the sample was evaporated to dryness by blowing a gentle stream of nitrogen gas over the sample. This sample then was weighted to obtain the total lipid content. Dried lipid content was re-suspended in 700 µL of deuterochloroform, sealed with a black rubber stopper (resistant to chloroform degradation), and stored at −20 °C for further processing (see 31P NMR and UPLC-MS/MS analyses below).

The aqueous phase was placed in a lyophilizer flask and lyophilized to dryness. The dried sample was dissolved in 2 ml of ddH2O and treated with a chelating agent (Chelex 100; 5 g chelex/100 ml) to remove all the divalent cations that broaden the NMR lines. The mixture was stirred for 1 h at 4 °C. The supernatant was then transferred into a 15 ml disposable centrifuge tube and centrifuged at 2000 x g (3000 rpm) for 10 minutes at 4 °C to remove the Chelex. The supernatant was transferred back into the lyophilizer. Fresh chelex was added to the sample and the process was repeated as described above. After centrifugation, the supernatant was transferred into a vial of known weight and placed in a refrigerator at 4 °C. The lyophilizer flask was rinsed three times with 2 ml of ddH2O. Each time, the washing was transferred into the same 15 ml disposable centrifuge tube and centrifuged at 2000 x g for 5 minutes at 4 °C. The supernatant was transferred each time to the same vial kept in a refrigerator at 4 °C. The supernatant was then
lyophilized to dryness. The final weight of the vial was measured to determine the mass of the dried sample. The vial was sealed with a grey rubber stopper and stored at –20 °C for ¹H NMR analyses.

3.1.7 Protein assays

Protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) in 96-well-plate format. Bovine serum albumin (BSA) was used as the standard. Briefly, a 0.5 ml aliquot was taken from a mixture of scraped cells suspended in methanol + water as described above (see lipid extraction section). To avoid methanol interference with the protein assay, methanol was evaporated together with water by attaching the sample-containing vial in the water bath (25 °C) and blowing a gentle stream of nitrogen gas over the top of the vial. The sample was reconstituted with 0.5 ml of PBS. A series of BSA concentrations was used to produce a calibration curve. A mixture of BCA reagent and copper (II) sulfate (BSA/CuSO₄ ratio of 50:1) was added to each well containing BSA and cell samples according to BCA working instructions. The plate was incubated at 37°C for 30 min, then the absorbance was measured with a microplate reader (FlexStation® 3 reader, Molecular Devices, LLC., CA, USA) set at 562 nm. The protein concentration in the samples was calculated based on the standard curve.
3.1.8 Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy ($^{31}$P NMR) of Lipid Extracts

Lipid extracts were removed from the -20 °C freezer and allowed to equilibrate to room temperature. As described above (see lipid and aqueous phase extraction section), the lipid extract for each sample was re-suspended in 700 µL of CDCl$_3$. A volume of 500 µL of lipids was transferred into a 5 mm NMR tube using a glass syringe and the remaining 200 µL was saved for UPLC-MS/MS analyses. A volume of 200 µL of 90 mM Cs$_2$EDTA-2H$_2$O solution (dissolved in CH$_3$OH:H$_2$O solution; 4:1, v/v) warmed to room temperature was added to the lipid solution in the NMR tube and mixed by inverting the tube. An internal standard was added to this sample (10 µL of 25 mM triphenylphosphosphate (TPP) in CDCl$_3$) for quantification purposes of the $^{31}$P metabolites. Proton-decoupled $^{31}$P NMR spectra of lipid extracts were acquired in field-lock mode using a 5 mm broadband probe operating at 242.8 MHz on a Varian Inova 600 NMR Spectrometer, using a 60° pulse, 6982 Hz bandwidth, 1.9 s acquisition time and 4 s interpulse delay and approximately 12 h of signal averaging (under partially saturated conditions). Spectral $^{31}$P data were processed using exponential multiplication with 0.5 Hz line broadening and Fourier transformation. Spectra then were baseline corrected (flattened) in MATLAB (The Mathworks, Inc. Natick, MA; v. R2013b) using an algorithm developed in our laboratory. The peak intensities for the metabolite signals of each spectrum then were summed to a constant value of 1000 (sum normalization). Quantitative measures of each phospholipid species were determined based on the known concentration of the TPP standard. Quantification of specific metabolite resonances was accomplished using an interactive spectral deconvolution algorithm in MATLAB as described by Anderson et al.
For quantitation purposes, the $^{31}$P signal intensities of all lipid metabolites were corrected for T1 saturation and NOE.

### 3.1.9 Proton ($^1$H) NMR of Aqueous Extracts

Dried aqueous extracts were dissolved in 700 μL of known concentration of a trimethylsilylpropanoic acid (TSP) solution in D$_2$O. All $^1$H NMR spectra were acquired on a Varian INOVA operating at 600 MHz, using a 61° pulse, 8000 Hz bandwidth, 4s acquisition time and 6s interpulse delay with the probe temperature set to 25 °C. A total of 1024 transients were collected per spectrum.

### 3.1.10 $^{31}$P NMR of Aqueous Extracts

The same samples prepared for $^1$H NMR of aqueous extracts (see section 2.8 above) were used to acquire $^{31}$P NMR spectra using the same Varian INOVA instrument as described above. H-1 and $^{31}$P NMR spectra were acquired in tandem experiments. P-31 spectra were acquired using a $^1$H-decoupling sequence with nuclear Overhauser enhancement (NOE) using a 60° pulse, 9708 Hz bandwidth, 1.6 s acquisition time and 3.6 s interpulse delay. The signal averaging for $^{31}$P aqueous data was approximately 12 h.

### 3.1.11 NMR data processing

NMR data were processed using Varian software (VNMR 6.1c) employing exponential multiplication (producing line-broadening of 0.30 Hz and 1 Hz for $^1$H and $^{31}$P spectral data respectively), Fourier transformation, and phase correction. Spectra were then baseline corrected (flattened) in MATLAB (The Mathworks, Inc. Natick, MA; v.
R2010b) using the Whittaker Smoother algorithm (with lambda value of 200) on selected spectral noise regions (167, 168). Spectral regions containing resonances for water (4.70 – 5.00 ppm) were removed from $^1$H spectral data. The peak intensities for the metabolite signals of each spectrum then were summed to a constant value (sum normalized). Quantification of the resonances for myo-inositol (4.07 ppm; triplet), phosophoethanolamine (PEtn; 3.87 ppm; multiplet) and glycerophosphocholine (GPC; 3.24 ppm; singlet) was achieved using $^1$H spectral data. GPC (3.082 ppm, singlet) was also quantified in $^{31}$P spectral data together with glycerophosphoethanolamine (GPE; 4.08 ppm; singlet). The quantification of all these metabolites was accomplished using an interactive spectral deconvolution algorithm in MATLAB adapted from our previously described methods (166). The deconvolution tool fits a defined spectral region using a combination of tunable baseline shapes (spline, v-shaped, linear, or constant) and a Gauss-Lorentz peak-fitting function. Integrated areas for peaks of interest then are output to a text file. For quantitation purposes, the $^1$H signal intensities of myo-inositol, PEtn, GPC and the $^{31}$P signal intensities for both GPC and GPE were corrected for T1 saturation and nuclear Overhauser enhancement (NOE). The GPC quantified using $^1$H signal was used as a concentration standard for GPE.

3.2 Ultra Performance Liquid Chromatography (UPLC)-Mass Spectrometry (LC-MS/MS) Analyses

3.2.1 Chemicals

Mass spectrometry grade methanol, chloroform, acetonitrile and ammonium acetate were purchased from Sigma (St. Louis, MO). All lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The internal standard 1,2-
dipentadecanoyl-\(sn\)-glycero-3-phosphoethanolamine (15:0/15:0 PE; \([\text{M} + \text{H}]^+ = 664.0\)) was used for quantification purposes of ethanolamine phospholipid molecular species. The following compounds were used as analytical standards for the development of the optimal eluent system, and the collision-induced dissociation (CID) energy and fragmentation patterns of PE-Pls, PE and LPE molecular species: 1-(1Z-octadecenyl)-2-docosahexaenoyl-\(sn\)-glycero-3-phosphoethanolamine (18:0p/22:6-PE plasmalogen; \([\text{M} + \text{H}]^+ = 776.0\)); L-\(\alpha\)-lysophosphatidylethanolamine plasmalogen (18:0p/lyso-PE; \([\text{M} + \text{H}]^+ = 465.6\)); and 1,2-dimyristoyl-\(sn\)-glycero-3-phosphoethanolamine (14:0/14:0-PE, \([\text{M} + \text{H}]^+ = 636.0\)).

3.2.2 Methods

Lipid extracts were dried by gently blowing a nitrogen gas stream over the sample in a 25 °C water bath, weighted, and reconstituted in 700 μL of CDCl₃. A 500 μL aliquot of each sample was used in \(^{31}\)P NMR analysis and the remaining 200 μL was stored for further mass spectrometry analyses. Each sample saved for these analyses was dried by blowing a gentle stream of nitrogen gas over the sample and re-suspended in ultrapure methanol/chloroform solution (1:1 ratio, v/v) to a final concentration of 2.5 mg/mL. For quantification purposes of ethanolamine phospholipid species, 30 μL of each sample was spiked with a known concentration of an internal standard (1,2-dipentadecanoyl-\(sn\)-glycero-3-phosphoethanolamine, 15:0/15:0 PE). Bugger (169) has reported significant differences in ionization efficiency between phospholipid classes of ESI-MS, requiring each phospholipid class to have its own internal standard. Some reports in the literature have suggested the use of two or more internal standards in the analyses of each phospholipid class since there may be a decrease in the detection efficiency as the length
of the acyl chains increases \((169-171)\). Deely et al. \((172)\) have investigated this issue and concluded that the acyl chain length has no effect on the detection efficiency. In the current study, our focus was to identify and quantify molecular species of ethanolamine phospholipid classes. We only used one internal standard for the quantification of the species for all the three sub-classes of ethanolamine phospholipids (PE-Ps, PE and LPE). Other studies have also used one internal standard to analyze each phospholipid class, sharing a similar head group \((169, 172-176)\). We used an internal standard that is not naturally occurring in any significant amount in our samples, as done in other studies \((177)\). The diluted lipid extracts were immediately subjected to mass spectrometry analyses. Ethanolamine phospholipids were separated from other phospholipid classes on a Waters ACQUITY UPLC system equipped with a BEH HILIC column (Hydrophilic interaction liquid chromatography; 1.7 µm, 100 mm x 2.1mm). The temperatures were set to 30 °C and 6 °C for column and autosampler, respectively. The mobile phase A consisted of 50% acetonitrile and 10 mM ammonium acetate solution pH 8.0 (acetonitrile/HPLC grade water, 50:50, v/v), while the mobile phase B contained 95% acetonitrile and 10 mM ammonium acetate solution pH 8.0 (acetonitrile/HPLC grade water, 95:5, v/v). Mobile phase A was prepared by adding 450 mL of water and 50 mL of 200 mM ammonium acetate solution (pH 8.0) to 500 mL of acetonitrile. Mobile phase B was prepared by adding 50 mL of 200 mM ammonium acetate solution (pH 8.0) to 950 mL of acetonitrile. Both mobile phases A and B were degassed in an ultrasonic bath for 15 min prior to use. The injection volume was set to 5 µL and the flow rate set to 0.5 mL/min. The time for UPLC ethanolamine phospholipid separations was 15 minutes per sample using the following scheme: the initial elution was set at 100% solvent B for 0.1
minute followed by a linear decrease to 80% during the next 10 minutes. This solvent was then decreased to 5% during the next 1 minute (10th to 11th minute) and maintained there for the subsequent 1 minute (11th to 12th minute). The solvent B was thereafter increased to 100% during 1 minute (12th to 13th minute) and it was maintained there till the end of the run (13th to 15th minute). The mass spectra were obtained by performing an electrospray ionization tandem quadruple mass spectrometry on a Thermo TSQ Quantum triple quadrupole LC/MS system (San Jose, CA), equipped with an electrospray ion source and controlled by Micromass Masslynx version V4.1 software. All the samples were run with the spray voltage and capillary temperature set to 4500 V and 200 °C, respectively. Nitrogen was used as the drying gas at a pressure of 100 ± 20 psi. The pressure for argon used as the collision gas was set to 1 mTorr.

All mass spectra were acquired in positive ion mode ([M + H]⁺). The collision-induced dissociation (CID) energy was set to 20 eV for PE-Pls species and 16 eV for both PE and LPE based on direct infusion data obtained for the standard compounds for each species. Initially, the molecular species of PE-Pls, PE and LPE in the sample mixture had to be identified to generate single reaction monitoring (SRM) transitions for each species. The identification of the molecular species for PE-Pls was carried out by scanning for their precursor and product ions (Table 1). When the precursor ion scan (PIS) is performed, the product ion of choice is selected in the third quadrupole (Q3) and the correlating precursor ion in the first quadrupole (Q1, from which the daughter ion originates) is scanned. This PIS mode enabled an identification of all the phospholipid parent masses for PE-Pls. Spectra for PE and LPE species were generated by scanning for their characteristic neutral loss of their phosphoethanolamine head group (141 Da).
Neutral loss scans correlate the precursor ion in the first quadrupole (Q1) and product ions in the third quadrupole (Q3) for a definite identification of the parent mass. Except for three compounds, the 141 Da neutral loss scanning was not used for PE-Pls analyses since it has been shown that these species exhibit a minimal loss of 141 Da. As a result of this method development, single reaction monitoring (SRM) transitions were generated for the identified molecular species of ethanolamine phospholipids and quantitation was performed by integrating peak intensities for each SRM transition. These experimental conditions have been established previously and used in other studies to analyze these phospholipids. Each sample was run in triplicate. Identification of 1-lyso-2-acyl-GPE species (lyso/16:0 and lyso/18:0) from the corresponding 1-acyl-2-lyso-GPE isomers (16:0/lyso and 18:0/lyso) was achieved based on the elution time for these species. Okudaira et al. have reported that 1-lyso-2-acyl-GPL species elute faster than 1-acyl-2-lyso-GPL isomers in a reverse-phase LC on CAPCELLPAK C18 ACR column. We ran our samples on a HILIC column and expected 1-acyl-2-lyso-GPE to migrate faster than 1-lyso-2-acyl-GPE. Thus, the slower peaks were attributed to lyso/16:0 and lyso/18:0 species whereas the faster peaks were assumed to be those for 16:0/lyso and 18:0/lyso. The peak area for each molecular ethanolamine PL species and that of the internal standard were determined using Thermo Xcalibur™ software. The concentration of the molecular species [A] (nmol/mg protein) in the sample was then calculated using the following equation:

\[
\text{Concentration of [A]} = \frac{\text{Peak area for X}}{\text{Peak area for internal standard}} \times \text{Concentration of internal standard}
\]
Table 1. Experimental conditions for identification of molecular species of AAPE and PE-Pls in N2A cells

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<td>16</td>
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e. ether linked (AAPE); p. plasmalogen. See abbreviations on pages Xiii - Xvi
Table 2. Experimental conditions for identification of molecular species of PE in N2A cells

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See abbreviations on pages Xiii - Xvi
**Table 3.** Experimental conditions for identification of molecular species of LPE in N2A cells

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<td>lyso/22:6</td>
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<td>Neutral loss</td>
<td>[M + H]^+</td>
<td>385.6</td>
<td>16</td>
</tr>
</tbody>
</table>

\* Peaks for these species were differentiated based on their elution times (see methods for details). See abbreviations on pages Xiii - Xvi
3.3 Statistical analyses

Levine’s and Welch’s tests were conducted to assess the equality of variances between experimental groups for each metabolite using statistical software package JMP® 11.0.0 (SAS Institute, Cary, NC, USA). If Levine’s test was significant \((p \leq 0.05)\), then a Welch’s test was used to determine if there were significant differences in the mean values between groups for the metabolite that was under consideration. If the Levine’s test was not significant (NS), then the data have equal variances between groups and Welch’s test was not applicable. Therefore, data were tested using a one-way ANOVA \((t\)-test). If both Levin’s and Welch’s tests were significant \((p \leq 0.05)\), a pairwise Welch test was performed for all pairs of groups. All the data were considered statistically significant at \(p \leq 0.05\). Data are expressed as mean ± S.E.M.
CHAPTER 4: RESULTS AND DISCUSSION FOR NMR DATA

4.1 RESULTS

4.1.1 Phospholipid profiles in Neuro-2A cells

To determine the phospholipid profile of N2A cells, we extracted lipids from these cells and acquired $^{31}$P NMR on the extracts (see methods for details). As shown in Fig. 8, these cells synthesize various types of phospholipids (PL), including phosphatidylethanolamine plasmalogens (PE-Pls) and phosphatidylcholine plasmalogens (PC-Pls). Choline phospholipids consisting of phosphatidylcholine (PC), PC-Pls, lysophosphatidylcholine (LPC) and 1-alkyl-2-acyl-sn-glycerophosphocholine (AAPC) constitute the most abundant PL class, accounting for 58.9% of the PL pool (Table 4). PC is the most abundant single PL sub-class accounting for more than half (52.8%) of the entire PL pool. Ethanolamine phospholipids make the second most abundant PL class in the cells, amounting to 25.1% of the PL pool. This class comprises PE-Pls, phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE), contributing 13.1%, 11.6% and 0.4% to the PL pool, respectively. Plasmalogens (PE-Pls and PC-Pls) account for 17.5% of the entire PL content. Besides being the second most prevalent PL in the cells, PE-Pls are also the most abundant plasmalogen moieties, accounting for 75% of the entire plasmalogen pool. Both choline and ethanolamine phospholipid classes accounts for 82% of the entire PL content of the cells. The cells also synthesize inositol PLs containing phosphatidylinositol (PI) and lysophosphatidylinositol (LPI). Their contribution to PL pool amounts to 7.5%. Phosphatidylserine and sphingomyelin constitute 3.2% and 3.3% of the pool, respectively.
Figure 8. $^{31}\text{P}$ NMR spectrum showing phospholipid profiles in N2A cells. 1. PC; 2. PC-Pls; 3. AAPC; 4. U; 5. PI; 6. LPC; 7. PS; 8. SM; 9. PE; 10. PE-Pls; 11. LPI; 12. PG; 13. LPE; 14. U. See abbreviations on pages Xiii – Xvi.
Table 4. Phospholipid contents of N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>PL</th>
<th>PPM</th>
<th>nmol/mg protein</th>
<th>PL pool (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0.84</td>
<td>73.3 ± 9.0</td>
<td>52.8 ± 0.6</td>
</tr>
<tr>
<td>PE</td>
<td>0.12</td>
<td>18.3 ± 2.3</td>
<td>13.1 ± 0.4</td>
</tr>
<tr>
<td>PE-PL</td>
<td>0.08</td>
<td>16.0 ± 1.9</td>
<td>11.6 ± 0.3</td>
</tr>
<tr>
<td>PC-PL</td>
<td>-0.78</td>
<td>6.1 ± 0.8</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>PI</td>
<td>-0.36</td>
<td>5.9 ± 0.7</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>LPI</td>
<td>0.17</td>
<td>4.5 ± 0.6</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>SM</td>
<td>-0.05</td>
<td>4.4 ± 0.5</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>PS</td>
<td>-0.08</td>
<td>4.4 ± 0.5</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>PA</td>
<td>0.25</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>LPC</td>
<td>-0.25</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>AAPC</td>
<td>-0.72</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>LPE</td>
<td>0.53</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.05</td>
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<tr>
<td>PG</td>
<td>0.51</td>
<td>0.4 ± 0.05</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>U</td>
<td>-0.53</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

