ABSTRACT

PRASHANT MONIAN
Analysis of Phospholipids in Neuronal Tissue Using Electrospray Ionization-Mass Spectrometry
(Under the Direction of DR. BRIAN S. CUMMINGS)

Phospholipids are an important constituent of all cell membranes, and are thought to play key roles in several physiological processes. This research focuses on the use of electrospray ionization-mass spectrometry (ESI-MS) in studying alterations in the phospholipid profiles of neuronal cells in response to different kinds of stress. First, alterations in phospholipid and fatty acid lipid profiles in primary neocortical cells during oxidant-induced cell injury were studied. Oxidant-induced alterations in phospholipid composition can lead to decreases in membrane integrity, cell injury and even death. Neurons are especially vulnerable to lipid peroxidation. Treatment with the oxidants hydrogen peroxide (H_2O_2) and *tert*-butylhydroperoxide (TBHP) was found to increase the abundance of phospholipids containing polyunsaturated fatty acids, but had minimal affect on those containing mono- or di-unsaturated fatty acids. In addition, Group IV and VI Phospholipase A_2 (PLA_2) were found to have differential roles in oxidant-induced neural cell injury based on the use of the group specific inhibitors, methyl arachidonyl flourophosphonate (MAFP) and bromoenol lactone (BEL) respectively. Secondly, the effects of cocaine withdrawal on the expression of glycerophospholipids in rat brain were studied. For the first time, differences in the expression of phospholipids between different areas of the rat brain were determined by ESI-MS. Withdrawal from cocaine appeared to effect expression of specific phospholipids in a site specific manner, with most of the changes taking place in the hippocampus, the region generally associated with long term memory and spatial navigation. This may explain the long term neuroadaptation associated with drug use.

INDEX WORDS: Phospholipids, Fatty Acids, Electrospray Ionization-Mass Spectrometry, Phospholipase A_2, Neurons, Oxidants, Cell Death, Cocaine Withdrawal, Long Term Potentiation, Neuroadaptation

ANALYSIS OF PHOSPHOLIPIDS IN NEURONAL TISSUE USING ELECTROSPRAY IONIZATION-MASS SPECTROMETRY

by

PRASHANT MONIAN

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DEDICATION

To my mom, brother, and late father. Your support and inspiration through the years have helped me become the person I am today. For this, I am immensely grateful.

ACKNOWLEDGEMENTS

I would like to thank Dr. Brian Cummings for his constant guidance and supervision throughout the course my research. His support as my research mentor has been instrumental in my growth as a student. He has instilled in me a strong passion for research, and for this I sincerely thank him. I would also like to thank the graduate students in his lab, Bin Sun and Xiaoling Zhang. They too have mentored me during my time in Dr. Cummings' lab, and have provided me with valuable skills that are sure to prove useful in future scientific endeavors. I am indebted to the Honors Program, especially CURO. The opportunities they have provided me with during my undergraduate career have laid the foundation for my graduate education at the Sloan-Kettering Institute. Finally, I would like to extend my gratitude to Dr. John Wagner both for his thoughtful input into my thesis as a reader, and for the projects we worked on in collaboration with his lab.

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CHAPTER 1
INTRODUCTION

Lipids are broadly defined as molecules that are insoluble in water and soluble in organic solvents (Peterson and Cummings, 2006). They play a variety of roles in biological systems, one of which is to maintain the structural integrity of cell and organelle membranes. All mammalian cells are enclosed by a plasma membrane, which separates the cytosolic components from the extracellular environment. In addition, eukaryotic cells possess specialized organelles like the Golgi apparatuses, mitochondria and endoplasmic reticulum, all of which are membrane-bound. Lipids are arranged on the membrane as a continuous double layer. This bilayer structure gives the membrane its fluid and dynamic nature, to which a number of membrane functions are attributable (Alberts et al., 2002 p.583-584).

Phospholipids are the most abundant membrane lipid, accounting for nearly 60% of the lipid mass of eukaryotic cell membranes (Peterson and Cummings, 2006). They are amphipathic molecules composed of a glycerol (3 carbon chain) backbone with fatty acids esterified at the *sn-1* and *sn-2* positions (**Figure 1.1**). The hydrophobic fatty acids vary in length (from 12 to 22 carbon atoms) and in saturation (from 0 to 6 double bonds). At the *sn-3* position is a phosphate head group linked to a polar head group (Peterson and Cummings, 2006). The amphipathic nature of phospholipid molecules causes them to spontaneously aggregate in aqueous environments, with the hydrophobic fatty acid tails oriented to the interior and the polar head groups facing aqueous environments. In addition, the cylindrical shape of phospholipid molecules ensures they form bilayers, with the hydrophobic tails sandwiched between the

hydrophilic head groups (the alternative arrangement would be spherical micelles) (Alberts et al., 2002 p.585).