See abbreviations on pages Xiii – Xvi.
4.1.2 *Myo*-inositol plus ethanolamine increases the levels of *myo*-inositol, phosphoethanolamine, phospholipid pool and ethanolamine phospholipids in N2A cells

Hoffman-Kuczynski and Reo (25, 155) have shown that rats treated with M plus Etn have elevated PE-Pls levels by 30 % in whole brain relative to vehicle-treated controls. In this study, we hypothesized that similar results can also be obtained in an N2A cell culture system. We tested our hypothesis using four experimental groups that involved culturing cells in: (1) growth medium (GM) only (control), (2) GM supplemented with 500 µM *myo*-inositol (M); (3) GM supplemented with 500 µM ethanolamine (Etn), and (4) GM supplemented with 500 µM *myo*-inositol plus 500 µM ethanolamine (ME) for 24 h (Fig. 9A). The concentration of *myo*-inositol selected was based on the findings from Novak *et al.* (185) who demonstrated that differentiated human central nervous system neurons (NT2-N neurons) have a maximal velocity of inositol uptake (Vmax) of 3.7 nmol/mg-protein/h when treated with various concentrations of inositol ranging from 0 to 500 µM. The concentration of ethanolamine

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**Experimental Design**

<table>
<thead>
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<th>B</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24 Time (h)</td>
<td>24</td>
</tr>
<tr>
<td>Treatment: C, M, Etn, ME</td>
<td>Treatment: C or H2O2</td>
</tr>
<tr>
<td>Protein assays</td>
<td>Cell viability assays</td>
</tr>
<tr>
<td>NMR</td>
<td>Protein assays</td>
</tr>
<tr>
<td></td>
<td>NMR</td>
</tr>
</tbody>
</table>

**Figure 9.** Schematic diagram of the experimental design depicting times of treatments and analyses performed on samples. C: Control (growth medium only); M: *Myo*-inositol (500 µM); Etn: Ethanolamine (500 µM); ME: *Myo*-inositol (500 µM) + Ethanolamine (500 µM). See text for details and abbreviations on pages Xiii - Xvi.
was chosen based on the report showing that the maximum ethanolamine uptake for neuronal cells is 41.4 pmol/mg-protein/min (186). A combination of myo-inositol plus ethanolamine treatment (ME) was investigated since PE-Pls biosynthesis requires the ethanolamine-derived CDP-ethanolamine in the pathway (86, 87), and our laboratory has previously shown that this combination treatment leads to enhanced biosynthesis of ethanolamine phospholipids in rat brain in vivo (25, 26, 155). The 24 h optimal incubation time was determined in a separate series of experiments in which cells were grown in growth medium supplemented with ME for 24, 48 and 72 h. The amounts of PE-Pls (nmol/mg protein) obtained with these incubation times were not significantly different (data not shown). Thus, we retained 24 h incubation time for the subsequent experiments. NMR spectroscopy of cell lipid extracts provided a measure of phospholipid composition. Phospholipids in each sample were normalized relative to protein level assayed in an aliquot of cell extract (aqueous + lipid) using the bicinchoninic acid (BCA) Protein Assay Kit (see Methods for details).

The effects of Etn, M and ME treatments on the cellular levels of myo-inositol are illustrated by Fig. 10A. The treatments increased the cellular levels of myo-inositol in the order: Etn < control < ME ~ M. Ethanolamine treatment alone did not have a significant effect on myo-inositol level in the cells. However, both M and ME treatments induced a 3-fold increase (p < 0.05) in myo-inositol content of the cells relative to that in the controls. Myo-inositol levels in these groups were also significantly higher (p < 0.05) than that displayed by Etn treated group. The effect of Etn, M and ME on phosphoethanolamine (PEtn) content of the cells was also determined. As shown in Fig. 10B, these treatments increased the cellular levels of PEtn in the order: control ~ M < Etn
ME mediated a 3.6-fold increase in PEtn levels in the cells relative to that obtained with the control cells (p < 0.05). A robust increase in the cellular level of PEtn was obtained with ME treatment relative to the control group (8-fold increase, p < 0.0001). The level of PEtn in ME treated cells was also elevated (p < 0.01) in comparison to those measured in M and Etn treated cells. M treatment had no significant effect on PEtn level in the cells. These effects observed with Etn, M and ME on the cellular levels of myo-inositol and PEtn were all expected.

We also investigated the effect of Etn, M and ME treatments on the PL pool in N2A cells. As shown in Fig. 10C, Etn and M have no significant effect on the total PLs. However, ME significantly enhanced the synthesis of PLs (2.4-fold increase, p = 0.0003) when compared to the control group. The PL content in ME treated cells was also significantly higher when compared to those in E and M groups (p = 0.0002 and p = 0.0009, respectively).
Figure 10. Effect of myo-inositol, ethanolamine and myo-inositol plus ethanolamine treatments on the level of (A) myo-inositol, (B) PEtn, (C) total PL, (D) PE-Pls and (E) PE in N2A cells (Mean ± SE). Etn: ethanolamine (500 μM) M: myo-inositol (500 μM), ME: myo-inositol plus ethanolamine (500 μM each). Significant differences between groups are indicated by the letters: a. differs from both control and Etn groups; b. differs from control and M treated groups; c. differs from all groups; p ≤ 0.05. See abbreviations on pages Xiii – Xvi.
We also determined the effects of myo-inositol and ethanolamine treatments on the levels of ethanolamine plasmalogens (PE-Pls) in N2A cells. As displayed in Fig. 10D, the treatments increased the cellular levels of PE-Pls in the order: control < Etn < M < ME. Etn and M treatments alone did not significantly influence the level of PE-Pls in the cells. Etn treated cells yielded a 21% increase in PE-Pls when compared to controls, but this was not significant. M alone induced a 47% increase in PE-Pls level relative to the control group, although this increase was not statistically significant due to the large variance in the data. The greatest increase in PE-Pls level was obtained with cells treated with ME (2.7-fold increase, p = 0.0008) when compared to controls. This was the same treatment that yielded a significant increase in the cellular levels of both M and PEtn (Fig. 10A & B). The PE-Pls content in this group was also significantly higher when compared to those in Etn and M groups (p = 0.002 and p = 0.007 respectively). CDP-ethanolamine and 1-alkyl-2-acyl-sn-glycerol are part of the substrates required for the biosynthesis of PE-Pls (5, 6, 39). PEtn is a substrate for ECT1 enzyme that catalyzes the synthesis of CDP-ethanolamine in the Kennedy pathway (86, 87). It has been shown that myo-inositol can be metabolized into dihydroxyacetone–3-phosphate (DHAP) (187, 188). As DHAP is the first substrate required in the pathway leading to the biosynthesis of 1-alkyl-2-acyl-sn-glycerol, it is probable that myo-inositol is upregulating this pathway. Combining M and Etn in one treatment (ME) upregulates the pathways leading to the synthesis of these two indispensable substrates that are required for the biosynthesis of PE-Pls.

Effect of treatments on the PE level in N2A cells was also assessed since the ethanolamine phosphotransferase (EPT), one of the enzymes involved in PE-Pls
biosynthesis (Figure 4), is also required for PE synthesis. EPT combines CDP-ethanolamine and diacylglycerol to form PE. Thus, both EPT and CDP-ethanolamine are required in PE and PE-Pls synthesis. As shown in Fig. 10E, the treatments increased the cellular levels of PE in the order: control < M < Etn < ME. M and E groups yielded 23% and 38% increases in PE contents of the cells, respectively, relative to control cells, although these increases were not statistically significant (Fig. 10E). Interestingly, ME treatment induced a 3-fold increase (p = 0.0003) in the cellular level of PE relative to the control group. The PE level in ME group was also significantly elevated when compared to those in Etn (p < 0.001) and M (p = 0.0007) groups. As shown in Fig. 10B, the concentrations of PEtn were only elevated in E and ME treated cells when compared to the controls. PEtn is a substrate for the ECT1 enzyme that catalyzes the synthesis of CDP-ethanolamine in the Kennedy pathway (86, 87). These data indicate that both PE and PE-Pls were increased only when a co-treatment (ME) was given. Although Etn treatment increases PEtn level in the cells, Etn alone does not significantly elevate the cellular content of PE and PE-Pls. This happens only when Etn is given together with M. These data suggest that M given in a co-treatment (ME) may be positively affecting the pathways leading to the production of diacylglycerol (required for PE synthesis) and 1-alkyl-2-acyl-sn-glycerol (required for PE-Pls synthesis).

The effect of Etn, M and ME treatments on the cellular levels of other phospholipid classes that do not carry ethanolamine as a head group was also investigated in this study. As shown in Table 5, M and Etn treatments did not significantly influence the levels of these PLs. However, it is noteworthy that Etn treatment displayed a trend by negatively affecting the levels of the PL that have choline as a head group (PC, PC-Pls,
LPC and AAPC). This was not surprising as it has been shown that ethanolamine exerts a negative effect on the cellular uptake of choline (189). The levels of PC, LPC, PI, LPI, PS and SM in the cells were significantly elevated ($p \leq 0.01$) by ME at 24 h post treatment when compared to controls. These data suggest that ME negated any effect that Etn alone exerted on the levels of the choline phospholipids.

Currently, no clear description of the pathway for PC-Pls biosynthesis is available (83, 190-192). However, it has been shown that PE-Pls is an essential precursor to PC-Pls in neuronal cells (85). To get an insight on how each treatment affected the balance between the levels of both plasmalogens in the cells, we calculated PE-Pls/PC-Pls ratio for each group. Although the ratio in M treated cells (ratio = 4.4) was slightly increased as compared to controls (ratio = 3.3), this increase was not statistically significant due to the large variance in the data. However, this trend suggests that the rate of PE-Pls synthesis was higher in M group relative to the control group. The PE-Pls/PC-Pls ratios were significantly increased in both ME and Etn groups (ratio = 8.5 and ratio = 9.2 respectively) when compared to control. The higher ratio obtained with Etn group can partially be justified by the observation that Etn treatment induced a slight decrease in PC-Pls and an increase in PE-Pls levels. An elevated ratio obtained with ME can be attributable to a strong increase (3-fold) in ME-mediated synthesis of PE-Pls in the cells.
Table 5. Effects of M, Etn and ME treatments on PL in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th></th>
<th>PL (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPM</td>
</tr>
<tr>
<td>PC</td>
<td>-0.84</td>
</tr>
<tr>
<td>PC-Ps</td>
<td>-0.78</td>
</tr>
<tr>
<td>AAPC</td>
<td>-0.72</td>
</tr>
<tr>
<td>U</td>
<td>-0.53</td>
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<tr>
<td>PI</td>
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</tr>
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<td>LPC</td>
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<tr>
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<td>SM</td>
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<tr>
<td>PE</td>
<td>0.08</td>
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<tr>
<td>PE-Ps</td>
<td>0.12</td>
</tr>
<tr>
<td>LPI</td>
<td>0.17</td>
</tr>
<tr>
<td>PA</td>
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</tr>
<tr>
<td>PG</td>
<td>0.51</td>
</tr>
<tr>
<td>LPE</td>
<td>0.53</td>
</tr>
</tbody>
</table>

a. differs from all groups at p ≤ 0.05. See abbreviations on pages Xiii – Xvi.
4.1.3 Cells treated with *myo*-inositol plus ethanolamine show elevated ethanolamine plasmalogens and are protected from H$_2$O$_2$-induced oxidative stress

To examine if increased ethanolamine phospholipids (in ME-treated cells) is also protective against oxidative stress, we used hydrogen peroxide (H$_2$O$_2$) as an oxidative stress-inducing agent. H$_2$O$_2$ has been used in various studies to induce oxidative stress in the cells (161-164). Studies have provided evidence that extracellular H$_2$O$_2$ can cross the cell membrane through specific aquaporin channels (193-195). The mechanisms through which H$_2$O$_2$ induces oxidative stress in the cells have also been described (196, 197). This involves the generation of hydroxyl radical, a highly reactive molecule (131) through the redox cycling by Fenton reaction (129, 198) (Fig. 11).

To determine a concentration of H$_2$O$_2$ that leads to approximately 50% cell death in 24 h, cells were cultured in the growth medium alone (control) or medium supplemented with H$_2$O$_2$ ranging from 0 to 2000 μM (0, 300, 400, 500, 600, 650, 800 and 2000 μM) for 24 h. Cell viability was assessed using trypan blue assay. This assay has also been used by other investigators to determine cell viability to different cell stressors (199-201). As illustrated in Fig. 12A, a concentration of 650 μM H$_2$O$_2$ yielded 53 ± 1.4%
(n=7) cell viability relative to control cells. This concentration was retained for subsequent experiments. The experimental design for these studies is shown in Fig. 9A. N2A cells were pre-treated with GM only (control; C), or medium supplemented with Etn, M, or ME for 24 h to increase the cellular level of Etn phospholipids. At 24 h, cells were rinsed with phosphate buffered saline (PBS) and incubated in fresh medium (control) or medium supplemented with 650 μM H2O2 for an additional 24 h (see methods for details). Each experimental group had its own control that did not receive H2O2. Cell viability then was assessed using trypan blue assay kits. The percent cell viability in each group was measured relative to its corresponding control group set to 100%. As illustrated in Figure 12B, exposure to 650 μM H2O2 for 24 h yielded 55.9 ± 1.1% (n = 16; p ≤ 0.0001) cell viability relative to corresponding control cells (n = 16). Pretreatment of cells with Etn alone (n = 5) did not protect cells against H2O2 toxicity (53 ± 1.4% cell viability; n = 5), but pretreatment with M increased cell viability significantly (62 ± 1.2%; n = 5; p = 0.0004), while ME treatment provided the greatest cell protection (79.5 ± 0.6%; n = 13; p ≤ 0.0001). These data suggest that M pretreatment can protect N2A cells from H2O2-induced oxidative damage, but a greater protection is obtained when the cells are pretreated with ME. ME is the same treatment that yielded a 2.7-fold increase in PE-Pls (Fig. 10D) and a 3-fold increase in PE (Fig. 10E) and also an increase in other non-ethanolamine lipids as shown in Table 5. It is interesting to note that the levels of PE-Pls in the cells prior to H2O2 treatment highly correlate ($r^2 = 0.95$) with the cell viability following this treatment (Fig. 12C). However, the concentrations values for PE-Pls used to run this correlation analysis were obtained from the measurements made at 24 h post ME treatment, prior to H2O2 exposure. Assessment on cell viability was
made at 24 h post H$_2$O$_2$ exposure (48 h post ME treatment). Thus, both measurements were not made from the same group of cells, but these cells were from the same batch.

**Figure 12.** N2A viability by trypan blue assay (Mean ± STDEV) (A) after exposure to H$_2$O$_2$ for 24 h at concentrations ranging from 0 – 800 μM. Data are normalized to the control group set at 100%. Sample size is n=1 at each concentration except at 500 μM (n=3), 600 μM (n=4), and 650 μM (n=7). A measurement was also made at 2,000 μM H$_2$O$_2$ yielding 2% cell viability (data not shown). (B) on cells pre-treated with growth medium only, myo-inositol, ethanolamine, myo-inositol plus ethanolamine for 24h followed by an exposure to 650 μM H$_2$O$_2$ yielding for 24h. Assessment of cell viability in each treatment group is relative to a corresponding control group (set at 100%) that received the pre-treatment, but not H$_2$O$_2$. Sample size is n = 16 (H$_2$O$_2$), n = 5 (Etn + H$_2$O$_2$), n = 5 (M + H$_2$O$_2$); n = 13 (ME + H$_2$O$_2$). (C) Correlation between PE-Pls level and neuroprotection. Cell viability assays and measurements on concentrations of PE-Pls were carried out on different batches of cells. See text for details. (D) Apoptosis on cells pre-treated as described in (B). The letter “a” denotes significant differences (p < 0.05) from H$_2$O$_2$ group, whereas “b” refers to significant differences from all the other groups. See abbreviations on pages Xiii – Xvi.
4.1.4 *Myo*-inositol plus ethanolamine blocks the H$_2$O$_2$-mediated decrease in ethanolamine plasmalogens in N2A cells

It has been suggested that the vinyl ether bond of plasmalogen is highly sensitive to oxidation (15-20, 145, 146) and may be involved in cell protection against oxidative stress (149, 150). Evidence that PE-Pls may be associated with cell protection is supported by the following observations: (1) treating N2A cells with ME yielded a 2.7-fold increase in PE-Pls content (Fig. 10D); (2) exposing these ME pretreated cells to H$_2$O$_2$ for 24 h resulted in a significant increase in cell viability (Fig. 12B); (3) cell viability and the level of PE-Pls are positively correlated (Fig. 12C); (4) previous studies in rats *in vivo* have shown protection against oxidative damage in brain regions where PE-Pls content is elevated (26, 146); and (5) the vinyl ether bond of PE-Pls is more prone to oxidation than ether or ester linkages (1). Therefore, we hypothesize that an increase in cell survival to H$_2$O$_2$-induced oxidative damage following ME treatment (79.5% cell viability, Fig. 12B) in the current study is a result of PE-Pls acting as a sacrificial trap of ROS, thus quenching oxygen free-radical propagation and shielding other crucial cellular molecules from oxidation. Consequently, we believe that by pre-treating the cells with ME, we enhanced the baseline level of PE-Pls in the cells, which served as ammunition stock that cells depended on when they were facing excessive oxidative stress. This hypothesis was tested using an experimental design that involved (1) pre-treating the cells with the growth medium only (C) or ME for 24 h to increase the level PE-Pls in the cells, (2) at 24 h, rinsing cells with PBS and incubating them in fresh medium (control) or medium supplemented with 650 μM H$_2$O$_2$ for an additional 24 h (Fig. 9B) and (3)
assessing the impact of H$_2$O$_2$ on the PL profile, with a special interest in PE-Pls (see methods for details).

As shown in **Fig. 13A** and **13B**, the levels of *myo*-inositol and PEtn in N2A cells were significantly increased by incubating cells in growth medium supplemented with ME over a 24 h period when compared to controls. However, the level of these metabolites returned to baseline (corresponding control values) after removing the growth medium that contained ME and re-suspending the cells in fresh growth medium for an additional 24 h period. The level of PEtn in ME group significantly decreased from 154 nmol/mg protein (at 24 h) to 49 nmol/mg protein (at 48 h) while the *myo*-inositol level decreased from 10 nmol/mg protein to 7 nmol/mg protein. H$_2$O$_2$ treatment had no significant effect on the levels of these metabolites. However, *myo*-inositol content in ME + H$_2$O$_2$ treated group showed a trend of further decreasing when compared to its control (ME), suggesting a role for *myo*-inositol in cell protection, possibly via an increase in its utilization for the synthesis of new PE-Pls. We also assessed the PL level in the cells treated with ME for 24 h and exposed to H$_2$O$_2$ for an additional 24 h (**Fig. 13C**). Stopping the ME treatment did not affect the cellular content of PL pool. The level of PL pool remained significantly elevated (p = 0.04) in the ME group relative to controls (both untreated controls and corresponding ME-treated controls). Although the H$_2$O$_2$ treatment did not statistically influence this pool, cells pre-treated with ME and dosed with H$_2$O$_2$ displayed an increasing trend in PL content when compared to corresponding controls (ME).

As shown in **Fig. 13D**, ME increased the PE-Pls levels at 24 h (36.6 ± 4.7 nmol/mg protein) by 2.7-fold (p = 0.0008) in comparison to the control group. This level
was not significantly different from that measured at 48 h (33.8 ± 2.9 nmol/mg protein, 24 h after stopping ME treatment). At 48h, however, the PE-Ps content in ME group was still significantly elevated (p = 0.005) relative to that measured in the control group (at 48 h). Cells pre-treated with growth medium only and exposed to H2O2 for 24 h (H2O2 group) displayed a 13% decrease in PE-Ps level when compared to corresponding control, although the decrease was not significant. Interestingly, this effect associated with H2O2 treatment was not observed in the cells that were pretreated with ME (ME + H2O2 group). In fact, the PE-Ps content in this group was increased by 8% when compared to corresponding control (ME). The treatments affected the levels of PE-Ps in the order: H2O2 < control < ME < ME + H2O2.

We also assessed the impact of exposing cells to H2O2 on the cellular level of PE. As shown in Fig. 13E, ME treatment elevated the PE levels, yielding a 3-fold increase (p = 0.0001) at the end of ME treatment (41.1 ± 5.8 nmol/mg protein at 24 h) as compared to that measured in the control group. The PE content dropped to 32.3 ± 2.8 nmol/mg protein at 48 h after stopping the ME treatment, although this decrease was not statistically significant (p = 0.09). However, this level (32.3 ± 2.8 nmol/mg protein) was significantly higher (p = 0.001) when compared to the corresponding control group (17.3 nmol/mg protein) at 48 h. Contrary to the observation that the cellular level of PE-Ps in the H2O2 group decreased by 13%, it is interesting to note that these cells displayed a 3% higher PE level relative to corresponding control, although this increase was not significant. These data suggest that PE-Ps were more sensitive to H2O2-induced degradation than PE species. The PE content in ME + H2O2 group increased by 17%
relative to that measured in ME group. The levels of PE measured in these experimental
groups were in the order: control < H$_2$O$_2$ < ME < ME + H$_2$O$_2$.

**Figure 13.** Effects of H$_2$O$_2$ treatment on the level of (A) myo-inositol, (B) PEtn, (C) PL pool, (D) PE-Pls and PE in N2A cells (Mean ± SE). Significant differences between groups are indicated by the letter: a. differs from control group; $p \leq 0.05$. See abbreviations on pages Xii - Xiii.

As shown in **Table 5**, the cellular levels of PC, LPC, PI, LPI, PS and SM were significantly elevated by ME at 24 h post treatment when compared to controls. It is also interesting to note that 24 h after stopping ME treatment (measurements taken at 48h), the levels of these PLs were similar to controls, except LPI and SM which remained significantly higher ($p \leq 0.01$) (**Table 6**). H$_2$O$_2$ treatment did not significantly affect the level of these PLs (**Table 6**). In ME + H$_2$O$_2$ group, the cellular contents of these
phospholipids were also similar to those in the corresponding control (ME group) except the levels for PS and AAPC which were significantly elevated (p < 0.02).

Table 6. Effects of H$_2$O$_2$ treatment on phospholipids in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>PL (nmol/mg protein)</th>
<th>PPM</th>
<th>Control</th>
<th>H$_2$O$_2$</th>
<th>ME</th>
<th>ME + H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>-0.84</td>
<td>80.3 ± 9.7</td>
<td>75.3 ± 10.9</td>
<td>104.5 ± 8.6</td>
<td>122.3 ± 7.8</td>
</tr>
<tr>
<td>PC-Pls</td>
<td>-0.78</td>
<td>7.3 ± 1.0</td>
<td>7.2 ± 1.3</td>
<td>3.6 ± 0.4</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>AAPC</td>
<td>-0.72</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.2$^b$</td>
</tr>
<tr>
<td>U</td>
<td>-0.53</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>PI</td>
<td>-0.36</td>
<td>6.5 ± 0.8</td>
<td>5.7 ± 0.6</td>
<td>7.4 ± 0.8</td>
<td>8.6 ± 0.7</td>
</tr>
<tr>
<td>LPC</td>
<td>-0.25</td>
<td>1.9 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>PS</td>
<td>-0.08</td>
<td>4.6 ± 0.4</td>
<td>4.9 ± 0.7</td>
<td>5.1 ± 0.5</td>
<td>7.1 ± 0.5$^b$</td>
</tr>
<tr>
<td>SM</td>
<td>-0.05</td>
<td>4.9 ± 0.6</td>
<td>4.4 ± 0.6</td>
<td>7.8 ± 1.0$^a$</td>
<td>7.6 ± 0.7</td>
</tr>
<tr>
<td>PE</td>
<td>0.08</td>
<td>17.3 ± 1.9</td>
<td>17.8 ± 2.4</td>
<td>32.3 ± 2.8$^a$</td>
<td>37.8 ± 2.8</td>
</tr>
<tr>
<td>PE-Pls</td>
<td>0.12</td>
<td>21.0 ± 2.6</td>
<td>18.3 ± 2.8</td>
<td>33.8 ± 2.9$^a$</td>
<td>36.4 ± 2.4</td>
</tr>
<tr>
<td>LPI</td>
<td>0.17</td>
<td>5.4 ± 0.7</td>
<td>6.2 ± 0.8</td>
<td>8.8 ± 0.6$^a$</td>
<td>10.7 ± 0.7</td>
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<tr>
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<tr>
<td>PG</td>
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<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>LPE</td>
<td>0.53</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.2$^a$</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

*a.* Differs from control; *b.* differs from ME group, p < 0.05. See abbreviations on pages Xiii - Xvi.
4.1.5 Myo-inositol plus ethanolamine blocks the H$_2$O$_2$-mediated increase in lyso-ethanolamine lipids and glycerophosphoethanolamine in N2A cells

In order to gain more insight on how H$_2$O$_2$ treatment affected ethanolamine phospholipids, we measured the levels of the degradation by-products of PE and PE-Pls by NMR. These included lysoglycerophosphoethanolamine (LPE) that is derived from hydrolysis of an acyl chain at the sn-1 (1-lyso) or sn-2 (2-lyso) position of the glycerol backbone of PE and PE-Pls. The 2-lyso species generated from PE and PE-Pls are structurally different. The 2-lyso that is from PE-Pls hydrolysis has an acyl chain attached via a vinyl ether linkage at the sn-1 carbon. The 2-lyso, derived from PE, has an acyl chain attached via an ester bond at the sn-1 carbon. The 1-lyso compounds derived from PE-Pls and PE may have similar chemical structures if the acyl chains attached to sn-2 are similar. Their structure will be different if these acyl chains are chemically different.

In this study, LPE represents a mixture of all these lyso species since they all give rise to a single peak in $^{31}$P NMR spectrum. The source of GPE includes the hydrolysis of both acyl chains in PE and PE-Pls or the removal of the acyl chain from LPE. Since both PE and PE-Pls contributed to LPE and GPE in our samples, we assessed the effect of treatments on LPE by calculating the ratio of the by-product to its precursors [(LPE/(PE + PE-Pls)) in each sample and comparing the ratio and GPE level across the treatment groups (Fig. 14A and 14B). The LPE/(PE + PE-Pls) ratio at 24 h post ME treatment did not differ from that calculated for the control group (Fig. 14A). These ratios were also not statistically different 24 h after stopping ME treatment (measured at 48 h). In contrast, the H$_2$O$_2$ group yielded a LPE/(PE + PE-Pls) ratio that was 3.2-fold (p = 0.005)
higher in comparison to control, suggesting that there was a H$_2$O$_2$-induced increase in LPE level and a decrease in PE and PE-Pls levels in the cells.

Figure 14. Effects of H$_2$O$_2$ treatment on the level of (A) LPE and (B) GPE in N2A cells (Mean ± SE). Significant differences between groups are indicated by the letters: a. differs from control group; b. differs from ME treated group; p ≤ 0.05. See abbreviations on pages Xiii - Xvi.

Our data show that PE-Pls level for the H$_2$O$_2$ group was decreased by 13% (Fig. 13D) while that of PE was increased by 8% (Fig. 13E), suggesting that an increase in LPE production in this group is predominately attributable to PE-Pls degradation. The LPE/(PE + PE-Pls) ratio for ME + H$_2$O$_2$ group was similar to that calculated for its corresponding control (ME), suggesting that ME treatment blocked the H$_2$O$_2$-mediated degradation of PE-Pls. The GPE level at 24 h post ME treatment was similar to that measured for the control group (Fig. 14B). These metabolites were also not statistically different 24 h after stopping ME treatment. In contrast, the GPE level for H$_2$O$_2$ group was increased by 4-fold (p = 0.003) when compared to control, suggesting a H$_2$O$_2$-induced increase in GPE production and a decrease in PE and PE-Pls degradation in the cells. Since the PE-Pls levels for the H$_2$O$_2$ group displayed a decrease while that for PE showed
an increase, the data suggests that an increase in GPE in this group is predominately attributable to PE-PIs degradation. Surprisingly, the GPE level for the cells pre-treated with ME was not affected by H$_2$O$_2$ treatment when compared to its corresponding control (ME) (p = 0.5). This suggests that ME treatment blocked the H$_2$O$_2$-mediated degradation of ethanolamine phospholipids.
4.2 DISCUSSION

4.2.1 Phospholipid profiles in N2A cells

The current study was conducted to (1) characterize the phospholipid profiles of N2A cells, (2) investigate the impact of \textit{myo}-inositol alone, ethanolamine alone and \textit{myo}-inositol plus ethanolamine (ME) treatments on the PL profiles in N2A cells, with a special interest in ethanolamine phospholipids, (3) determine how these treatments affect the cell’s ability to respond to H$_2$O$_2$-induced oxidative stress, and (4) determine how H$_2$O$_2$ affects the cellular contents of PL, with a special interest in ethanolamine phospholipids.

Although N2A cells have been extensively used to study various biological processes (14, 156, 202-205), their phospholipid compositions have not yet been reported. We therefore characterized the PL profile of the cells as shown in Table 4. These cells synthesize different PL classes. It is worth noting that we were not able to measure the levels of AAPE species by NMR but we were able to measure them by mass spectrometry. It has been reported that the central nervous system is predominantly rich in PE-Pls and PE and poor in AAPE moieties (206). AAPE species gets converted into PE-Pls by delta-1-alkyl-desaturase enzyme when it inserts a double bond between C1 and C2 of its alkyl chain (Fig. 4) (6). Two possibilities may explain why we were unable to detect AAPE species by NMR. First, it could be that these species were much lower in concentrations, thus, yielding very weak peak intensities below the level of detection. The other possibility is that they accumulated to an appreciable concentration, but the resonance was buried under the tall and broad peaks for PE-Pls and PE. AAPE, PE-Pls
and PE have structural similarities and the only feature that distinguish them is the linkage of their acyl chains at sn-1 position to the glycerol backbone (ether, vinyl ether and ester linkages for AAPE, PE-Pls and PE, respectively).

Plasmalogens are the major phospholipid constituents of the neural membranes (55). PE-Pls and PC-Pls are the only vinyl ether linked species synthesized by these cells. PE-Pls are three times more abundant (18.3 ± 2.3 nmol/mg protein) than PC-Pls (6.1 ± 0.8 nmol/mg protein). These are the two major plasmalogens species that have been reported to be the constituents of the mammalian cell membranes (55). PE-Pls are the most dominant plasmalogens in most cells, except in skeletal and cardiac muscle where PC-Pls is prevalent (55). PE-Pls accounted for 13.1% of the total PL pool, and 54% and 75% of the ethanolamine lipids and plasmalogen pool, respectively. Comparing the phospholipid compositions of N2A cells to those for mixed murine spinal cord neuronal cultures (207), the spinal cord cells contribute fewer amounts of choline glycerophospholipids (43.5%), but a higher amount of ethanolamine glycerophospholipids (44.8%) to the PL pool. The contribution of PE-Pls to this pool (13.0%) is consistent with what we measured in N2A cells (13.1%). However, in spinal cord cells, PE-Pls are a smaller fraction of total ethanolamine phospholipids (29.1%) compared to that recorded with N2A cells (54%). Likewise, N2A cells contain greater amounts of PC-Pls (4.4%, compared to 1.9%) and PI (4.3%, compared to 1%), but more comparable amounts of PS (3.2%, compared to 2.5%) and SM (3.3%, compared to 2.9%) than those measured in spinal cord cells. The spinal cells, however, have higher levels in PE (25.9%, compared to 11.6%), AAPC (3.6%, compared to 0.5%), and LPE (1.5% compared to 0.4%) than those measured in N2A cells. Two possible reasons may explain
the differences in phospholipid compositions between these two cell cultures. First, the
differences may result from alterations in phospholipid metabolism inherent to different
culture conditions. Second, we cannot also rule out the possibility that differences may
derive from tissue sources of the cells. Pertinent questions that our dataset does not
provide answers to are (1) how the phospholipid compositions in N2A cell culture system
reflect those generated from in vivo settings, and (2) how they relate to those in human
neurons. It has been shown that PE-Pls are the most abundant plasmalogens in human
brain (5) with an abundance ranging between 15 and 20% of the PL pool in the cell
membranes (5). The level of PE-Pls in N2A cells (13.1%) is comparable.

Studies have suggested that plasmalogens may possess antioxidant properties,
protecting the cells against oxidative damage (12, 15, 40, 146). This is partially due to
their vinyl ether linkage at the sn-1 position of the glycerol backbone that may be more
susceptible to oxidation than the corresponding ester bond in other glycerophospholipid
moieties (16, 18, 40, 208). If plasmalogens are potentially serving as antioxidants in the
cells, then establishing the mechanisms that can increase their cellular baseline levels will
be beneficial against excessive oxidative stress. In this regard, studies have shown that
myo-inositol increases the levels of PE-Pls in rat brain (25, 26, 155, 209) and in serum
for hyperlipidemic patients with metabolic syndrome (46). A systemic administration of
PE-Pls can abolish the lipopolysaccharide-induced neuroinflammation and β-amyloid
protein accumulation in the mouse brain (10, 11). All these studies have been conducted
in vivo, where the system is more complex to pinpoint exactly the source of plasmalogens
measured in these tissues. Reactive oxygen species (ROS) and a deficiency in cellular
plasmalogen levels have been implicated in neuronal cell death and pathogenesis of
neurodegenerative diseases (21, 22, 24, 111, 117, 123, 124, 210-213). There is an urgent need for designing neuroprotective interventions that can stop or reverse the ROS-mediated progression of neuronal death. If plasmalogens possess the antioxidant properties as studies have suggested (12, 15, 40, 146), then increasing their levels in neuronal cells may be a potential intervention mechanism that can slow, stop or rescue cells from ROS-induced death. Thus, this could reverse the course of diseases/disorders by stopping or slowing its progression.

In the current study, our goal was to determine if treating N2A cells with myo-inositol +/- ethanolamine would increase levels in ethanolamine phospholipids, especially PE-Pls. We hypothesized that a co-treatment (ME) would elevate the levels of these phospholipids, possibly via enhanced biosynthesis. ME would yield more PE-Pls since the biosynthesis of these species requires ethanolamine-derived CDP-ethanolamine (Kennedy pathway) (87), and our laboratory has previously shown that this combination treatment leads to enhanced biosynthesis of ethanolamine phospholipids in rat brain in vivo.

Myo-inositol is an essential organic osmolyte molecule found in brain, kidney and retina (214). In brain and cerebrospinal fluid, its levels increase with age (215). Humans obtain myo-inositol from the diet such as fruits, vegetables, and wheat germ. The daily dietary intake ranges between 500 mg to 2000 mg (46). The body can also make it through de novo synthesis that requires glucose 6-phosphate as the first substrate (216). Myo-inositol plays an essential role in eukaryote cells, where it is used in various biological processes such as the synthesis of phosphatidylinositol (217), a known structural neural membrane component serving as a precursor for inositol-containing
membrane phospholipids such as glycerophosphoinositol and a wide variety of the membrane-bound signaling molecules as well as the soluble second messengers (218-220). Our data show that the concentration of myo-inositol in N2A cells was 3.6 nmol/mg protein (Fig. 10A). Ethanolamine treatment alone did not influence the level of myo-inositol in the cells. However, both M and ME treatments induced a 3-fold increase (p < 0.05) in the cellular level of myo-inositol relative to that in the control group. This increase was reversed by removing the medium that contained ME and re-suspending the cells in fresh medium for an additional 24 h (Fig. 13A). Some literature reports indicate that the concentrations of myo-inositol in rat brain range between 6 – 8 μmol/mg tissue (155, 221, 222), while others report that concentrations vary between 2 - 4 μmol/mg tissue (223). Although these discrepancies exist on the actual cellular levels of myo-inositol, there seems to be a common agreement that ethanolamine alone does not impact the cellular levels of myo-inositol. Hoffman and Reo (155) have also shown that myo-inositol and ethanolamine do not elevate the cellular levels of myo-inositol in rat brain unless they are given together as a co-treatment.