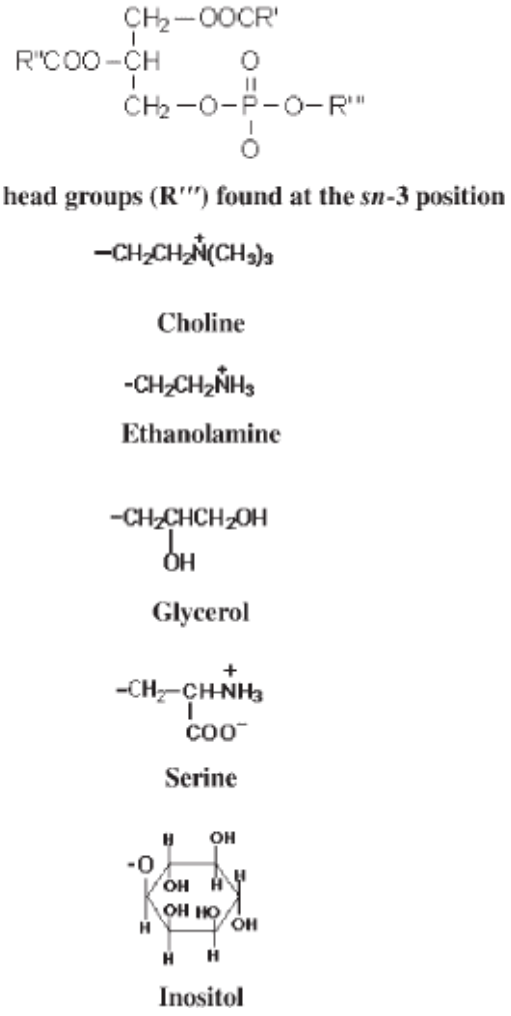

Figure 1.1. Basic structure of a phospholipid**.** Consists of a glycerol backbone with fatty acids (R' and R") linked at the *sn-1* and *sn-2* positions (**A**). Various polar head groups link (R'") link to the phosphate group at the *sn-3* position (**B**). Taken from Peterson and Cummings, 2006.

The membranes of eukaryotic cells contain a variety of phospholipids, with head groups that differ in shape, size and charge. The five phospholipids most common to plasma membranes of mammalian cells are phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEth), phosphatidylserine (PtdSer), phosphatidylglycerol (PtdGly), and phosphatidylinositol (PtdIns) (**Figure 1.1**). These phospholipids all share the typical glycerol backbone attached to two fatty acids, but differ in the polar head group present, each conferring a unique identity and nomenclature (Peterson and Cummings, 2006). Also commonly present are

sphingolipids, another class of lipids that are derived from the amino alcohol sphingosine (instead of the typical glycerol backbone) (**Figure 1.2.**). An important member of this class of lipids is sphingomyelin(SM), which has a phosphocholine head group attached to the sphingosine backbone (Sonnino et al., 2006).

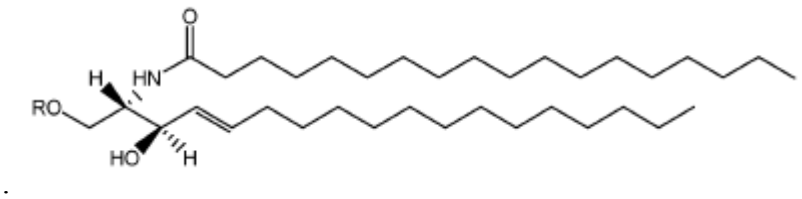

Figure 1.2. Basic structure of sphingolipids. Taken from Sonnino et al., 2006.

A characteristic feature of the lipid bilayer is its striking asymmetry. For example, in most eukaryotic membranes, PtdCho and SM are present in the outer monolayer, while PtdEth and PtdSer are confined mostly to the inner monolayer. This asymmetry is functionally important since many proteins bind to specific lipid head groups, allowing for targeted cell signaling. For example, in cells undergoing apoptosis, PtdSer is translocated from its normal position in the inner leaflet to the outer leaflet, where it signals macrophages to digest the dead cell. Because of the importance of bilayer asymmetry, cells have specialized ways to maintain this asymmetry once generated. This includes slow trans-bilayer movement of lipids and selective lipid transporters (e.g. flippases, scramblases) that move lipids and their precursors from one side of the membrane to the other (Daleke, 2007).