As expected, only ethanolamine and ME treatments increased the levels of PEtn in N2A cells. In fact, a 3.6-fold increase was observed in ethanolamine treated cells relative to the controls (p < 0.05). The increase was even more robust (8-fold increase, p ≤ 0.0001) for the cells treated with ME relative to control cells. The level of PEtn in the ME group significantly decreased after removing the medium that contained ME and re-suspending the cells in fresh medium for an additional 24 h period (Fig. 13B). Marshall et al. (224) have also investigated the effect of exogenous ethanolamine on its concentrations in arterial plasma, brain extracellular fluid and the whole brain of rats.
Their findings indicated that ethanolamine treatment increased its levels in these tissues and fluid in a dose-dependent fashion. Ethanolamine is an indispensable molecule in the Kennedy pathway (87) for the synthesis of CDP-Ethanolamine, a substrate required for de novo synthesis of both PE-Ps and PE (6, 87). Myo-inositol alone and ethanolamine alone did not have a significant impact on the levels of PL pool, PE-Ps and PE in N2A cells (Fig. 10C, D & E). Myo-inositol and ethanolamine elevated the cellular contents of PE-Ps by 47% and 21%, respectively. They also increased the levels of PE by 23% and 38%, respectively. Only co-treating cells with both myo-inositol and ethanolamine (ME) yielded a significant increase in the total PL pool (2.2-fold), PE-Ps (2.7-fold) and PE (3-fold) relative to the control group. The levels of PL pool, PE-Ps and PE remained significantly elevated (p = 0.005) after replacing the growth medium that contained ME with fresh growth medium for an additional 24 h. It is also noteworthy that the levels of myo-inositol and PEtn dropped and were similar to those measured in the control group, suggesting they were actively being used to sustain the levels of these phospholipids. Although it is not clear how ME influenced the PL levels in N2A cells, a possible explanation for PE-Ps and PE could be that myo-inositol and ethanolamine provided substrates required for their synthesis. It has also been suggested that the effect of myo-inositol on PE-Ps levels may be associated with an increase of NADPH since its catabolism through the pentose phosphate cycle yields 2 molecules of NADPH (55). It is well established that myo-inositol can be metabolized into dihydroxyacetone-3-phosphate (DHAP) via the pentose phosphate pathway. DHAP is the first substrate required in the peroxisome for de novo synthesis of PE-Ps (6). From our data, it is possible that myo-inositol influenced the level of PE-Ps by increasing the cellular content of peroxisomal
DHAP available to the PE-Pls pathway. In PE-Pls synthesis pathway, 1-alkyl-2-acyl-glycerol combines with CDP-ethanolamine (metabolized from ethanolamine) (see Fig. 4) to form 1-alkyl-2-acyl-GPE. Then, 1-alkyl-2-acyl-GPE is converted into PE-Pls by a desaturase enzyme. Therefore, the importance of PEtn (ethanolamine metabolite) for the synthesis of PE-Pls is understandable. Similarly, the CDP-Etn can also combine with diacylglycerol to form PE. Other studies that have investigated the effect of myo-inositol with or without ethanolamine on the PL contents in rat brain (25, 155) reported a 47% increase in newly synthesized ethanolamine phospholipids in the whole rat brain and an 89% increase in the cerebellum region following ME treatment. Additionally, they also observed a 30% increase in PE-Pls in the cerebellum region of the rat brain following this treatment. Our findings are in agreement with Hoffman-Kuczynski and Reo’s reports (25, 26, 155) which show that the co-treatment of ME is an essential recipe for increasing PE-Pls levels in rat brain.

We also investigated the effect of myo-inositol, ethanolamine and ME treatments on the other phospholipid classes. As shown in Table 5, myo-inositol and ethanolamine treatments did not significantly influence the levels of these PLs. However, it is noteworthy that ethanolamine treatment displayed a trend in negatively affecting the levels of choline-containing PL (PC, PC-Pls, LPC and AAPC). However, this effect was not observed when ethanolamine was given together with myo-inositol, suggesting that ME negated any effect that ethanolamine alone exerted on the levels of the choline phospholipids. The effect of ethanolamine on choline phospholipids was not surprising as it has been shown that ethanolamine exerts an inhibitory effect on the cellular uptake of choline (189). We also observed a significant increase (p ≤ 0.01) in the levels of PC,
LPC, PI, LPI, PS and SM for the cells that were treated with ME for 24 h relative to the controls. It is also worthy to note that 24 h after stopping ME treatment and incubating them in fresh growth medium for an additional 24h, their contents in these PLs were similar to those in the controls, except for LPI and SM levels, which remained significantly elevated ($p \leq 0.01$; Table 5).

4.2.2 Myo-inositol plus ethanolamine protects N2A cells against $H_2O_2$-induced oxidative stress

It is interesting to highlight that the levels of PE-Pls in the cells prior to $H_2O_2$ treatment highly correlate ($r^2 = 0.95$) with the cell viability following this treatment (Fig. 12C). This suggests that increasing the baseline level of PE-Pls can help the cells cope with oxidative stress. However, the mechanism behind this protection is not known. $H_2O_2$ is classified as an oxidant with the potential to inflict damage to nucleic acids, proteins, as well as lipids (193). Studies have provided evidence that extracellular $H_2O_2$ can cross the cell membrane through specific aquaporin channels (193-195). The intracellular $H_2O_2$ concentration is tightly controlled (193). Mechanisms through which $H_2O_2$ induces oxidative stress in the cells have been described (196, 197). It has been reported that $H_2O_2$ itself is not a very potent harmful agent to the cells (194), but can easily get metabolized into the highly reactive hydroxyl radical molecule (‘OH) via the Fenton reaction (198) and this moiety can oxidize lipids, nucleic acids and proteins (194). Our data clearly show that pre-treating N2A cells with ME prior to $H_2O_2$ exposure significantly limits the $H_2O_2$-mediated N2A cell death ($79.5 \pm 0.6\%$ cell viability) as compared to the control cells ($55.9 \pm 1.1\%$ cell viability). Hardaway et al. (160)
investigated the N2A cells viability to ascorbic acid and H$_2$O$_2$ and reported that exposing these cells up to 300 μM H$_2$O$_2$ does not produce a significant cell death. At a dose of 300 μM, they recorded 92.0 ± 4.3% cell viability. The highest dose of H$_2$O$_2$ that was used in their study was 400 μM, which significantly reduced the cell survival to 73 ± 4.3%. The estimated LC$_{50}$ was determined to be above 400 μM H$_2$O$_2$. In the current study, the cell viability at 300 μM and 400 μM H$_2$O$_2$ are 89% (n = 1) and 82% (n = 1), respectively. Thus, data for Hardaway et al. (160) and ours are very similar. Cells pre-treated with ME prior to H$_2$O$_2$ exposure tolerate 650 μM H$_2$O$_2$ like control cells treated with 400 μM H$_2$O$_2$. Although the mechanisms of ME-induced cell protection have not yet been elucidated, it is possible that the protection may be linked to changes in the cellular levels of ethanolamine phospholipids such as PE-Pls mediated by this treatment (Figs. 10D & E). This is also supported by the observation that levels of PE-Pls in the cells prior to H$_2$O$_2$ treatment highly correlate ($r^2 = 0.95$) with cell viability following H$_2$O$_2$ exposure (Fig. 12C). Hossain et al. (13) have shown that plasmalogens (consisting of 96.5% ethanolamine plasmalogens and 2.5% choline plasmalogens) are capable of rescuing N2A from serum starvation-induced apoptosis. They treated N2A cells with 10% FBS (fetal bovine serum), 0.4% FBS and 0.4% FBS plus a mixture of plasmalogens (consisting of 96.5% ethanolamine plasmalogens and 2.5% choline plasmalogens) at concentrations of 5 μg/ml and 20 μg/ml for 72 hours and determined the cell survival rate and the activity of caspases involved in serum starvation-induced apoptosis. Their data demonstrate that plasmalogen concentrations of 5 μg/ml and 20 μg/ml significantly increased cell survival and enhanced phosphorylation of AKT and ERK 1/2, which are known to inhibit the cleavage of pro-apoptotic caspase-9 and caspase-3 (157-159). Our
data show that 650 μM H₂O₂ induced a significant decrease in apoptosis, while this effect was not observable in the cells pre-treated with ME prior to being exposed to this dose of H₂O₂. It has been shown that a moderate dose of H₂O₂ can trigger apoptosis, whereas an elevated dose induces necrotic cell death (225-227). For example, when T-lymphoma cells are treated with a dose of H₂O₂ that is equal or less than 300 μM for 6 h they display activation of caspase-9 and caspase-3 activity, resulting in apoptotic cell death (228-230). However, when these cells are exposed to 500 μM H₂O₂ for 6 h, they don’t display caspase activity and they die from necrosis (230). In the current study, N2A cells also responded to a dose of 650 μM H₂O₂ by decreasing apoptosis. This effect was not observed with ME + H₂O₂ treatment. In fact, the level of apoptosis was similar to that observed in the corresponding control (ME), suggesting that ME decreased necrosis. It has been reported that a lower concentration of H₂O₂ temporally inhibits the caspases enzymes, giving the cells the possibility to repair them for the apoptosis to resume (229). However at a higher dose of H₂O₂, the caspases remain inhibited, resulting in necrosis event (229). Caspase enzymes are sensitive to an increase in ROS and the cell must maintain an intracellular reducing environment for apoptosis to proceed (229). This suggests that ME+ H₂O₂ may be providing this environment, thus preventing cells from undergoing necrosis.

It has been suggested that the vinyl ether bond of plasmalogens is highly sensitive to oxidation than ether or ester linkages (16, 18, 40, 208, 231) and may be involved in cell protection against oxidative stress (12, 15, 40, 146). Although we cannot draw conclusion on the involvement of PE-PLs species in N2A cell protection against H₂O₂-induced oxidative damage, our data suggest that these species may be among the targets
since their levels decreased following H$_2$O$_2$ exposure. Interestingly, the cellular levels of PE were not decreased by H$_2$O$_2$ treatment. The mechanism by which ME treatment blocked the H$_2$O$_2$-induced decrease in PE-Pls levels in the cells is unclear and requires further investigation. The significance of PE-Pls in neuropathologies is highlighted by findings that neurodegenerative disorders/diseases are associated with a decrease in PE-Pls levels \((23, 24, 119)\). However, it remains to be elucidated if a decrease in PE-Pls is a contributing cause or downstream effect of pathology of these diseases/disorders \((232)\).

4.2.3 Myo-inositol plus ethanolamine blocks the H$_2$O$_2$-induced degradation of ethanolamine phospholipids

In order to get more insight on how H$_2$O$_2$ treatment affected ethanolamine phospholipids, we measured the levels of the degradation by-products (LPE and GPE) of PE and PE-Pls. Since ME treatment enhanced the PE-Pls levels and increased the cell viability \((80\%)\) following H$_2$O$_2$-induced oxidative damage, we expected a decrease in PE-Pls and an increase in both LPE and GPE levels if PE-Pls were being sacrificed. Thus, not observing these effects was a surprising outcome. LPE can give rise to GPE when the remaining acyl chain is hydrolyzed. Lysoplasmalogenase enzyme also known as alkenyl ether hydrolase (alkenylglycerophosphoethanolamine hydrolase) hydrolyzes lysoplasmalogens to yield GPE \((233-235)\). This enzyme is highly specific for glycerophospholipids containing a vinyl-ether linkage at sn-1 and a hydroxyl (lyso) at sn-2, and acts with nearly equal efficiency on ethanolamine and choline classes \((236)\). It is possible that H$_2$O$_2$ has an effect on the activity this enzyme and that effect was inhibited by ME treatment. The other possible explanation for these results is that the cells
preserve homeostasis through balancing PE-Pls degradation and synthesis/remodeling. The level of myo-inositol in this group dropped to the level below that of the control (ME), suggesting that it was actively being utilized, presumably for the synthesis of PE-Pls. In the neural membranes, the homeostasis of plasmalogens is controlled by a balance that must exist between their degradation, *de novo* synthesis and re-synthesis (re-acylation/de-acylation cycle) (52). We therefore cannot rule out the possibility that 1-alkenyl-2-lyso-GPE (lyso-plasmalogens) were re-acylated to form new PE-Pls, while the 1-acyl-2-lyso-GPE and 1-lyso-2-acyl-GPE were utilized to synthesize PE. This is supported by the observations that ME + H₂O₂ induced a 28% and 33% decrease in the cellular levels of 16:0/lyso and 18:0/lyso species, respectively, while the PE level was increased by 11% relative to the corresponding controls (ME treated cells). When the levels of LPE become high enough, they are capable of lysing the cell membranes because of their amphiphilic nature (237, 238). They are, therefore, maintained at very low levels (239). Their adversary effects involve modulating the activities of various enzymes (240), increasing the membrane fluidity (241), inhibiting cholinergic signal transduction (242) and activating cAMP dependent protein kinase A (243). In brain tissues, lyso-plasmalogens are rapidly used by CoA-independent transacylase enzymes in the remodeling pathway to re-synthesize plasmalogens (244, 245) in order to maintain their normal levels in neural membranes (3).
CHAPTER 5: RESULTS AND DISCUSSION FOR MASS SPECTROMETRY DATA

5.2 RESULTS

5.1.1 Molecular species composition of ethanolamine phospholipids in N2A cells

Different molecular species of PE-Pls, PE and LPE present in N2A cells were examined by electrospray ionization tandem quadrupole mass spectrometry (ESI-MS/MS) run in the positive ion mode (see methods for details).

A summary of all Etn PL subclasses detected by ESI-MS/MS in N2A cells is shown in Fig. 15. Molecular species in each subclass and their abundance are shown in Table 7-9. We identified and quantified a total of 57 molecular species of Etn PL, comprising 3 species of AAPE (Table 7), 19 species of PE-Pls (Table 7), 23 of PE (Table 8) and 12 species of LPE (Table 9). As illustrated in Fig. 15, PE-Pls and their precursors (AAPE) combined account for 54% of the entire Etn PL pool. The cellular contents of PE and LPE constitute 46% and 1.3% of this pool, respectively. Three different molecular species of AAPE (34:1-e; 36:1-e and 36:2-e) were identified (Table 7), which constitute 8.1% of the total Etn PL. The most abundant AAPE species is 34:1-e contributing 3.2% and 39.6% of total Etn PL and AAPE pool, respectively. This species (34:1-e) is the precursor for 34:1p PE-Pls species. Interestingly, 34:1p is also the most prevalent PE-Pls species in the cells, accounting for 10.4% and 23.0% of total Etn PL and PE-Pls pool, respectively. Both 36:1-e and 36:2-e are also the precursors for PE-Pls (36:1p and 36:2p PE-Pls species, respectively). They contribute 2.6% and 2.3% to entire Etn PL pool, respectively. In fact, 36:2p is the second most abundant PE-Pls in
N2A cells, constituting 11.8% and 2.7% of the entire PE-Pls pool and total ethanolamine PL, respectively. The first four most prevalent PE-Pls species (34:1p, 36:2p, 36:4p and 38:5p) combined account for 57% and 26% of PE-Pls pool and total Etn PL, respectively.

Table 8 shows the molecular species of PE synthesized by N2A cells. The first three most predominant species of PE are 34:1, 36:2 and 36:1, making 40.6% of the entire PE pool and about 18.1% of the total Etn pool.

As shown in Table 9, the contribution of LPE species to the entire pool of Etn PL in N2A cells is relatively minimal. LPE species only make 1.3% of all Etn PL contents in the cells. The sn-2 lyso products produced by the cells included 16:0/lyso, 18:0/lyso and 18:0p/lyso (Table 7). These are generated by phospholipase A2 (PLA2) enzymes that cleave the fatty acyl chains attached to sn-2 position of glycerophospholipids. Both 16:0/lyso and 18:0/lyso are generated from PE species, whereas 18:0p is synthesized from PE-Pls by plasmalogen-selective PLA2 (2). N2A cells also synthesize sn-1 lyso products. These can be produced by the phospholipase A1 enzymes (PLA1) (99) or the oxidation by the reactive oxygen species (15, 16). Nine out of twelve different LPE species synthesized by the cells are sn-1 lyso products (Table 7). These account for 91% of the entire LPE pool. Both 16:0/lyso and 18:0/lyso combined contribute 8% to the entire LPE pool while the contribution of 18:0p/lyso to this pool is only 2%. The most predominant LPE species in N2A cells are
lyso/18:0 and lyso/16:0, which account for 40.5% and 16.1% of the LPE pool, respectively.

**Table 7.** Molecular species of AAPE and PE-Pls in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>nmol/mg protein</th>
<th>% Etn Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>34:1-e</td>
<td>1.11 ± 0.12</td>
<td>3.20 ± 0.48</td>
</tr>
<tr>
<td>36:1-e</td>
<td>0.92 ± 0.12</td>
<td>2.58 ± 0.37</td>
</tr>
<tr>
<td>36:2-e</td>
<td>0.80 ± 0.09</td>
<td>2.25 ± 0.26</td>
</tr>
<tr>
<td>32:0p</td>
<td>0.02 ± 0.002</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>32:1p</td>
<td>0.36 ± 0.04</td>
<td>0.98 ± 0.09</td>
</tr>
<tr>
<td>34:1p</td>
<td>3.77 ± 0.47</td>
<td>10.43 ± 0.96</td>
</tr>
<tr>
<td>36:1p</td>
<td>0.99 ± 0.16</td>
<td>2.69 ± 0.31</td>
</tr>
<tr>
<td>38:1p</td>
<td>0.11 ± 0.2</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>34:2p</td>
<td>1.09 ± 0.14</td>
<td>2.98 ± 0.23</td>
</tr>
<tr>
<td>36:2p</td>
<td>1.96 ± 0.26</td>
<td>5.36 ± 0.36</td>
</tr>
<tr>
<td>36:3p</td>
<td>0.35 ± 0.05</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>38:3p</td>
<td>0.43 ± 0.07</td>
<td>1.17 ± 0.10</td>
</tr>
<tr>
<td>40:3p</td>
<td>0.03 ± 0.004</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>36:4p</td>
<td>1.95 ± 0.25</td>
<td>5.39 ± 0.56</td>
</tr>
<tr>
<td>38:4p</td>
<td>1.43 ± 0.21</td>
<td>3.87 ± 0.41</td>
</tr>
<tr>
<td>40:4p</td>
<td>0.14 ± 0.02</td>
<td>0.39 ± 0.06</td>
</tr>
<tr>
<td>36:5p</td>
<td>0.17 ± 0.02</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>38:5p</td>
<td>1.68 ± 0.22</td>
<td>4.59 ± 0.40</td>
</tr>
<tr>
<td>40:5p</td>
<td>0.27 ± 0.04</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>38:6p</td>
<td>1.00 ± 0.15</td>
<td>2.73 ± 0.31</td>
</tr>
<tr>
<td>40:6p</td>
<td>0.56 ± 0.09</td>
<td>1.52 ± 0.21</td>
</tr>
<tr>
<td>40:7p</td>
<td>0.41 ± 0.6</td>
<td>1.10 ± 0.09</td>
</tr>
</tbody>
</table>

* e. ether linked (AAPE); p. plasmalogen.

See abbreviations on pages Xiii – Xvi.
Table 8. Molecular species of PE in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>nmol/mg protein</th>
<th>% Etn Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>28:0</td>
<td>0.007 ± 0.006</td>
<td>0.016 ± 0.11</td>
</tr>
<tr>
<td>34:0</td>
<td>0.31 ± 0.06</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>36:0</td>
<td>0.28 ± 0.05</td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td>34:1</td>
<td>2.56 ± 0.45</td>
<td>6.72 ± 0.71</td>
</tr>
<tr>
<td>36:1</td>
<td>1.95 ± 0.36</td>
<td>5.03 ± 0.47</td>
</tr>
<tr>
<td>34:2</td>
<td>1.38 ± 0.22</td>
<td>3.69 ± 0.43</td>
</tr>
<tr>
<td>36:2</td>
<td>2.42 ± 0.41</td>
<td>6.38 ± 0.69</td>
</tr>
<tr>
<td>38:2</td>
<td>0.76 ± 0.14</td>
<td>2.00 ± 0.25</td>
</tr>
<tr>
<td>34:3</td>
<td>0.08 ± 0.01</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>36:3</td>
<td>0.59 ± 0.10</td>
<td>1.55 ± 0.17</td>
</tr>
<tr>
<td>38:3</td>
<td>0.87 ± 0.16</td>
<td>2.26 ± 0.24</td>
</tr>
<tr>
<td>40:3</td>
<td>0.13 ± 0.03</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>36:4</td>
<td>0.20 ± 0.04</td>
<td>0.52 ± 0.08</td>
</tr>
<tr>
<td>38:4</td>
<td>1.64 ± 0.31</td>
<td>4.27 ± 0.51</td>
</tr>
<tr>
<td>40:4</td>
<td>0.24 ± 0.05</td>
<td>0.61 ± 0.06</td>
</tr>
<tr>
<td>36:5</td>
<td>0.11 ± 0.02</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>38:5</td>
<td>1.22 ± 0.22</td>
<td>3.19 ± 0.37</td>
</tr>
<tr>
<td>40:5</td>
<td>0.70 ± 0.13</td>
<td>1.82 ± 0.18</td>
</tr>
<tr>
<td>38:6</td>
<td>0.11 ± 0.02</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>40:6</td>
<td>1.07 ± 0.21</td>
<td>2.79 ± 0.33</td>
</tr>
<tr>
<td>38:7</td>
<td>0.04 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>40:7</td>
<td>0.43 ± 0.08</td>
<td>1.13 ± 0.14</td>
</tr>
<tr>
<td>40:8</td>
<td>0.06 ± 0.01</td>
<td>0.15 ± 0.02</td>
</tr>
</tbody>
</table>

See abbreviations on pages Xiii – Xvi.
Table 9. Molecular species of LPE in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>nmol/mg protein</th>
<th>% Etn Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0/lyso*</td>
<td>0.015 ± 0.008</td>
<td>0.045 ± 0.028</td>
</tr>
<tr>
<td>18:0/lyso*</td>
<td>0.026 ± 0.013</td>
<td>0.081 ± 0.049</td>
</tr>
<tr>
<td>18:0p/lyso</td>
<td>0.011 ± 0.007</td>
<td>0.035 ± 0.024</td>
</tr>
<tr>
<td>lyso/14:0</td>
<td>0.004 ± 0.002</td>
<td>0.014 ± 0.009</td>
</tr>
<tr>
<td>lyso/16:0*</td>
<td>0.072 ± 0.019</td>
<td>0.187 ± 0.039</td>
</tr>
<tr>
<td>lyso/18:0*</td>
<td>0.180 ± 0.043</td>
<td>0.468 ± 0.086</td>
</tr>
<tr>
<td>lyso/20:3</td>
<td>0.035 ± 0.017</td>
<td>0.088 ± 0.037</td>
</tr>
<tr>
<td>lyso/20:4</td>
<td>0.045 ± 0.027</td>
<td>0.103 ± 0.051</td>
</tr>
<tr>
<td>lyso/22:4</td>
<td>0.052 ± 0.017</td>
<td>0.121 ± 0.035</td>
</tr>
<tr>
<td>lyso/20:5</td>
<td>0.028 ± 0.019</td>
<td>0.069 ± 0.042</td>
</tr>
<tr>
<td>lyso/22:5</td>
<td>0.030 ± 0.014</td>
<td>0.078 ± 0.030</td>
</tr>
<tr>
<td>lyso/22:6</td>
<td>0.016 ± 0.007</td>
<td>0.040 ± 0.016</td>
</tr>
</tbody>
</table>

* Peaks for these species were differentiated based on their elution times (see methods for details);

p. plasmalogen. See abbreviations on pages Xiii – Xvi.
5.1.2 **Myo-inositol plus ethanolamine preferentially elevates the levels of ethanolamine plasmalogen species containing saturated and monounsaturated fatty acyl chains in N2A cells**

We determined the effect of ME treatment on the Etn PL pool. As shown in **Fig. 16A**, ME treatment increased Etn PL content by 47% relative to control cells (p ≤ 0.05). This treatment induced an overall 73% increase in the AAPE pool, a 62% increase in PE species, a 26% increase in PE-Pls, and a 125% increase in LPE species (p ≤ 0.05) (**Fig. 16B**; **Table 10**).

![Figure 16](image-url)

*Figure 16.* Effects of ME and H₂O₂ treatments on (A) Etn PL pool; (B) Etn PL subclasses in N2A cells (Mean ± SE). a. denotes significant differences (p < 0.05; n = 7-8) from control group. See abbreviations on pages Xi–Xvi.

For visualization on how each treatment affected the molecular species of Etn PL, we constructed a heatmap using the Logarithm-base 2 transformed concentration values for each species measured (**Fig. 17A**). In this heatmap, the species with an abundance that is less than 1 nmol/mg protein are shown in blue, whereas those with an abundance that is above 1 nmol/mg protein are shown in red. The cellular concentration of those that are shown in white color is equal to 1 nmol/mg protein. Comparing ME treatment versus the control group shows an effect on some of the Etn PL species. However, the figure does
not display the magnitude of the changes relative to the control. To have a clear insight on the magnitude of changes induced by treatments, we calculated the fractional change (FC) induced by each treatment relative to the standard deviation of the corresponding control group as shown in Equation (1) and constructed another heatmap figure using these values (Fig. 17B).

\[
FC = \frac{\text{Treated} - \text{Control}}{\text{SDEV for control}}
\]  

(1)

As illustrated in Fig. 17B, ME increased the levels of all the AAPE species in the cells. Changes in their cellular levels ranged from 1.5-fold to 2-fold increase and 34:1 was the most affected AAPE species by the treatment \( (p = 0.0004, \text{Table 11}) \). Exposing cells to ME preferentially enhanced the levels of all PE-Pls species rich in SFA (32:0p) and MUFA (32:1p; 34:1p; 36:1p and 38:1p) relative to PUFA species \( (p \leq 0.05; \text{Fig. 17B; Table 11}) \). The increase ranged from 26% to 220%. The most affected PE-Pls species were 32:0p and 32:1p (2-fold increase).

Figures 18B and 18C show the effect of treatments on the abundance (nmol/mg protein) of PE-Pls species containing SFA + MUFA and those containing PUFA, respectively. ME increased the levels of PE-Pls species containing SFA + MUFA by 60% relative to those measured in control group (Fig. 18B) and their relative proportion of the pool size increased from 32% to 40% (Fig. 18A). Interestingly, the cellular levels of PE-Pls species that contain

<table>
<thead>
<tr>
<th>Etn Subclass</th>
<th>ME</th>
<th>H(_2)O(_2)</th>
<th>ME + H(_2)O(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAPE</td>
<td>+ 72.7</td>
<td>-7.1</td>
<td>+ 9.9</td>
</tr>
<tr>
<td>PE</td>
<td>+ 61.5</td>
<td>+ 0.1</td>
<td>+ 10.8</td>
</tr>
<tr>
<td>PE-Pls</td>
<td>+ 25.8</td>
<td>-27.2</td>
<td>+ 14.7</td>
</tr>
<tr>
<td>LPE</td>
<td>+1 25.4</td>
<td>+67.6</td>
<td>-12.2</td>
</tr>
</tbody>
</table>

Table 10. Effects of ME and H\(_2\)O\(_2\) on PE and PE-Pls levels (% change relative to control) in N2A cells
PUFA were only increased by 10% relative to those measured in the control cells (Fig. 18C, \( p \leq 0.05 \)), and their relative proportion of the pool size decreased from 68% to 60% (Fig. 18A). Most of these species showed little or no change in their levels following ME treatment, except 36:2p species which increased by 49% (\( p = 0.006 \)). It is worthy to note that the precursor for this species (36:2-e) was also among the species that were significantly increased by ME (\( p = 0.01 \)). Nevertheless, four species in this category slightly decreased by less than 10% (Table 11), while those for 38:3p and 40:3p decreased by 10.7% and 18.5%, respectively, although the decrease was not significant.

![Heatmap showing effects of ME and H₂O₂ treatments on the abundance of different molecular species of Etn PLs in N2A cells. The heatmap was generated using the log-base 2 transform of the concentration values (nmol/mg protein) for each species. See abbreviations on pages Xiii – Xvi.](image)

**Figure 17 A.** Heatmap showing effects of ME and H₂O₂ treatments on the abundance of different molecular species of Etn PLs in N2A cells. The heatmap was generated using the log-base 2 transform of the concentration values (nmol/mg protein) for each species. See abbreviations on pages Xiii – Xvi.
**Figure 17 B.** Heatmap showing changes induced by ME and H$_2$O$_2$ treatments on different molecular species of Etn PLs in N2A cells. The heatmap was generated using the log-base 2 transform of the changes in concentration for each species in a treated group relative to its corresponding control. These changes were calculated using equation (1). See abbreviations on pages Xiii – Xvi.
<table>
<thead>
<tr>
<th>Assignment</th>
<th>Control</th>
<th>$\text{H}_2\text{O}_2$</th>
<th>ME</th>
<th>ME + $\text{H}_2\text{O}_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>34:1-e</td>
<td>1.11 ± 0.12</td>
<td>1.11 ± 0.29</td>
<td>2.17 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.40 ± 0.50</td>
</tr>
<tr>
<td>36:1-e</td>
<td>0.92 ± 0.12</td>
<td>0.83 ± 0.22</td>
<td>1.54 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.72 ± 0.37</td>
</tr>
<tr>
<td>36:2-e</td>
<td>0.80 ± 0.09</td>
<td>0.68 ± 0.18</td>
<td>1.16 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.25</td>
</tr>
<tr>
<td>32:0p</td>
<td>0.02 ± 0.002</td>
<td>0.01 ± 0.003</td>
<td>0.04 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>32:1p</td>
<td>0.36 ± 0.04</td>
<td>0.27 ± 0.07</td>
<td>0.74 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76 ± 0.19</td>
</tr>
<tr>
<td>34:1p</td>
<td>3.77 ± 0.47</td>
<td>2.84 ± 0.72</td>
<td>5.92 ± 0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.16 ± 1.41</td>
</tr>
<tr>
<td>36:1p</td>
<td>0.99 ± 0.16</td>
<td>0.77 ± 0.20</td>
<td>1.53 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.62 ± 0.37</td>
</tr>
<tr>
<td>38:1p</td>
<td>0.11 ± 0.2</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>34:2p</td>
<td>1.09 ± 0.14</td>
<td>0.78 ± 0.22</td>
<td>1.33 ± 0.10</td>
<td>1.52 ± 0.38</td>
</tr>
<tr>
<td>36:2p</td>
<td>1.96 ± 0.26</td>
<td>1.39 ± 0.36</td>
<td>2.93 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.05 ± 0.77</td>
</tr>
<tr>
<td>36:3p</td>
<td>0.35 ± 0.05</td>
<td>0.23 ± 0.06</td>
<td>0.45 ± 0.04</td>
<td>0.50 ± 0.13</td>
</tr>
<tr>
<td>38:3p</td>
<td>0.43 ± 0.07</td>
<td>0.28 ± 0.08</td>
<td>0.39 ± 0.02</td>
<td>0.41 ± 0.10</td>
</tr>
<tr>
<td>40:3p</td>
<td>0.03 ± 0.004</td>
<td>0.02 ± 0.004</td>
<td>0.02 ± 0.002</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>36:4p</td>
<td>1.95 ± 0.25</td>
<td>1.41 ± 0.40</td>
<td>1.80 ± 0.12</td>
<td>2.41 ± 0.65</td>
</tr>
<tr>
<td>38:4p</td>
<td>1.43 ± 0.21</td>
<td>1.06 ± 0.30</td>
<td>1.37 ± 0.08</td>
<td>1.76 ± 0.46</td>
</tr>
<tr>
<td>40:4p</td>
<td>0.14 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.14 ± 0.01</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>36:5p</td>
<td>0.17 ± 0.02</td>
<td>0.12 ± 0.03</td>
<td>0.16 ± 0.01</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>38:5p</td>
<td>1.68 ± 0.22</td>
<td>1.14 ± 0.33</td>
<td>1.62 ± 0.12</td>
<td>2.13 ± 0.60</td>
</tr>
<tr>
<td>40:5p</td>
<td>0.27 ± 0.04</td>
<td>0.19 ± 0.05</td>
<td>0.27 ± 0.01</td>
<td>0.34 ± 0.09</td>
</tr>
<tr>
<td>38:6p</td>
<td>1.00 ± 0.15</td>
<td>0.76 ± 0.22</td>
<td>1.11 ± 0.08</td>
<td>1.48 ± 0.41</td>
</tr>
<tr>
<td>40:6p</td>
<td>0.56 ± 0.09</td>
<td>0.44 ± 0.12</td>
<td>0.61 ± 0.04</td>
<td>0.80 ± 0.21</td>
</tr>
<tr>
<td>40:7p</td>
<td>0.41 ± 0.06</td>
<td>0.27 ± 0.07</td>
<td>0.44 ± 0.05</td>
<td>0.60 ± 0.18</td>
</tr>
</tbody>
</table>

<sup>e</sup>. ether linked (AAPE); <sup>p</sup>. plasmalogen; <sup>a</sup>. differs from control group ($p \leq 0.05$).

See abbreviations on pages Xiii – Xvi.
Figure 18. Effects of ME and H$_2$O$_2$ treatments on the (A) proportion of SFA + MUFA-rich PE-Pls and PUFA-rich PE-Pls relative to PE-Pls pool; (B) abundance of SFA + MUFA-rich PE-Pls; (C) abundance of PUFA-rich PE-Pls in N2A cells. a. denotes significant differences (p < 0.05; n = 7-8) from control group. See abbreviations on pages Xiii – Xvi.
5.1.3 *Myo*-inositol plus ethanolamine enhances the levels of phosphatidylethanolamine in N2A cells

ME preferentially increased the PE pool relative to that for PE-Ps (Fig. 16B). This treatment elevated the PE contents of the cells by 62% relative to the control cells (p = 0.01). As illustrated in Fig. 17B and Table 12, ME increased significantly the cellular levels of all PE species (p ≤ 0.05), except those for 28:0, 34:3, 40:3, 36:5, 38:5 and 40:8 species. In fact, the increase in the level of 40:3 was nearly significant (p = 0.08). However, 28:0 was the only PE species that displayed a decrease (30%) in its cellular level associated with ME treatment, although the decrease was not significant. The increase in PE levels ranged from 20% (for 34:3 species) to 200% (for 34:0 species). Interestingly, ME preferentially elevated the levels of PE species that contain SFA and MUFA (Fig. 19A-B; Table 12), except for 28:0 (Fig. 17B). Their contribution to the PE pool changed from 30% (control) to 34% (ME group) (Fig. 19A). The abundance (nmol/mg protein) of PE species containing SFA + MUFA displayed an overall level increase of 87% relative to those measured in the control group (p < 0.003) (Fig. 19B). The abundance of PE species that contain PUFA increased by 51% relative to those measured in the control cells (Fig. 19C, p ≤ 0.05).

We also investigated the effect of ME treatment on the concentration of LPE species in N2A cells. ME treatment elevated the cellular levels of LPE by an average of 2.3-fold relative to control. ME also preferentially elevated the LPE species that contain SFA (Table 13). The most predominant LPE species were lyso/18:0 and lyso/16:0, which constituted 46.2% and 20.6% of LPE pool, respectively. ME elevated their cellular
contents by 2.5-fold and 2.8-fold, respectively. Lyso/18:0 and lyso/16:0 are also the most predominant LPE species in the control group (Table 13).

5.1.4 Myo-inositol plus ethanolamine blocks the H$_2$O$_2$-induced degradation of ethanolamine plasmalogens in N2A cells

We determined the impact of H$_2$O$_2$ treatment on the levels of PE-Pls and PE species in N2A cells. As shown in Fig. 16A, exposing N2A cells to H$_2$O$_2$ decreased their Etn PL contents by 12% relative to control cells, but the decrease was not significant. H$_2$O$_2$ induced an overall 7% decrease in the cellular levels of AAPE pool, although the decrease was not significant. The levels of all PE-Pls species dropped without exception (Fig. 17B). The decrease in AAPE levels ranged from 20% (for 40:6p) to 42% (for 40:3p). It appeared that all the PE-Pls were prone to degradation regardless of the degree of saturation and length of the fatty acyl chains. H$_2$O$_2$ induced a 7% decrease in AAPE pool, a 27% decrease in PE-Pls pool, a 0.1% increase in PE pool and a 68% increase in LPE pool (Table 10). The cellular levels of PE-Pls species that contain SFA and MUFA were decreased by 28%, while those that contain PUFA dropped by 25% (Fig. 18A & B). Interestingly, all the effects associated with H$_2$O$_2$ on the cellular levels of AAPE and PE-Pls species were blocked when the cells were pre-treated with ME prior to H$_2$O$_2$ exposure (ME + H$_2$O$_2$ group) (Fig 17B and Table 11). Exceptionally, the levels for 32:0p and 38:1p species decreased by 9 and 13%, respectively, while all other species were either unaffected or displayed an increase in response to H$_2$O$_2$. In fact, ME + H$_2$O$_2$ group displayed a 15% average increase in PE-Pls pool relative to that measured in the corresponding control (ME), although this increase was not significant (Table 10).

Another interesting observation that is worthy to highlight is that ME + H$_2$O$_2$ group
preferentially increased the PE-Pls that contain PUFA (4-7 double bonds). However, these species were among those that displayed little or no change following ME treatment (Fig. 17B, Table 11). These data suggest that pretreating the cells with ME inhibits the H$_2$O$_2$-induced degradation of PE-Pls in N2A cells.

It was remarkable to observe that H$_2$O$_2$ did not impact the concentration of PE species in N2A cells (Fig 17B, Table 10, Table 12). In fact, the overall influence of this treatment on these species was negligible (0.1% increase). Regardless of the degree of saturation and length of their fatty acyl chains, the PE species are more resilient to H$_2$O$_2$-induced degradation (Fig. 19B-C) than the PE-Pls species.