Lipids also play a role in cell signaling. Because of their ability diffuse freely through membranes, lipid signaling is thought to be qualitatively different from other classical signaling paradigms. For example, rather than being stored in vesicles prior to release, lipid messengers

are usually synthesized at their site of action only when required (Carrasco-Pancorbo et al., 2009). One of the key regulators of lipid signaling are phospholipases; enzymes that cleave specific phospholipid molecules in response to various extracellular and intracellular signals, generating short-lived mediators that can modify specific cellular processes (Alberts et al., 2002 p.591).

Among the different phospholipases found in the cell, phospholipase A_2 (PLA_2) are ubiquitous in nature and play key roles in several biological processes including inflammation, necrosis, apoptosis, and maintenance of membrane lipids. PLA_2 represent a superfamily of esterases that mediate the release of free fatty acids and lysophospholipids by hydrolyzing the *sn-2* ester bond in phospholipids. These products can in turn generate potent lipid signals such as prostaglandins, leukotrienes and lysophosphatidic acid (LPA). PLA_2 are a diverse family and have traditionally been classified into three categories (Cummings, 2007):

- Secretory Ca^{2+} - dependent PLA_2 ($sPLA_2$)
- Cytosolic Ca^{2+} - dependent PLA_2 ($cPLA_2$)
- Ca^{2+} - independent PLA_2 ($iPLA_2$)

$sPLA_2$ are the oldest class of PLA_2. They require millimolar amounts of Ca^{2+} for activity, have low molecular masses (13-19 kDa), and utilize histidine to hydrolyze the *sn*-2 ester bond of the glycerol backbone. $cPLA_2$ and $iPLA_2$ isoforms have higher molecular masses (66-90 kDa) and utilize serine to facilitate the hydrolytic cleavage of the *sn*-2 fatty acid. In addition, micromolar amounts of Ca^{2+} are required for translocation of $cPLA_2$ to membrane phospholipids. $iPLA_2$ isoforms are located in the cytosol and in organelles and do not require Ca^{2+} for activity or translocation to the membrane (Cummings, 2007).

While all three above PLA_2 isoforms cleave phospholipids at the *sn-2* position, they show some specificity for the phospholipids they act on. For example, unlike $iPLA_2$, $cPLA_2$ prefer arachidonic acid containing phospholipids (Balsinde et al., 2002). Arachidonic acid (AA) is a 20-carbon chain omega-6 fatty acid with 4 double bonds. AA, along with its metabolites, has several physiological roles. For example, metabolism of AA produces lipid mediators like thromboxanes, leukotrienes, prostacyclins and prostaglandins. These lipids mediate various cellular processes including cell proliferation, cell death, inflammation, vasoconstriction, and vasodilatation in numerous tissues including platelets, endothelium, and smooth muscle (Cummings 2007). Studies also show there to be a correlation between AA metabolites and carcinogenesis (Hughes-Fulford et al., 2005). Given the widespread effects of AA metabolites, it makes sense that the concentration of free AA in normal cells is very low, thus limiting the formation of these metabolites (Balsinde et al., 1999). Cleavage and release of AA from glycerophospholipids is responsible for a majority of mitogenic signals derived from PLA_2 activity, and all PLA_2 isoforms are capable of releasing AA from phospholipids (Cummings, 2007).

As mentioned before, in contrast to $cPLA_2$, $iPLA_2$ does not show preference for AA containing phospholipids. Yet $iPLA_2$ has been identified as the key PLA_2 involved in maintenance of membrane phospholipids. $iPLA_2$ performs this 'housekeeping' role as part of the Lands cycle; a cycle involving the release of fatty acids (including AA) from the *sn-2* position of membrane phospholipids, creating a lysophospholipid that is re-esterified with another fatty acid. This process allows the incorporation of free fatty acids into membrane phospholipids and is key to maintaining the structural integrity of the membrane (Cummings, 2007).

Several efforts have been made to further characterize the activities of each of the three above PLA_2 isoforms, particularly concerning their roles in phospholipid remodeling. Inhibition of PLA_2 using chemical inhibitors represents the most clear-cut approach to studying the involvement of these enzymes in cellular processes (Balsinde et al., 1999). The same approach could be used to study the activities of PLA_2 towards phospholipids. The availability of isoform-specific inhibitors has been particularly beneficial towards this goal. Theoretically, inhibition of a specific isoform of PLA_2 would cause alterations in the abundance of those phospholipids that are normally targets of that specific PLA_2. This principle has been demonstrated in several studies. For example, inhibition of $iPLA_2$ in HEK293 and Caki-1 cells led to decreases in abundance of certain phospholipids (Zhang et al., 2005). It is hypothesized that under normal conditions, $iPLA_2$ cleaves these lipids at the *sn*-2 position to release fatty acids that undergo reacylation and re-insertion into the membrane. Inhibition of $iPLA_2$ thus prevents such re-insertion, causing the overall level of that phospholipid to decline (Zhang et al., 2005). Studies have indicated that methyl arachidonyl fluorophosphonate (MAFP) is a potent inhibitor of $cPLA_2$ as well as $iPLA_2$. Selective inhibition of $iPLA_2$ can be achieved by the use of bromoenol lactone (BEL) (Hooks and Cummings, 2008).