Figure 19. Effects of ME and H$_2$O$_2$ treatments on the (A) proportion of SFA + MUFA-rich PE and PUFA-rich PE relative to PE pool; (B) abundance of SFA + MUFA-rich PE; (C) abundance of PUFA-rich PE in N2A cells. a. denotes significant differences (p < 0.05; n = 7-8) from control group. See abbreviations on pages Xiii – Xvi.
Table 12. Effects of ME and H₂O₂ treatments on molecular species of PE in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Control</th>
<th>H₂O₂</th>
<th>ME</th>
<th>ME + H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>28:0</td>
<td>0.007 ± 0.006</td>
<td>0.005 ± 0.003</td>
<td>0.01 ± 0.002</td>
<td>0.01 ± 0.008</td>
</tr>
<tr>
<td>34:0</td>
<td>0.31 ± 0.06</td>
<td>0.30 ± 0.08</td>
<td>0.61 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63 ± 0.13</td>
</tr>
<tr>
<td>36:0</td>
<td>0.28 ± 0.05</td>
<td>0.30 ± 0.09</td>
<td>0.52 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.54 ± 0.10</td>
</tr>
<tr>
<td>34:1</td>
<td>2.56 ± 0.45</td>
<td>2.76 ± 0.77</td>
<td>4.91 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.07 ± 0.89</td>
</tr>
<tr>
<td>36:1</td>
<td>1.95 ± 0.36</td>
<td>2.07 ± 0.55</td>
<td>3.49 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.82 ± 0.82</td>
</tr>
<tr>
<td>34:2</td>
<td>1.38 ± 0.22</td>
<td>1.37 ± 0.37</td>
<td>1.93 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.27 ± 0.47</td>
</tr>
<tr>
<td>36:2</td>
<td>2.42 ± 0.41</td>
<td>2.32 ± 0.59</td>
<td>3.78 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.24 ± 0.92</td>
</tr>
<tr>
<td>38:2</td>
<td>0.76 ± 0.14</td>
<td>0.69 ± 0.18</td>
<td>1.18 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.18 ± 0.22</td>
</tr>
<tr>
<td>34:3</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.10 ± 0.01</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>36:3</td>
<td>0.59 ± 0.10</td>
<td>0.53 ± 0.14</td>
<td>0.82 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95 ± 0.22</td>
</tr>
<tr>
<td>38:3</td>
<td>0.87 ± 0.16</td>
<td>0.79 ± 0.20</td>
<td>1.35 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.43 ± 0.30</td>
</tr>
<tr>
<td>40:3</td>
<td>0.13 ± 0.03</td>
<td>0.11 ± 0.03</td>
<td>0.18 ± 0.02</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td>36:4</td>
<td>0.20 ± 0.04</td>
<td>0.20 ± 0.06</td>
<td>0.31 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td>38:4</td>
<td>1.64 ± 0.31</td>
<td>1.63 ± 0.45</td>
<td>2.43 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.87 ± 0.65</td>
</tr>
<tr>
<td>40:4</td>
<td>0.24 ± 0.05</td>
<td>0.23 ± 0.06</td>
<td>0.37 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.43 ± 0.09</td>
</tr>
<tr>
<td>36:5</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.15 ± 0.02</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>38:5</td>
<td>1.22 ± 0.22</td>
<td>1.11 ± 0.30</td>
<td>1.69 ± 0.19</td>
<td>1.94 ± 0.43</td>
</tr>
<tr>
<td>40:5</td>
<td>0.70 ± 0.13</td>
<td>0.76 ± 0.21</td>
<td>1.13 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.32 ± 0.27</td>
</tr>
<tr>
<td>38:6</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.19 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>40:6</td>
<td>1.07 ± 0.21</td>
<td>1.18 ± 0.33</td>
<td>1.78 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.09 ± 0.42</td>
</tr>
<tr>
<td>38:7</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>40:7</td>
<td>0.43 ± 0.08</td>
<td>0.42 ± 0.11</td>
<td>0.66 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74 ± 0.16</td>
</tr>
<tr>
<td>40:8</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>. differs from control group (p ≤ 0.05). See abbreviations on pages Xiii – Xvi.
Table 13. Effects of ME and H$_2$O$_2$ treatments on molecular species of LPE in N2A cells (Mean ± SE)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>% Total ethanolamine phospholipids</th>
<th>Control</th>
<th>H$_2$O$_2$</th>
<th>ME</th>
<th>ME + H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0/lyso*</td>
<td>0.015 ± 0.008</td>
<td>0.017 ± 0.007</td>
<td>0.029 ± 0.010</td>
<td>0.021 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>18:0/lyso*</td>
<td>0.026 ± 0.013</td>
<td>0.037 ± 0.016</td>
<td>0.055 ± 0.024</td>
<td>0.037 ± 0.012</td>
<td></td>
</tr>
<tr>
<td>18:0p/lyso</td>
<td>0.011 ± 0.007</td>
<td>0.022 ± 0.009</td>
<td>0.018 ± 0.010</td>
<td>0.015 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>lyso/14:0</td>
<td>0.004 ± 0.002</td>
<td>0.004 ± 0.001</td>
<td>0.006 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td></td>
</tr>
<tr>
<td>lyso/16:0*</td>
<td>0.072 ± 0.019</td>
<td>0.051 ± 0.016</td>
<td>0.205 ± 0.054$^a$</td>
<td>0.196 ± 0.061</td>
<td></td>
</tr>
<tr>
<td>lyso/18:0*</td>
<td>0.180 ± 0.043</td>
<td>0.121 ± 0.037</td>
<td>0.459 ± 0.102$^a$</td>
<td>0.434 ± 0.119</td>
<td></td>
</tr>
<tr>
<td>lyso/20:3</td>
<td>0.035 ± 0.017</td>
<td>0.131 ± 0.062</td>
<td>0.071 ± 0.046</td>
<td>0.055 ± 0.029</td>
<td></td>
</tr>
<tr>
<td>lyso/20:4</td>
<td>0.045 ± 0.027</td>
<td>0.211 ± 0.099</td>
<td>0.106 ± 0.063</td>
<td>0.091 ± 0.044</td>
<td></td>
</tr>
<tr>
<td>lyso/22:4</td>
<td>0.052 ± 0.017</td>
<td>0.019 ± 0.004</td>
<td>0.061 ± 0.027</td>
<td>0.043 ± 0.015</td>
<td></td>
</tr>
<tr>
<td>lyso/20:5</td>
<td>0.028 ± 0.019</td>
<td>0.054 ± 0.031</td>
<td>0.026 ± 0.017</td>
<td>0.013 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>lyso/22:5</td>
<td>0.030 ± 0.014</td>
<td>0.131 ± 0.061</td>
<td>0.068 ± 0.042</td>
<td>0.058 ± 0.029</td>
<td></td>
</tr>
<tr>
<td>lyso/22:6</td>
<td>0.016 ± 0.007</td>
<td>0.067 ± 0.031</td>
<td>0.039 ± 0.024</td>
<td>0.032 ± 0.016</td>
<td></td>
</tr>
</tbody>
</table>

* Peaks for these species were differentiated based on their elution times (see methods for details); p. plasmalogen; a. differs from control group (p ≤ 0.05). See abbreviations on pages Xiii – Xvi.
5.1.5 Myo-inositol plus ethanolamine blocks the H$_2$O$_2$-induced increase in lysoglycerophosphoethanolamine levels in N2A cells

The effect of H$_2$O$_2$ on the concentration of LPE species in N2A cells was also assessed. As shown in Fig. 20A-C, H$_2$O$_2$ changed the balance between the levels of LPE that contain PUFA and those that contain SFA. This treatment induced a 19% decrease in the levels of LPE containing SFA relative to those in the control group, although this decrease was not significant (Fig. 20B). Conversely, the levels of LPE containing PUFA displayed a 3-fold increase relative to those in the control group (p ≤ 0.05) (Fig. 20C). In this category, the species that were preferentially affected include lyso/20:4, lyso/22:5, lyso/22:6 and lyso/20:3, which increased by 4.7-fold, 4.4-fold, 4.2-fold and 3.7-fold, respectively (Table 13). Lyso/22:4 is the only PUFA LPE species that decreased in concentration following H$_2$O$_2$ treatment. Interestingly, in response to H$_2$O$_2$ the levels of all the $sn$-1 lyso products that contain SFA decreased (lyso/14:0, lyso/16:0 and lyso/18:0), while those for $sn$-2 lyso products (18:0p/lyso, 16:0/lyso and 18:0/lyso) were slightly increased (Fig. 17B, Table 13). In fact, exposing cells to H$_2$O$_2$ increased the level of 18:0p/lyso product by 2-fold relative to that measured in the control group. Our data show that only PE-Pls species dropped in their levels by 27% following exposure to H$_2$O$_2$, whereas those for PE species remained unchanged (Fig. 20D), suggesting that most of the LPE were generated from the degradation of PE-Pls. Table 14 identifies the probable parents (PE-Pls) for LPE species in H$_2$O$_2$ group and ME + H$_2$O$_2$ group based on the reports showing that $sn$-1 position of PE-Pls contains C16:0, C18:0, C18:1 acyl chains (48-50). Microsoft Excel software was used to perform the correlation analyses between LPE species and their probable PE-Pls parents (using concentrations values).
The Table 14 only highlights correlations where $r^2$ was $\geq |0.5|$. The unique species of lysoplasmalogen (18:0p/lyso) strongly correlated with nine out of twelve probable PE-Pls parents in H$_2$O$_2$ group, while in ME + H$_2$O$_2$ group this species only correlated with five PE-Pls species. These data suggest that plasmalogen-selective PLA$_2$ was relatively more active in H$_2$O$_2$ group than in ME + H$_2$O$_2$ group. For instance, 18:0p/lyso correlated well with 34:1p in H$_2$O$_2$ group ($r^2 = -0.9$), while displaying no correlation with this same PE-Pls species in ME + H$_2$O$_2$ group ($r^2 = -0.3$). This may not be surprising since H$_2$O$_2$ induced a 2-fold increase in 18:0p/lyso level (Fig. 23), while the level of this species decreased by 17% in ME + H$_2$O$_2$ group (Fig. 23). In order to assess how H$_2$O$_2$ treatment affected the balance between the levels of PE-Pls and those of LPE in the cells, we calculated the LPE/PE-Pls ratio. As illustrated in Fig. 20E, this ratio increased by 2.3-fold relative to that calculated for the control group ($p \leq 0.05$). Since our data show that H$_2$O$_2$ preferentially increased the cellular levels of LPE containing PUFA (from 34% to 52%) (Fig. 20A), we then calculated two ratios: (1) the LPE/PE-Pls ratio for PUFA-containing species (Fig. 20F) and (2) the LPE/PE-Pls ratio for (SFA+MUFA)-containing species (Fig. 20G). As shown in Fig. 20F, the first ratio increased by 4.5-fold relative to that calculated for the control group ($p \leq 0.05$), while the second ratio (Fig. 20G) did not change, suggesting that H$_2$O$_2$ preferentially induced oxidation of PE-Pls rich in PUFA.

Amazingly, the H$_2$O$_2$-induced degradation of PE-Pls was not observed in the cells pre-treated with ME prior to H$_2$O$_2$ exposure (ME + H$_2$O$_2$ group) (Fig. 17B, Table 13). Additionally, no significant changes in the cellular levels of LPE were observed with ME + H$_2$O$_2$ treatment when compared to control cells (Fig. 20C, Fig. E-G). It is interesting to note that this treatment yielded a 13% decrease in LPE pool, while that in H$_2$O$_2$ group
increased by 39% relative to corresponding control (Fig. 20D). Additionally, while the levels of PE species were mostly unaffected by H$_2$O$_2$ treatment, those cells pretreated with ME and then exposed to H$_2$O$_2$ showed an 11% increase in the level of these species (although not statistically significant), and an even larger increase (15%) in PE-Pls levels (Fig. 20D). These data suggest that PE species are more resistant to H$_2$O$_2$-mediated degradation. The data also implies that the levels of PE-Pls species are not affected by H$_2$O$_2$ only when the cells are pre-treated with ME.
Figure 20A-C. Effects of ME and H$_2$O$_2$ treatments on the (A) proportion of SFA-rich LPE and PUFA-rich LPE relative to LPE pool; (B) abundance of LPE containing SFA; (C) abundance of LPE containing PUFA in N2A cells. α denotes significant differences ($p < 0.05$; $n = 7-8$) from control group. See abbreviations on pages Xiii – Xvi.
Figure 20D-G. Effects of ME and H$_2$O$_2$ treatments on the (D) LPE levels (shown here as % change relative to corresponding control in N2A cells. The % change for PE in H$_2$O$_2$ group is near zero (0.1%), thus only error bar is showing. (E) LPE/PE-Pls ratio; (F) LPE/PE-Pls ratio for PUFA-containing species; (G) LPE/PE-Pls for (SFA + MUFA)-containing species; a. denotes significant differences (p < 0.05; n = 7-8) from control group. See abbreviations on pages Xiii – Xvi.
Table 14. Probable PE-Pls parents of LPE for control cells (C) exposed to H$_2$O$_2$ and ME-treated cells (T) exposed to H$_2$O$_2$ (T) based on the assumption that the sn-1 position of PE-Pls contains C16:0, C18:0 and C18:1 acyl chains (48-50). The values highlighted in yellow indicate the length of acyl chains that PE-Pls must lose in order to yield the corresponding LPE species. The negative values indicate the correlation coefficients between LPE species and probable PE-Pls parent. Only correlations where $r^2$ was $\geq 0.5$ are shown in the table. L: lyso; C: control; T: treated.
5.2 DISCUSSION

5.2.1 Composition of Ethanolamine phospholipids in N2A cells

We have demonstrated that treating N2A cells with ME increases PE-Pls, PE, and other non-Etn PL pools, and provides protection against H$_2$O$_2$-mediated oxidative stress. We have also shown that exposing these cells to H$_2$O$_2$ reduces the level of the PE-Pls pool and increase that of LPE without significantly affecting PE levels. The main emphasis of this section of the study was to determine (1) which Etn PL molecular species are synthesized by N2A cells and (2) how they are affected by ME treatment and H$_2$O$_2$–induced oxidation. In regard to molecular species composition within Etn PL subclasses, PE-Pls and their precursor species (AAPE) combined contribute 54% to the entire pool of Etn PLs. This contribution is similar to what Han et al. (246) have reported in neurons. Han et al., (246) have indicated that PE-Pls constitute about 60 mol% of Etn PL pool in neurons and synaptosomes, while in non-neuronal brain cell membranes, their contribution can even exceed 80%. We found that 34:1p is the most abundant PE-Pls species in the cells, representing 23% of the entire PE-Pls pool. Interestingly, 34:1-e is also the most predominant AAPE species (40% of the AAPE pool), which is an immediate precursor for 34:1p PE-Pls species. We also found that 34:1 is the most predominant PE species constituting 15% of the PE pool. Each of these three species has 34 carbons and one double bond in their acyl chains. The only distinguishing feature is the type of bonds attaching their acyl chains at the sn-1 position to the glycerol backbone. It has been reported that the acyl chain at the sn-1 position is typically C16:0, C18:0 or C18:1 (3, 5, 39-41). Additionally, substrate specificity studies which investigated the selectivity of alkylglycerophosphate acyltransferase (LPAAT) also known as alkyl/acyl-
glycero-3-phosphate acyltransferase (AAG3P-AT; EC 2.3.1.51) on different acyl-coA species (16:0, 18:0, 18:2, 20:4, 22:4, 22:6) have shown that LPAAT specificity for acyl chains depends on the concentration of 1-alkyl-\(sn\)-glycero-3-phosphate (its substrate) (79). This enzyme prefers using polyunsaturated acyl-CoA over saturated species when 1-alkyl-\(sn\)-glycero-3-phosphate is available at low concentrations (79). If these conditions are also applied to N2A cells, the concentration of 1-alkyl-\(sn\)-glycero-3-phosphate is probably at a high level to the point where LPAAT actively esterifies C16:0 and C18:0 acyl chains at the \(sn\)-2 position of 34:1-e and 36:1-e AAPE species. Therefore, the 34:1-e and 36:1-e AAPE species would correspond to 18:1-e/16:0 and 18:1-e/18:0, giving rise to 34:1p and 36:1p PE-Pls species (corresponding to 18:1p/16:0 and 18:1p/18:0 species). Fleming and Hajra (79) carried out investigations on the specificity of this enzyme in kidney, spleen, heart, lung, liver and brain rat tissues and concluded that the activity of acyltransferase enzyme could partially determine the composition of fatty acyl groups esterified in lipids. We cannot conclude for sure that the acyltransferase in N2A cells is also behaving in the similar manner to this enzyme in rat tissues. Horrocks and Sharma (247) reported that the \(sn\)-2 position of PE-Pls in gray matter is rich in 20:4, 22:4 and 22:6 acyl chains, whereas in white matter, 18:1, 20:1 and 22:4 are the most abundant acyl chains attached at this position. This is another example of variations in composition of acyl chains in phospholipids which again depend on the tissue types and the acyl chain specificity for the enzymes involved in acylating the phospholipids.
5.2.2 *Myo*-inositol plus ethanolamine preferentially increases ethanolamine plasmalogens containing saturated and monounsaturated fatty acids in N2A cells

To our best knowledge, this is the first study to reveal that SFA + MUFA-rich PE-Pls and PE species in N2A cells are more sensitive to ME treatment than PUFA-rich species. Exposing the cells to ME increased the cellular levels of PE-Pls and PE pools containing SFA and MUFA. Although the mechanism behind these changes remains to be elucidated, it has been shown that *myo*-inositol can be metabolized into DHAP via the pentose pathway (188). DHAP is also the first building block for PE-Pls biosynthesis (Fig. 4). Although it is unclear why N2A cells respond to ME treatment by preferentially elevating the levels of SFA + MUFA-rich PE-Pls and PE species, we can speculate that this treatment is probably inducing an increase in the cellular level of 1-alkyl-2-acyl-

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affected by the lack of a carbonyl group at the sn-1 position and the structural orientation of the acyl chain attached at the sn-2 position \(249\). This conformation promotes a strong intermolecular hydrogen bonding between the head groups and allows the plasmalogen species to adopt an inverse hexagonal phase. This has a profound implication on the cell membrane processes such as lipid packing, fluidity, and how these species interact with other molecules anchored in the neural membranes such as ion channels and receptors \(249\). There are reports that describe the role of plasmalogens in shaping the cell membrane. In fact, cell membranes rich in plasmalogens with a high percentage of MUFA at the sn-2 position such as myelin are more compact and stable \(42, 241\). Conversely, those that are highly rich in unsaturated acyl chain at the sn-2 position, mostly in gray matter, are thought to be involved in facilitating membrane fusion \(250, 251\), intercellular communication and serve as storage for bioactive signaling lipid molecules \(246, 249, 252\). This indicates the importance of the degree of saturation in the fatty acyl chains attached to plasmalogens as it relates to cell membrane function. Plasmalogens are known to have a tendency of packing more closely with one another, thus reducing the membrane fluidity and passive ion permeability \(43\). Moreover, SFA pack more tightly than PUFA species. It is possible that ME treatment enhances these properties, thus decreasing the membrane fluidity, which may affect access of ROS to membrane lipids.

5.2.3 Myo-inositol plus ethanolamine protects against the \(H_2O_2\)-induced degradation of ethanolamine plasmalogens in N2A cells

Our data indicate that \(H_2O_2\) induced a 27% loss in PE-Pls pool and 39% increase in LPE levels in N2A cells. These were the same cells that displayed 56% survival rate
when they were exposed to H$_2$O$_2$. Strikingly, this loss was not observed when the cells were pre-treated with ME prior to H$_2$O$_2$ exposure (ME + H$_2$O$_2$ group). Additionally, ME + H$_2$O$_2$ was the same treatment that elevated the cell survival to 79.5%. There seems to be a link between H$_2$O$_2$-induced decrease in the cellular levels of PE-Pls species and an increase in their degradation by-products (LPE). Most importantly, only PE-Pls species appeared to be affected by this treatment as the levels of PE remained unchanged in the cells. PE-Pls and PE have similarities in the chemical structure. The only distinguishing feature is the types of linkage that attaches the acyl chain at their sn-1 position to the glycerol backbone. The acyl chain in PE-Pls is attached through a vinyl ether bond while that for PE is esterified to the backbone via an ester linkage. Between these two linkages, investigators have demonstrated that the vinyl ether bond is the most sensitive to oxidation (3, 5, 39-41). We have also shown that this bond is indeed the most vulnerable to hydrochloric acid hydrolysis (Fig. 21A-B). Figure 21A shows a $^{31}$P NMR spectrum generated using the lipid extract, while Figure 21B shows a spectrum generated using the same lipid extract but exposed to hydrochloric acid (12 M) fumes for 1 h. As the Figure 21B shows, none of the peaks for other phospholipids were affected by HCl, including that for PC-Pls, except the peak for PE-Pls. Hydrolysis of this PL gave rise to LPE peak in the spectrum which was not apparent before subjecting this extract to HCl fumes. We also used these two samples to run MS/MS analyses for Etn PL species (data not shown). We observed that all the LPE species generated by HCl hydrolysis were lacking the acyl chain at sn-1 position (data not shown). The peak for PE species was not affected by HCl treatment, suggesting that these LPE species were formed from PE-Pls hydrolysis. These observations highlight the sensitivity of the vinyl ether bond at the sn-1 position of PE-
Pls to HCl hydrolysis as compared to ester linkage at the \( sn-2 \) position. This constitutes another piece of evidences that this bond is more vulnerable to hydrolysis as compared to an ester or ether bond. Similar observations on the vinyl ether bond’s susceptibility to HCl hydrolysis have been reported (178). All these observations strongly support the hypothesis that an increase in LPE levels in the cells primarily resulted from the degradation of PE-Pls, and not the PE species. Although the cells are constantly and actively recycling membrane phospholipids, a homeostasis of these lipids must be maintained for their survival (29). Establishing this homeostasis requires a balance between phospholipid biosynthesis, degradation and remodeling (52), which also requires a considerable amount of ATP (89). Both a loss of PE-Pls and an increase in LPE in the cell membranes will surely inflict a significant impact on the integrity of the cell membranes and their functions. It is not clear if a decrease in the PE-Pls levels following an H\(_2\)O\(_2\)-induced oxidative stress in N2A cells resulted from an increase in (a) oxidation of these species (b) the activity of

**Figure 21.** \(^{31}\)P NMR spectra showing the phospholipid profiles of lipid extract (A) before and (B) after being exposed to HCl (12 M) fume for 1 h. See abbreviations on pages Xiii – Xvi.
phospholipase A$_1$, which enzymatically cleaves the acyl chains of PL at the $sn$-1 position \(^{(99)}\) or (c) a combination of both. Regardless of the trigger for PE-Pls degradation, this effect was blocked by pre-treating the cells with ME prior to H$_2$O$_2$ treatment.

**Figure 22.** A schematic diagram showing the degradation and re-acylation pathways of ethanolamine phospholipids. See abbreviations on pages Xiii – Xvi.

The cells control the levels of LPE by two different mechanisms as shown in Fig. 22. First, cells can re-acylate the LPE, thus forming a new PE-Pls or PE species. This process is carried out by acyltransferase enzymes \(^{(51)}\). The second option involves further hydrolyzing the LPE by lysophospholipases to generate free fatty acids and glycerophospho-bases \(^{(96, 99-101)}\). Our data show that H$_2$O$_2$ uniformly induced a degradation of PE-Pls regardless the levels of saturation of their acyl chains \(\text{Fig. 17B}\). However, we were surprised to observe that the LPE/PE-Pls ratio for SFA + MUFA species remained unchanged \(\text{Fig. 20F}\), but the LPE/PE-Pls ratio for PUFA species
increased about 4-fold following H$_2$O$_2$ treatment (Fig. 20E). This indicates that the cells failed to re-acylate the LPE containing PUFA, and/or inhibited the further degradation of these species into glycerophosphoethanolamine (GPE). This suggests that H$_2$O$_2$ negatively affected the activities of the enzymes involved in both processes. Moreover, there was a significant increase in the cellular levels of GPE following H$_2$O$_2$ exposure (Fig. 14, see results for NMR data). The increase in GPE production likely resulted from the degradation of LPE species containing SFA since H$_2$O$_2$ treatment yielded a 19% decrease in the level of these species. It is worthy to note that the LPE/PE-Pls ratio for PUFA species (Fig. 20E) and SFA + MUFA species (Fig. 20F), as well as the GPE (Fig. 14B) levels were all normal in the ME + H$_2$O$_2$ group when compared to the corresponding control (ME). The mechanisms by which LPE and GPE levels were controlled in ME + H$_2$O$_2$ group and how those in H$_2$O$_2$ group were altered are not known.

We showed that ME treatment preferentially increased the levels of PE-Pls species containing SFA + MUFA, which presumably strengthens the integrity of cell membrane. Neural cells are particularly vulnerable to oxidative injury because they contain high levels of PUFAs (51) and don’t possess sufficient reduced glutathione (139). Our data show that cells pre-treated with ME prior to H$_2$O$_2$ exposure had more PE-Pls containing SFA + MUFA to spare under oxidative stress, presumably preserving PE-Pls species that contain PUFA. The only PE-Pls species that decreased under ME + H$_2$O$_2$ treatment were 32:0p (9% decrease) and 38:1p (13% decrease). It is possible that these species were preferentially targeted by oxidative damage. If degrading PE-Pls was the mechanism of ME-induced protection against oxidative stress, we would have observed a significant increase in LPE and/or GPE. However, this wasn’t the case. A couple of explanations...
could explain these observations. Assuming that PE-Pls were also degraded under this treatment, (1) the resulting LPE by-products were re-acylated to re-form PE-Pls (from lysoplasmalogens) and PE (from 1-lyso-2-acyl-GPE) to maintain the normal levels of Etn PLs. In fact, both LPE species containing PUFA (Fig. 20B) and those containing SFA (Fig. 20A) decreased by 20% and 8%, respectively, although the decrease was not significant. This suggests that ME + H₂O₂ treatment increased the acyltransferase activities and decreased the activities of hydrolase enzymes. Additionally, 18:0p/lyso species decreased by 17%, suggesting that the treatment inhibited the activity of lysoplasmalogenase. All these observations are supported by a decrease in GPE levels (64%). (2) It is possible that the pool was replenished through the de novo biosynthesis pathway. This is supported by the observation that the levels of AAPE species increased by 10% following ME + H₂O₂ treatment. (3) We cannot rule out that the activities of PLA₁ and PLA₂ were increased, but PE-Pls pool was maintained at the normal levels through the mechanisms laid out under scenarios (1) and (2). The homeostasis of Etn PLs in ME + H₂O₂ was maintained at the normal level. This could probably explain why a high rate of cell survival to H₂O₂ exposure was observed in ME treated group.

We only measured one species of lysoplasmalogens (18:0p/lyso) in N2A cells. H₂O₂ treatment induced a 2-fold increase in the cellular level of this species when compared to that in the control cells. Strikingly, the level of this species in ME + H₂O₂ cells was unchanged relative to corresponding control (ME). It has been reported that oxidized phospholipids resulting from ROS are capable of inducing changes in the micro viscosity of the cell membranes due to changes in structural composition of oxidized/unoxidized species, thus increasing the substrate availability for phospholipase
A2 \((153, 253, 254)\). In brain tissues, lysoplamalogens are generated by plasmalogen-selective phospholipase A\(_2\) \((3)\) when it cleaves the acyl chain esterified at the \(sn\)-2 position of the plasmalogen’s glycerol backbone. In these tissues lysoplamalogens are either rapidly used by CoA-independent transacylase enzyme in the remodeling pathway to re-synthesize plasmalogens \((244, 245)\) in order to maintain their normal levels in neural membranes \((3)\) or cleaved by lysoplasmalogenase to form GPE. When the levels of lysoplamalogens become high enough, they are capable of lysing the cell membranes because of their amphiphilic nature \((237, 238)\). They are therefore, maintained at very low levels \((239)\). Their adversary effects include modulating the activities of various enzymes \((240)\), increasing the membrane fluidity \((255)\), inhibiting the cholinergic signal transduction \((242)\) and activating the cAMP dependent protein kinase A \((243)\).

Alternative routes for lysoplamalogen utilization include being enzymatically hydrolyzed by phospholipase C to form 1-alkenyl-glycerol \((256)\) and phospholipase D that yield plasmenic acid (alk-1-enyl-glycerophosphatidic acid) \((257)\). Lysoplamalogens can also be degraded by lysoplasmalogenase enzyme also known as alkenyl ether hydrolase \((\text{alkenylglycerophosphoethanolamine hydrolase, alkenylglycerophosphocholine hydrolase, and alkenylglycerohydrolase}) (233-235)\). This enzyme is highly specific for glycerophospholipids containing a vinyl-ether linkage at \(sn\)-1 and a hydroxyl (lyso) at \(sn\)-2, and acts with nearly equal efficiency on ethanolamine and choline classes \((236)\). It has been proposed the lysoplasmalogenase serves at maintaining lysoplamalogens at low levels in order to prevent their toxicity \((234, 236)\). It has been established that lysoplasmalogenase catalyzes the hydrolysis of the vinyl ether linkages on the lysoplamalogens (lysoplasmenylcholine or lysoplasmenylethanolamine).
to generate long chain fatty aldehydes and glycerophosphoethanolamine or glycerophosphocholine moieties depending on the substrate (233-235). They are also involved in the hydrolysis of the vinyl ether bond on 1-alkenyl-glycerol to form a fatty aldehyde and glycerol (233, 234, 258). Our data suggest that the H₂O₂ treatment may have inhibited the activity of lysoplasmalogenase since the level of lysoplasmalogen (18:0p/lyso) was elevated (2-fold increase) with this treatment. Conversely, we showed that ME + H₂O₂ treatment may have enhanced the activity of this enzyme since the level of 18:0p/lyso was decreased by 17%. Although the mechanism involved is not known, a simple speculation would suggest that ME probably blocked the H₂O₂-mediated effect on lysoplasmalogenase activity.
CHAPTER 6: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

6.1 SUMMARY

The main goal of this study was to (1) assess if myo-inositol +/- ethanolamine treatments increase ethanolamine plasmalogens in N2A cells and provide protection against H₂O₂-induced oxidative stress, and (2) determine the molecular species of ethanolamine phospholipids (PE-Pls, PE and LPE) that are preferentially affected by these treatments. These studies included four specific aims. NMR and mass spectrometry were used to analyze the samples. Table 16 illustrates how the measurements from both analytical methods compare.

Table 15. Etn PLs measured with NMR and Mass spectrometry (nmol/mg protein)

<table>
<thead>
<tr>
<th>Group</th>
<th>AAPE</th>
<th>PE-Pls</th>
<th>LPE</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMR</td>
<td>MS</td>
<td>NMR</td>
<td>MS</td>
</tr>
<tr>
<td>Control</td>
<td>- 2.8 ± 0.3</td>
<td>21.0 ± 2.6</td>
<td>16.7 ± 2.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>- 2.6 ± 0.7</td>
<td>18.3 ± 2.8</td>
<td>12.2 ± 3.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>ME</td>
<td>- 4.9 ± 0.4</td>
<td>33.8 ± 2.9</td>
<td>21.0 ± 1.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>ME + H₂O₂</td>
<td>- 5.4 ± 1.1</td>
<td>36.4 ± 2.4</td>
<td>24.1 ± 6.1</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

As shown in Table 15, AAPE were not quantified by NMR. It is possible that their resonance in 31P NMR spectrum was buried under the tall and broad peaks for PE-Pls and PE since they share structural similarities and the only feature that distinguish them is the linkage of their acyl chains at sn-1 position (ether, vinyl ether and ester linkages for AAPE, PE-Pls and PE, respectively). In general, concentrations for PLs measured with NMR are slightly higher than those measured with mass spectrometry, but the differences are not that great (20% average difference).
**Specific aim 1.** This aim was designed to assess the phospholipid contents of N2A cells, with specific interest in PE-Pls. The data show that N2A cells synthesize a variety of phospholipids including PE-Pls, PE and LPE. Choline phospholipids constitute the most abundant phospholipid class, accounting for more than 50% of the entire PL pool. Although the contribution of ethanolamine phospholipids to this pool is about half of that of choline PL, they are also the second most contributor to this pool. PE-Pls and PE contribute equally to the Etn PL pool (45.8% and 44.9%, respectively). A small portion of this pool (1.3%) is made by the degradation by-products (LPE) of PE-Pls and PE. PE-Pls precursors (AAPE) are also less abundant (8% of total Etn PLs) in N2A cells.

**Specific aim 2.** This aim was intended to examine the effects of myo-inositol alone (M), ethanolamine alone (Etn) and myo-inositol plus ethanolamine (ME) treatments on the level of myo-inositol, phosphoethanolamine (PEtn) and phospholipids in N2A cells, with a special interest in PE-Pls. As expected, M and ME increased myo-inositol content of the cells (3-fold increase) relative to that in the controls, while Etn and ME enhanced that of PEtn (3.6-fold and 8-fold increase, respectively). Etn and M had no significant effect on the total PLs. However, ME significantly enhanced the synthesis of PLs (2.4-fold increase), PE-Pls and PE (3-fold increase) relative to control group. Most of the other non-Etn PLs were increased by 2-fold, except for LPI which increased by 4-fold. Taken together, ME significantly enhanced the levels of myo-inositol, PEtn, Etn PLs and other non-Etn lipids in the cells.

**Specific aim 3.** This aim was designed to assess the effects of M, Etn and ME treatments on N2A cell protection against hydrogen peroxide-induced oxidative stress. A dose response curve for H₂O₂ indicated that exposing the cells to 650 μM H₂O₂ for 24 h yields
53% ± 1.7 cell viability. Pre-treating N2A cells with Etn alone for 24 h before exposing them to \( \text{H}_2\text{O}_2 \) for an additional 24 h did not provide protection against \( \text{H}_2\text{O}_2 \)-induced oxidative stress. However, pre-treating these cells with M improved significantly the cell viability (62% ± 1.2), and pretreatment with ME yielded a robust cell survival to \( \text{H}_2\text{O}_2 \) exposure (79.5% ± 0.6). ME is the same treatment that displayed a significant 3-fold increase in PE-Pls and PE and about 2-fold increase in most other non-Etn lipids. It is also noteworthy that PE-Pls levels in the cells prior to \( \text{H}_2\text{O}_2 \) treatment highly correlated \((r^2 = 0.95)\) with the cell viability following this treatment, suggesting that increasing the baseline level of PE-Pls can help the cells cope with oxidative damage. Taken together, it appears that ME treatment provided protection against \( \text{H}_2\text{O}_2 \)-induced death.

**Specific aim 4.** The purpose of this aim was to examine the impact of \( \text{H}_2\text{O}_2 \) on the molecular species of ethanolamine phospholipids in N2A cells with and without ME treatment. We were especially interested in the effects on PE-Pls species and their degradation by-products (LPE and GPE). The molecular species of Etn phospholipids measured in N2A cells include 3 species of AAPE, 19 species of PE-Pls, 23 of PE and 12 species of LPE. PE-Pls species and their precursors (AAPE) combined account for approximately 54% of the entire Etn PL pool in N2A cells, while PE and LPE constitute about 45% and 1% of this pool, respectively. The most abundant AAPE species is 34:1-e (a precursor for 34:1p PE-Pls species). Interestingly, 34:1p is also the most prevalent PE-Pls species in the cells. The PE-Pls species that are rich in PUFA account for 68% of the entire PE-Pls pool. The most predominant species of PE is 34:1 and accounts for 15% of the entire PE pool. PE species containing PUFA make 70.1% of the PE pool. Nine out of twelve different LPE species measured in N2A cells are \( sn-1 \) lyso products and account
for 91\% of the entire LPE pool. The most predominant LPE species in N2A cells are lyso/18:0 and lyso/16:0, which account for 40.5\% and 16.1\% of the LPE pool, respectively. It is also worthy to highlight that 66\% of LPE pool purely consists of the LPE-SFA rich species.

6.1.1 Myo-inositol plus ethanolamine preferentially increases ethanolamine plasmalogens containing SFA + MUFA in N2A cells

ME increased the PE-Pls pool by 200\% relative to the control. Although the mechanism by which this treatment enhances the levels of PE-Pls in N2A cells is not known, it is possible the treatment increases the substrates required in the biosynthesis pathway of PE-Pls. Here is the supporting evidence. First, ME treatment induced an 8-fold increase in PEtn pool (p < 0.0001) relative to the control group. PEtn is a substrate for ECT1 enzyme that synthesizes CDP-ethanolamine (CDP-Etn) in the Kennedy pathway (Fig. 4) (86, 87). CDP-Etn combines with 1-alkyl-2-acyl-sn-glycerol to form 1-alkyl-2-acyl-GPE (AAPE), a precursor for PE-Pls (Fig. 4). Second, ME mediated a 3-fold increase in myo-inositol content (p < 0.05) of the cells. It has been shown that myo-inositol can be metabolized into DHAP (187, 188), a first substrate required in the pathway leading to the biosynthesis of 1-alkyl-2-acyl-sn-glycerol and PE-Pls (Fig. 4). From our data, it is possible that myo-inositol influenced the level of PE-Pls by increasing the peroxisomal DHAP pool used in the biosynthesis of PE-Pls. Etn alone induced a 3.6-fold increase in PEtn levels in the cells (p < 0.05) but failed to enhance the cellular levels of myo-inositol and PE-Pls. Similarly, myo-inositol alone induced a 3-fold increase in the cellular level of myo-inositol (p < 0.05), but did not significantly affect the levels of PEtn.
and PE-Pls in the cells. Both, CDP-Etn (metabolite of PEtn) and 1-alkyl-2-acyl-sn-glycerol (probable metabolite of myo-inositol) molecules are indispensable substrates for the biosynthesis of PE-Pls (5, 6, 39). Only combining myo-inositol and Etn in one treatment (ME) upregulated the pathways leading to the synthesis of substrates that are required for the biosynthesis of PE-Pls. Third, ME increased the cellular levels of AAPE species (precursors for PE-Pls) by 73%. Other investigators have suggested that the effect of myo-inositol on PE-Pls levels may be associated with an increase in NADPH pool since myo-inositol’s metabolism into DHAP generates 2 molecules of NADPH (55). However, our data do not support this hypothesis since myo-inositol alone failed to enhance the levels of PE-Pls in N2A cells.