Given the abundance and importance of phospholipids in mammalian cells, analysis of phospholipid species composition and structure has emerged as an active area of research. Unlike proteins, lipids display little specific binding, and so many of the techniques used to study proteins and DNA do not work as well with phospholipids. Traditional methods to study phospholipids included thin layer chromatography, gas chromatography and high-performance liquid chromatography. In recent years however, mass spectrometry has proven to be a most priceless tool in phospholipid analysis. Specifically, electrospray ionization-mass spectrometry

(ESI-MS) offers increased sensitivity for detection of phospholipids (Pulfer and Murphy, 2003). Briefly, in ESI-MS, the lipid samples are ionized and then subjected to an applied charge that creates negative or positive ions. Phospholipid samples are then separated by mass analyzers and identified based on their mass-to-charge ratio (*m/z*) (Peterson and Cummings, 2006). ESI-MS allows for high throughput screening and relative quantification of phospholipid samples in a single step, as opposed to conventional methods that employ separate quantification and analytical techniques. ESI-MS has thus clearly advanced the field of lipidomics (Peterson and Cummings, 2006).

The goal of this work was to analyze the expression of phospholipids in neuronal tissue in two different scenarios. The first part of this work focuses on alterations in phospholipid and fatty acid profiles in primary neocortical cells during oxidant-induced cell injury. One way in which phospholipids are often damaged is through oxidation of fatty acid chains. Oxidation induced alterations in phospholipid composition can lead to decreased membrane integrity, cell injury and even death. Oxidative stress occurs as a result of an imbalance between generation of toxic free radicals and cellular antioxidant defenses. The exact mechanisms that participate in development of oxidative stress are not defined (Ratan et al., 1994). The reactive oxygen species (ROS) generated during oxidative stress could induce lipid peroxidation as a result of attack on membrane lipids. Recent studies indicate that lipid peroxidation could be involved in several cellular stress responses. For example, exposure of human neuroblastoma cells to oxidized polyunsaturated fatty acids was shown to promote phosphorylation of the key tumor suppressor protein p53, thus activating p53-dependent apoptosis (Shibata et al., 2006). Phospholipids containing polyunsaturated fatty acids are believed to be the most susceptible to oxidation. These types of phospholipids are present to a higher degree in the brain, making it more susceptible to

oxidant-induced cell injury. In addition, arachidonic acid containing phospholipids are thought to play a role in oxidant-induced cell death. Despite the clear involvement of lipid peroxidation in oxidant-induced cell death, the exact phospholipids involved during ROS injury and lipid peroxidation are not known (Peterson et al., 2008).

Neurons are especially vulnerable to oxidative stress and lipid peroxidation, owing to their high levels of oxygen consumption, high iron content, and low levels of antioxidants such as catalase, glutathione peroxidase, and superoxide dismutase (El-Kossi and Zakhary, 2000). Oxidative stress is often observed in the neurons of patients following stroke or other types of brain injury, where the increase in free radicals combined with lack of oxygen causes a decrease in ATP levels. This in turn leads to the failure of ATP-dependent pumps, allowing an influx of sodium, calcium, and chlorine into the neurons. Increased intracellular calcium could lead to activation of lipid metabolism enzymes such as PLA_2 (Peterson et al., 2008).

As mentioned before, the exact phospholipids involved in oxidant-induced neuronal cell death are unknown. Given the alteration of phospholipids in neurodegenerative conditions such as stroke and Alzheimer's disease, there is a need to investigate the specific phospholipids and fatty acids altered in oxidant-induced neuronal cell injury (Peterson et al., 2008). In this study, mouse primary neocortical cells were treated with varying concentrations of two model oxidants, hydrogen peroxide (H_2O_2) and *tert*-butylhydroperoxide (TBHP). The phospholipid and fatty acid profiles of treated cells were compared with those from untreated cells to identify altered species. Possible clinical applications that could result from this work include the identification of specific lipid species that could serve as biomarkers for oxidant-induced neuronal cell death. Not surprisingly, studies have indicated that PLA_2 also play a key role in the membrane remodeling that occurs following oxidant-induced cell death. For example, inhibition of endoplasmic

reticulum-iPLA$_2$ (ER-iPLA$_2$; now characterized as iPLA$_2\gamma$) with BEL prior to oxidant exposure increases oxidant-induced phospholipid loss in rabbit renal proximal tubule cells (RPTC) (Kinsey et al., 2005). Thus the role of cPLA$_2$ and iPLA$_2$ in the context of oxidant-induced cell death was also investigated in this study, aided by the use of the group specific inhibitors BEL and MAFP.

The second part of this work investigated the expression of glycerophospholipids in rat brain after cocaine withdrawal. Cocaine is drug of abuse that is thought to indirectly enhance levels of dopamine, norepinephrine, and serotonin, by inhibiting the reuptake of these key neurotransmitters in the brain. This leads to "activation of dopaminergic and noradrergic pathways associated with mood and activation of serotonergic systems associated with mood and arousal" (Gavin, 1991). Long term abuse of cocaine is associated with neuroadaptation, a condition where the nervous system undergoes a compensatory adaption in response to persistent, drug-induced neurochemical perturbation (Gavin, 1991). The molecular and cellular mechanisms underlying learning and memory processes have for long been associated with the neuroadaptations following exposure to cocaine. One of the most commonly accepted cellular mechanisms for learning and memory involves long term potentiation (LTP) (Thompson et al., 2005). LTP refers to an abrupt and sustained increase in the efficiency of synaptic transmission and has become the dominant model of activity-dependent synaptic transmission in the mammalian brain. LTP was first observed and can most reliably be generated in the hippocampus, a region of the brain that is essential for learning and memory (**Figure 1.3.**)

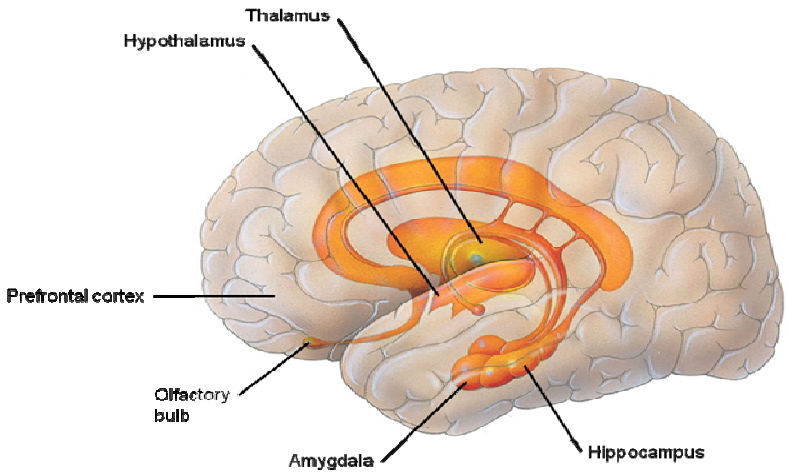

Figure 1.3. Location of the hippocampus in the human brain. Taken from Campbell and Reece, 2004.

LTP is triggered within seconds and can last for hours (acute) to days (chronic) (Malenka and Nicoll, 1999). Recent studies have indicated that cocaine abuse has multiple actions on LTP. For example, intermediate doses of cocaine have been shown to lead to significant increases in LTP in the CA1 region of the rat hippocampus, while extremely high doses lead to inhibition of LTP in the same region (Thompson et al., 2005). Thus, the alterations in LTP induced by cocaine may be responsible for the neuroadaptations associated with the abuse of the drug (Thompson et al., 2004).

Given the importance and sustained nature of LTP, it has been hypothesized that several signaling processes play a role in the establishment and maintenance of LTP (Malenka and Nicoll, 1999). However, the exact mechanisms involved have not been identified. Several forms of PLA_2 are known to occur in mammalian brain (Farooqui and Horrocks, 1994). In the hippocampus (the region where LTP is best observed), $iPLA_2$ represents the predominant constitutive phospholipase activity, and can thus be hypothesized to play a role in the induction and maintenance of LTP. This is strongly supported by evidence from recent studies where BEL

(a selective inhibitor of $iPLA_2$) was found to disrupt LTP induced in CA1 hippocampal slices from rat brain (Martel et al., 2006). Studies also show that stimulation of dopamine receptors can release arachidonic acid via activation of PLA_2 in various cell culture systems, while dopaminergic drugs can alter the rate of arachidonic acid turnover in vivo (Ross et al., 2002). Furthermore, $cPLA_2$ activity was found to be decreased in the brains of chronic cocaine users, specifically in dopamine-rich regions (Ross et al., 1996). This change is thought to "represent a compensatory response to over-stimulation of the enzyme by elevated dopamine receptor occupancy", i.e. neuroadaptation (Ross et al., 2002). Given the clear involvement of PLA_2 in membrane remodeling, as well as its close association with LTP (and consequently neuroadaptation), it can be hypothesized that significant alterations of membrane phospholipids must be involved with the establishment and maintenance of LTP. Specifically, any alterations in $iPLA_2$ activity in the brain (bought upon by drug abuse) could lead to alterations in phospholipid remodeling, and these changes may be responsible for or coincide with the induction of LTP and neuroadaptation. However the exact lipids involved in this process have not been identified.