It was interesting to observe that ME treatment changed the proportion of PUFA versus SFA+MUFA present in plasmalogens. The PUFA:SFA+MUFA-rich PE-Pls changed from 68/32% (control) to 60/40% (ME). ME treatment preferentially enhanced the levels of PE-Pls species containing SFA+MUFA. The mechanism involved in this preferential change in the PE-Pls pool is not known. However, it is known that (1) predominant fatty acyl chains attached to sn-1 position of plasmalogens in mouse and human brain are SFA (C16:0 and C18:0) and MUFA (C18:1) accounting for 98-99% of the acyl chains at this position (48-50), (2) FAR1 enzyme that provides fatty alcohols esterified at the sn-1 position prefers these fatty acids as substrates (64), and (3) AAG3P-AT enzyme that esterifies acyl chains at the sn-2 position prefers using saturated acyl-CoA over polyunsaturated species when 1-alkyl-sn-glycero-3-phosphate (a precursor for AAPE) is available at high concentrations (79). Our data show that ME led to an increase in AAPE (by 73%), suggesting that this treatment increased the concentrations of 1-alkyl-
sn-glycero-3-phosphate in N2A cells leading to increased cellular levels of PE-Pls species rich in SFA + MUFA over species containing PUFA.

6.1.2 Myo-inositol plus ethanolamine inhibits H$_2$O$_2$--induced increase in degradation of ethanolamine plasmalogens and their degradation by-products, and increases N2A cell viability against oxidative stress

Exposing N2A cells to H$_2$O$_2$ yielded a poor cell survival (56%) and an overall decrease in PE-Pls level by 27%. This treatment did not significantly affect the proportion of PUFA-rich PE-Pls and SFA+MUFA-rich PE-Pls (68/32% for control vs. 67/33% for H$_2$O$_2$, Fig. 18A), suggesting that these PE-Pls pools were uniformly affected by the treatment (28 mole % and 25 mole % decrease, respectively). H$_2$O$_2$ treatment did not have an effect on PE pool in the cells. However, a completely different response to H$_2$O$_2$ was observed when the cells were pre-treated with ME prior to H$_2$O$_2$ exposure (Fig. 23). First, pre-treating cells with ME provided protection against H$_2$O$_2$-induced cell death (80% cell survival). Second, instead of losing PE-Pls as seen with H$_2$O$_2$ treatment, ME + H$_2$O$_2$ induced (1) an overall 15% increase in PE-Pls pool relative to corresponding control (ME), (2) a 22% increase in the pool of PE-Pls species containing PUFA, (3) and a 4% increase in PE-Pls species rich in SFA+MUFA. It is worthy to note that prior to exposing ME treated cells to H$_2$O$_2$, the pool of PE-Pls containing SFA+MUFA was greater than that containing PUFA (SFA+MUFA > PUFA). But after H$_2$O$_2$ treatment, the abundance of these pools of PE-Pls flipped (PUFA > SFA+MUFA). The possible explanations could be that (1) H$_2$O$_2$ exposure induces an increase in synthesis of PUFA-rich PE-Pls species, (2) the elevated levels of SFA+MUFA pool are preferentially sacrificed,
and/or (3) the turnover rate for PUFA species is increased. Cells treated with ME + H₂O₂ increased their PE content by 11% relative to their corresponding controls (ME treated cells), suggesting that biosynthesis of PE was increased or the turnover rate was influenced by exposure to H₂O₂.

A decrease in PE-Pls levels for the cells treated with H₂O₂ was associated with an overall 39% increase in LPE content. Nine out of twelve LPE species measured lack acyl chains at sn-1 position of their glycerol backbones, suggesting an increase in vinyl ether bond oxidation, PLA₁ activity or both. The observation that H₂O₂ treatment did not affect the cellular level of PE suggests that LPE species were mostly generated from the degradation of PE-Pls species. The proportion of PUFA-rich LPE:SFA-rich LPE changed from 34/66% (control group) to 52/48% (H₂O₂ group) (Fig. 20A), suggesting an H₂O₂-induced preferential degradation of PUFA-rich PE-Pls species. However, data on PE-Pls indicate a uniform degradation of both PE-Pls pools. Given that (1) LPE pool containing PUFA increased by 3-fold, (2) LPE rich in SFA decreased by 19%, (3) lyso-PE-Pls (18:0p/lyso species) increased by 2-fold, and (4) GPE increased by 4-fold (Fig. 23), it is fair to suggest that LPE-rich SFA were preferentially further degraded into GPE. An increase in GPE level suggests that the balance between LPE hydrolysis and re-acylation processes were affected in favor of hydrolysis. Moreover, an increase in the cellular level of lyso-PE-Pls suggests an increase in PLA₂ activity and a decrease in the activity of lysoplasmalogenase that attaches an acyl chain on this species to re-form PE-Pls (Fig. 23). Interestingly, cells pre-treated with ME displayed a completely opposite response to H₂O₂ for their contents in PUFA-rich LPE, lyso-PE-Pls, and GPE (Fig. 23). In fact, these cells displayed a decrease in PUFA-rich LPE (20%), lyso-PE-Pls (17%), and GPE (64%)
relative to corresponding controls (ME treated cells; Fig. 23). The pool of SFA-rich LPE decreased by 8%. Thus, the ME + H₂O₂ group shows low levels of PE-Pls degradation by-products and relatively high cellular levels of PE-Pls as described above. The probable explanations could be that ME + H₂O₂ treatment (1) increased the activity of lyso-acyltransferase enzymes that re-acylate LPE species to generate PE, (2) decreased the activity of PLA₂ or increased the activity of lysoplasmalogenase that attaches an acyl chains to lyso-PE-Pls species to form PE-Pls, and/or (3) decreased the activity of hydrolases that catalyze the hydrolysis of LPE to form GPE and fatty aldehydes (Fig. 23).

It has been reported that high levels of lysoplasmalogens are capable of lysing the cell membranes because of their amphiphilic nature (237, 238). In order to prevent toxicity, lysoplasmalogens are maintained at very low levels by lysoplasmalogenase (234, 236) (239). Other adversary effects of elevated lysoplasmalogens include modulating the activities of various enzymes (240), increasing the membrane fluidity (255), inhibiting the cholinergic signal transduction (242) and activating the cAMP dependent protein kinase A (243).

6.1.3 Hypothetical mechanisms of myo-inositol plus ethanolamine-mediated N2A cell protection against H₂O₂-induced oxidative stress

H₂O₂ treatment decreased the cellular levels of PE-Pls and increased their degradation by-products (LPE and GPE). Nine out of twelve LPE species measured lacked acyl chains at sn-1 position of their glycerol backbones, suggesting an increase in vinyl ether bond oxidation, PLA₁ activity or both. Since the PE pool was not affected by H₂O₂ treatment, we suggest that majority of LPE and GPE generated were from the
degradation of PE-Pls species. It has been reported that vinyl ether bond is more susceptible to oxidation than the corresponding ester bond linking the phospholipid moieties to their glycerol backbone \((3, 5, 39-41)\). This bond may preferentially undergo ROS-mediated hydrolysis as compared to free radical attack of olefinic residues in the \(sn-2\) position of diacyl-phospholipids that are embedded in the hydrophobic domain of cell membranes \((19, 147, 148)\). Neural cells are particularly vulnerable to oxidative injury because they contain high levels of PUFAs \((51)\) and don’t possess sufficient reduced glutathione \((139)\). It is possible that all these factors contributed to PE-Pls loss in \(H_2O_2\) treated cells. Elevated levels of ROS inflict damage to neural membranes, changing not only their fluidity \((140)\), but also decreasing activities of other membrane actors such as

![Diagram summarizing the effects of \(H_2O_2\) treatment on PE-Pls species and the hypothetical mechanisms for ME-mediated protection against \(H_2O_2\)-induced PE-Pls degradation. See abbreviations on pages Xii and Xiii.]

\[ \text{Figure 23.} \]
membrane-bound enzymes, ion channels, and receptors \((141)\). It has also been reported that they are capable of inducing alterations in cell signaling \((142, 143)\) and surface charge \((51)\). Although ME pre-treatment protected N2A cells against H\textsubscript{2}O\textsubscript{2}-induced toxicity, it was not known by which mechanisms this happened. However, we cannot rule out the possibility that by increasing the cellular levels of PE-Pls, these species served as a sacrificial trap for ROS when cells were exposed to H\textsubscript{2}O\textsubscript{2}. It is also possible that ME enhanced the cell viability to H\textsubscript{2}O\textsubscript{2}-induced toxicity by maintaining Etn phospholipid homeostasis. This is supported by the observations that cell viability strongly correlated with the baseline level of PE-Pls \((r^2 = 0.95)\) prior to H\textsubscript{2}O\textsubscript{2} exposure. Pre-treating cells with ME provided substrates for the synthesis of new PE-Pls to replenish those lost due to H\textsubscript{2}O\textsubscript{2}-induced degradation. This assertion is supported by our data showing that ME increased the pool of PE-Pls precursors (AAPE) by 73\% prior to H\textsubscript{2}O\textsubscript{2} exposure. Maintaining a normal level of PE-Pls and LPE after exposure to H\textsubscript{2}O\textsubscript{2} supports our assertion that pre-treating cells with ME preserved homeostasis through balancing PE-Pls degradation, \textit{de novo} synthesis and remodeling. This is supported by our data which show that the level of myo-inositol in ME + H\textsubscript{2}O\textsubscript{2} group dropped to the level below that of the corresponding control (ME), suggesting that it was actively being utilized, presumably for the synthesis of PE-Pls.

Establishing homeostasis of phospholipids requires a balance between biosynthesis, degradation and remodeling \((52)\). If degrading PE-Pls was the mechanism of ME-induced protection against oxidative stress, we would have observed a significant increase in LPE and/or GPE. However, this wasn’t the case. Assuming that PE-Pls were also degraded under ME + H\textsubscript{2}O\textsubscript{2} treatment, a couple of possibilities could explain these
observations. (1) Increasing PE-Pls containing SFA+MUFA may have strengthened the membrane integrity, decreasing its susceptibility to oxidative damage. Peroxidized phospholipids in neural membranes can result in a membrane-packing defect, thus, facilitating PLA₂ access to the sn-2 ester bond for hydrolysis (51). Our data indicate that lyso-PE-Pls strongly correlated ($r^2 > 0.5$) with nine out of twelve probable PE-Pls parents in H₂O₂ group, whereas in ME + H₂O₂ group this species only correlated with five PE-Pls species. These data suggest that plasmalogen-selective PLA₂ was relatively more active in H₂O₂ group than in ME + H₂O₂ group. Plasmalogens are known to have a tendency of packing more closely with one another, thus reducing the membrane fluidity and passive ion permeability (43). Additionally, SFA pack more tightly than PUFA. Maybe both factors combined serve to decrease membrane fluidity, which may affect access of ROS to membrane lipids. (2) An increase in SFA + MUFA-rich PE-Pls may have also served as a reservoir that can be sacrificed in response to H₂O₂ and that is protective.

6.2 CONCLUSIONS

Our data demonstrate that the levels of PE-Pls in neural cells can be enhanced by ME treatment. They also indicate that increasing the baseline levels of PE-Pls in these cells can provide protection against oxidative stress. Although the mechanisms behind the ME-mediated cell protection against oxidative stress are not known, these show that there may be a link between PE-Pls levels and cells protection, more specifically the oxidation of vinyl ether linkage. Understanding this mechanism has an enormous significance for the researchers and pharmaceutical industries as well. The ME-mediated increase in cellular levels of PE-Pls may serve as a potential approach to enhance the brain’s ability to reduce neurological sequelae of hazardous chemical exposure due to oxidative stress.
More research could be tailored to designing new preventive and therapeutic strategies that increase the PE-Pls levels in the cells to combat pathophysiology associated with cellular PE-Pls deficiency, and deleterious effects that may result from oxidative damage or toxicity. A neuroprotective approach could be developed to address a wide spectrum of stressors on brain and cognitive function, enhancing brain resiliency.

### 6.3 FUTURE DIRECTIONS

It has been demonstrated that the rate-limiting step in the biosynthesis of plasmalogens is catalyzed by a peroxisomal fatty acyl-CoA reductase 1 (FAR 1) enzyme (63). This enzyme supplies the fatty alcohols required for the formation of ether-linked bonds that will become vinyl ether-linked bonds in plasmalogens (Fig. 4). Reports show that the activity of FAR 1 is increased in plasmalogen-deficient cells which can be normalized by supplying the cells with plasmalogens (63, 259). The cellular activity of this enzyme is controlled in the response to the levels of plasmalogens (through a negative feedback mechanism) by increasing the rate of its degradation (63). One of the future directions include inhibiting the FAR 1 and test if (a) the increase in PE-Pls and PE seen with ME treatment is reduced; (2) the protection against oxidative stress is eliminated. This will be achieved by establishing two N2A cell colonies: a colony of cells that lack FAR 1 enzyme (knocked out) and a normal colony (control). It is expected that the effects of ME on Etn PLs in FAR 1-deficient cells will be reduced in comparison to the control cells. Similarly, it is also expected that the protection to H₂O₂ exposure seen with ME pre-treatment will also be eliminated.
Other important issues that need to be addressed include testing the activities of plasmalogen-selective-PLA₂, PLA₁, lyso acyltransferase and lysoplasmalogenase enzymes under ME and H₂O₂ treatments. Our data show that H₂O₂ increased the levels of lyoplasmalogen (18:0p/lyso species) by 2-fold relative to the control. This species is generated by plasmalogen-selective-PLA₂ when it cleaves the acyl chain at the sn-2 position of plasmalogen. Interestingly, this effect was inhibited in ME + H₂O₂ treatment. It has been shown that H₂O₂ induces the translocation of cytosolic PLA2 (cPLA2) to the cell membrane of fibroblasts and increases the activity due to substrate availability by the action of ROS on membranes (153, 260). However, it is not known if plasmalogen-selective PLA₂ responds in the same way to H₂O₂. Our data suggest that H₂O₂ increased the activity of plasmalogen-selective-PLA₂ and the effect was inhibited under ME + H₂O₂ treatment. It is worthy to run enzyme activity assays and check the status of this enzyme under these treatments. Similarly, the status of PLA₁ needs to be assessed. An increase in PLA₁ activity may probably be linked to a decrease in PE-Pls levels observed under H₂O₂.

Lyso acyltransferases, hydrolases and lysoplasmalogenases are among the enzymes that control the levels of LPE in the cells. Our data show an overall 39% increase in LPE and a 4-fold increase in GPE levels following H₂O₂ exposure. However, ME + H₂O₂ displays opposite effects on these metabolites (an overall 13% and 64% decrease in LPE and GPE, respectively). These differences could reflect how each treatment affected the enzyme activities. Our data suggest that H₂O₂ enhanced the enzyme activities while the ME + H₂O₂ blocked this effect. It will be interesting to carry
out the activity assays for lyso acyltransferases, hydrolases and lysoplasmalogenases under these treatments.

In our study, we assessed how each treatment affected the levels of Etn PLs in N2A cells. However, we only measured LPE and GPE as the degradation by-products of PE-Pls and PE. We did not assess the levels of other products. Studies have shown that oxidation by-products of PE-Pls’s vinyl ether bond include LPE \((19, 178, 261-264)\) formyl-GPE \((19, 264)\) fatty aldehydes and hydroxy aldehydes \((19)\). However, the study done by Wynalda and Murphy \((19)\) reported that the formation of formyl products from PE-Pls’s oxidation is negligible. Stadelman-Ingrand et al. \((18)\) observed that fatty aldehydes and hydroxy aldehydes generated from \(sn-1\) position of plasmalogen following a UV irradiation are further oxidized as soon as they are produced. Although these observations suggest that a quantification of formyl-GPE, fatty and hydroxy aldehydes cannot give an accurate account on PE and PE-Pls’s degradation patterns, at least this information combined with the LPE measurements can enhance the evaluation on how the treatments affected these Etn PL in N2A cells. Therefore, it will be worthy to also examine the levels of formyl-GPE, fatty aldehydes and hydroxy aldehydes in the cells following \(H_2O_2\) and ME + \(H_2O_2\) treatments.

Lastly, other set of experiments that are worthy to carry out include checking the levels of ROS in the cells following \(H_2O_2\) and ME + \(H_2O_2\) treatments. Procedures for assessing the generation of superoxide radicals and \(H_2O_2\) in the cells have been published \((265)\). These experiments will provide additional information on the level of oxidants in the cells which can be linked to the levels of LPE.
REFERENCES


the acyl dihydroxyacetone phosphate pathway, *Annals of the New York Academy of

plasmalogen biosynthesis and peroxisome assembly, *Proceedings of the National

catalyzing the biosynthesis of glycerol-ether lipids in Zellweger syndrome. A new
category of metabolic disease involving the absence of peroxisomes, *The New

catalyzing the biosynthesis of lysophosphatidate and its ether analog in cultured
fibroblasts from Zellweger syndrome patients and normal controls, *Archives of

76. Aguado, B., and Campbell, R. D. Characterization of a human lysophosphatidic
acid acyltransferase that is encoded by a gene located in the class III region of the
human major histocompatibility complex, *The Journal of Biological Chemistry*
**273**, 4096-4105.

and chromosomal localization of two human lysophosphatidic acid acyltransferases,

78. West, J., Tompkins, C. K., Balantac, N., Nudelman, E., Meengs, B., White, T.,


Biomarker Detection and Identification in Osteoarthritis Patients, *Journal of Proteome Research* 9, 2377-2389.


lysophospholipids and 1-acyl-2-lysophospholipids in biological samples by LC-

Differentiated human NT2-N neurons posses a high intracellular content of myo-

186. Massarelli, A. C., Dainous, F., Hoffmann, D., Mykita, S., Freysz, L., Dreyfus, H.,
and Massarelli, R. (1986) Uptake of ethanolamine in neuronal and glial cell cultures

*Biochimica et Biophysica Acta* **28**, 666-667.

Conversion of myo-inositol to d-glucuronic acid and l-gulonic acid in the rat

ethanolamine on choline uptake and incorporation into phosphatidylcholine in

biosynthesis of plasmenylcholine from plasmenylethanolamine in HL-60 cells,
*Biochimica et Biophysica Acta* **1166**, 309-312.

2- acyl-sn-glycerol in myocardium and its effective utilization by choline
2650.


learned to stop worrying and love glycopospholipids, *Journal of Lipid Research* 48, 993-1011.


alkenylhydrolase (lysoplasmalogenase) from rat liver microsomes, *Biochimica et Biophysica Acta* **1002**, 302-312.


deficiency have impaired muscarinic cholinergic signal transduction activity and
amyloid precursor protein secretion, *Biochemical and Biophysical Research
Communications* **248**, 57-61.


Coenzyme A-independent transfer of arachidonate from phosphatidylcholine to
lysoplasmenylethanolamine, *The Journal of Biological Chemistry* **258**, 13806-
13811.

bioactive species, *Biochemistry of Lipids, Lipoproteins and Membranes, 4th edition*
**36**, 233–262.

early Alzheimer's disease subjects and in animal models: molecular characterization
using electrospray ionization mass spectrometry, *Journal of Neurochemistry* **77**, 
1168-1180.

Elsevier Biomedical Press, Amsterdam, 4, 51-93.


255. Han, X. L., and Gross, R. W. (1991) Proton nuclear magnetic resonance studies on the molecular dynamics of plasmenylcholine/cholesterol and