The goal of this part of the work was to study phospholipid alterations in different regions of the rat brain in response to cocaine withdrawal. Three specific regions of the brain were studied, namely the hippocampus, the cerebellum and the frontal lobe. As previously mentioned, the hippocampus is associated with primary functions like long term memory and spatial navigation, both of which are thought to be based on induction of LTP (Thompson et al., 2005). The cerebellum is important for coordination and error checking during motor, perceptual and cognitive functions. These include proper hand-eye coordination, learning and remembering motor skills, smooth movement of muscles etc. (Campbell and Reece, 2004). Finally, the frontal lobe is located in the anterior part of the cerebral cortex, and is associated with higher order

functions like speech, language, motor functions, etc. (Campbell and Reece, 2004). Rats were subjected to acute cocaine withdrawal after which the phospholipid and sphingomyelin profiles of these three regions were analyzed using ESI-MS and compared to control tissue. Additionally, the effects of two different withdrawal protocols (extinguished vs. abstinent) were compared. In one scenario, animals were injected with saline during periods of withdrawal (extinguished). In the other scenario, animals were left untreated during periods of withdrawal (abstinent).

Thus the overall goal of this project was to use ESI-MS to analyze alterations in phospholipids in neuronal cells in two different scenarios, namely oxidant-induced injury in primary neocortical cells and cocaine withdrawal in rat brain.

CHAPTER 2
MATERIALS AND METHODS

Materials

Horse serum and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA). Neurobasal medium and B-27 supplement were obtained from Invitrogen (Carlsbad, CA). Bromoenol lactone (BEL) was obtained from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Methods

2.1. Isolation and culture of mouse neocortical primary cells

Primary cultures were prepared from embryonic day 17 Swiss Webster mice as described previously (Dravid and Murray, 2004). Pregnant mice were sacrificed by CO_2 asphyxiation, and embryos removed by laparotomy under sterile conditions. Cortices were collected in an isolation buffer consisting of 15 mL Krebs buffer (1 M NaCl; 0.045 M KCl, 8.5 mM $NaH_2PO_4 \cdot H_2O$, 0.12 M d-glucose, 0.2 mM phenol red, and 0.2 M HEPES, pH 7.4), 135 mL distilled H_2O, 0.45 g bovine serum albumin and 1.2 mL of 3.82% $MgSO_4$. The cortex was stripped of meninges, and the neocortex collected from each hemisphere. The neocortices were minced by trituration with a Pasteur pipette and centrifuged at 1334 × *g* for 5 min. The supernatant was aspirated and the pellet was incubated for 25 min at 37 °C in 30 mL of isolation buffer containing 7.5 mg trypsin. A solution of 15 mL of isolation buffer containing 1.2 mg DNase (150 KU) and 9.0 mg soybean trypsin inhibitor (>7700 BAEE units/mg) was added and the cells were centrifuged at 1334 × *g* for 5 min. The supernatant was aspirated and cells were dissociated by two successive trituration

and sedimentation steps in isolation buffer containing soybean trypsin inhibitor and DNase. The cells were centrifuged at 1334 × *g* for 7 min and resuspended in Eagles' minimal essential medium supplemented with 2 mM l-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin and 0.10 mg/mL streptomycin, pH 7.4. Cells were plated onto poly-l-lysine coated plates at a density of 1.45×10^6 cells/mL and incubated at 37 °C in a 5% CO_2 and 95% O_2 humidity atmosphere. Cytosine arabinoside (10 μM) was added to culture medium on day 2 to prevent proliferation of non-neuronal cells. Media was changed on day 7 and 10 by half addition with Neurobasal media supplemented with 2% B-27 supplement, 1% penicillin/streptomycin and 0.1% glutamine.

For treatment, cells were grown in T25 flasks for 12 days. Cells were pretreated with 2.5 μM BEL or MAFP for 30 min and then dosed with H_2O_2 (0-1000 μM) and TBHP (0-400 μM) for 0-24 h. After treatment, media was removed and cells were washed with PBS and 3 ml of methonal:water (2.0:0.8 v/v) was added to the flask and cells were removed with a cell scraper. Samples were frozen at -20°C after which cellular phospholipids were extracted using Bligh-Dyer lipid extraction (described below).

2.2. Animals

Male Sprauge-Dawley rats (Harlan) weighing approximately 300 g at the beginning of the experiments and were housed individually in a temperature and humidity controlled vivarium having a 12 h light: 12 h dark cycle. They were given access to food and water ad libitum and were handled daily for 5 days prior to injection to diminish stress associated with handling. The housing and experiment procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local Animal Care and Use Committee (ACUC) at the

University of Georgia. Rats were injected i.p. once daily with either saline or cocaine (15mg/kg) over 4 days in a conditioned place preference (CPP) protocol prior to a withdrawal period of either confinement in the home cage (abstinent) or injection of saline (extinguished). After 4 days of abstinence or extinction, a reinstatement CPP test was given in which a 5th cocaine injection was delivered. Tissues were collected 1 week following this final cocaine exposure (**Figure 2.1**). This protocol has previously been shown to induce neuroadaptation.

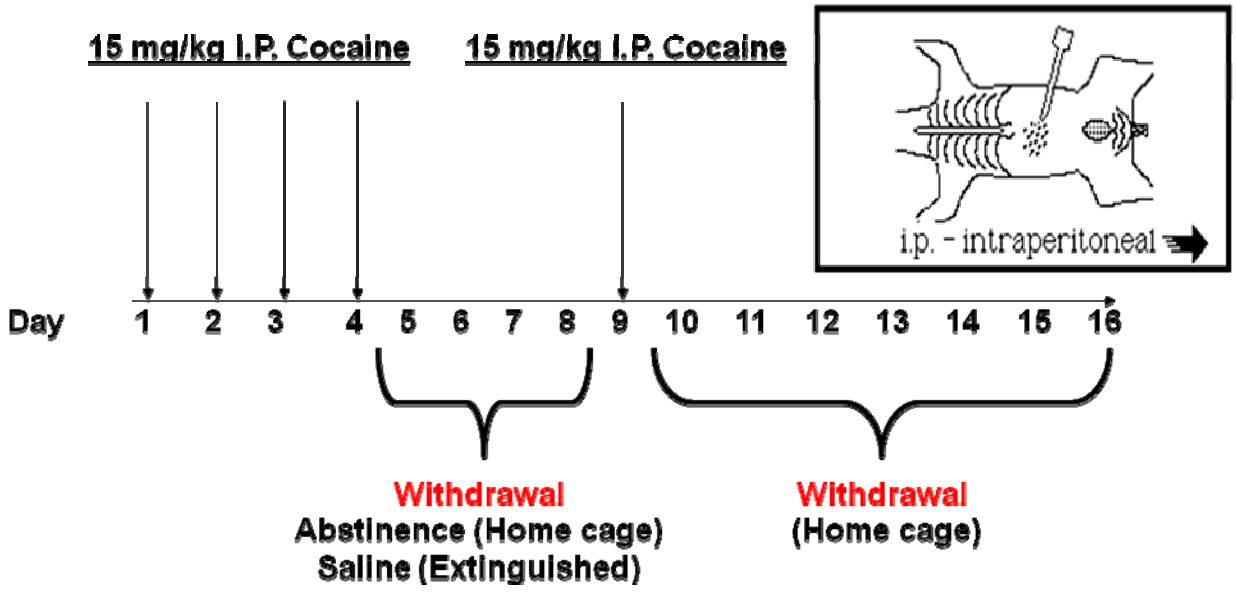

Figure 2.1. Study Design

2.3. Bligh-Dyer lipid extraction

Cellular phospholipids were extracted using chloroform and methanol according to the method of Bligh and Dyer (Bligh and Dyer, 1959). After treatment, brain tissue was removed and flash-frozen in liquid N_2 while cell culture samples were frozen at -20°C . After thawing, tissue was washed with PBS and homogenized in 3 mL of methanol: water (2.0:0.8 v/v). The

homogenate was transferred to a glass test tube and 1.25 mL chloroform was added. Tubes were vortexed for 30 seconds and allowed to sit for 10 minutes on ice. Tubes were centrifuged at 213 x *g* for 1 minute and the bottom chloroform layer was transferred to a new test tube. The extraction steps were repeated a second time and the chloroform layers combined. The collected chloroform layers were dried under argon, reconstituted with 50 mL of methanol: chloroform (2:1 v/v), and stored at -20°C.

2.4. Lipid phosphorus assay

Lipid phosphorus was quantified using malachite green (Zhou and Arthur, 1992). 10 mL of lipid extract was dried down under argon in a test tube. 200 mL of perchloric acid was added to the tube, and heated at 130°C for 2-3 hours. After this time, 1 mL of dH_2O was added to the tube while vortexing. 1.5 mL of reagent C (4.2 g Ammonium Molybdate Tetrahydrate in 100 mL 5 N HCl and 0.15 g Malachite Green Oxalate in 300 mL dd dH_2O) was added and vortexed. 200 mL of 1.5% v/v Tween 20 was added and vortexed. After 25 minutes of sitting at room temperature, a 200 mL aliquot was used to measure the absorbance at 590 nm.

2.5. Characterization and quantitation of cellular phospholipids using electrospray ionization-mass spectrometry (ESI-MS)

Lipid extract samples (500 pmol/μL) were prepared by reconstituting frozen samples in chloroform: methanol (2:1, v/v). Mass spectrometry was performed as previously described (Taguchi et al., 2000). Samples were analyzed using a Trap XCT ion-trap mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an ESI source. Samples (5 μL) were introduced by means of a flow injector into the ESI source at a rate of 0.2 mL/min. The elution solvent was acetonitrile:methanol:water (2:3:1, v/v/v) containing 0.1% (w/v) ammonium formate

(pH 6.4). The mass spectrometer was operated in the positive ionization mode. The flow rate of nitrogen drying gas was 10 L/min at 80 °C. The capillary and cone voltages were set at 2.5 kV and 30 V, respectively. As previously described (Taguchi et al., 2000), qualitative identification of individual phospholipid molecular species was based on their calculated theoretical monoisotopic mass values and quantification was done by comparison to the most abundant phospholipid in each sample, which corresponded to *m/z* 760 or [(34:1 (16:0-18:1)] phosphatidylcholine (PtdCho). MassLynx 4.0 software was used for data analysis.

MS^{nth} fragmentation was performed on a Trap XCT ion-trap mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an ESI source. The analyte was introduced by direct injection from the HPLC system. The nitrogen drying gas flow rate was 8.0 L/min at 350 °C, nebulizer pressure was 30 psi, and capillary voltage was 3 kV. The ion source and ion optic parameters were optimized with respect to the positive molecular ion of interest.

2.6. Characterization and quantification of fatty acids using ESI-MS

Lipid extracts (500 pmol/µl) were prepared by reconstituting in chloroform:methanol (2:1, v/v). A deuterated palmitic acid solution (5 µL of 0.12 mM) was added as an external standard to each sample. Samples were analyzed using a LCT Premier time of flight mass spectrometer (Waters, Milford, MA) equipped with an electrospray ion source. Samples (5 µL) were introduced by means of a flow injector into the ESI source at a rate of 0.2 mL/min. The elution solvent was acetonitrile:methanol:water (2:3:1, v/v/v) containing 0.1% (w/v) ammonium formate (pH 6.4). The mass spectrometer was operated in the negative ionization mode. The ESI conditions were as follows: nitrogen flow rate, 10 L/min at 80 °C; capillary voltage, 2.5 kV; source block temperature, 120 °C; desolvation temperature, 350 °C; cone voltage, 40 V.

2.7. Statistical Analysis

Data are represented as the average ± the S.E.M. of 2-4 separate experiments (n = 2-4). The appropriate analysis of variance (ANOVA) was performed for each data set using SAS software. Individual means were compared using either a Fishers protected least significant test, or a Student T-Test with $P < 0.05$ being considered indicative of a statistically significant difference between mean values.

CHAPTER 3
RESULTS

3.1. Phospholipid and fatty analysis of treated primary neocortical cells

ESI-MS was used to identify phospholipids altered in primary neocortical cells after exposure to oxidants. As previously mentioned, ESI-MS allows for rapid and simultaneous quantification and identification of phospholipids in small amounts of sample (Peterson and Cummings, 2006). Additionally, the increased sensitivity of ESI-MS over conventional methods like high pressure liquid chromatography (HPLC) allows for the analysis of phospholipids whose abundance is as low as 1% of total cellular phospholipids (Zhang et al., 2005).

The two oxidants H_2O_2 and TBHP induced concentration- and time-dependent changes in the phospholipid profile of primary neocortical cells. The characteristic positive-ion ESI-MS spectra for phospholipids extracted from control (untreated) cells is shown in **Figure 3.1.A**, while **Figure 3.1.B** shows the spectra for cells exposed to 500μM H_2O_2 for 24 hours. Preliminary analysis of phospholipids was based on theoretical *m/z* values reported in Taguchi et al. (2000), and Lipid Maps (www.lipidmaps.org).

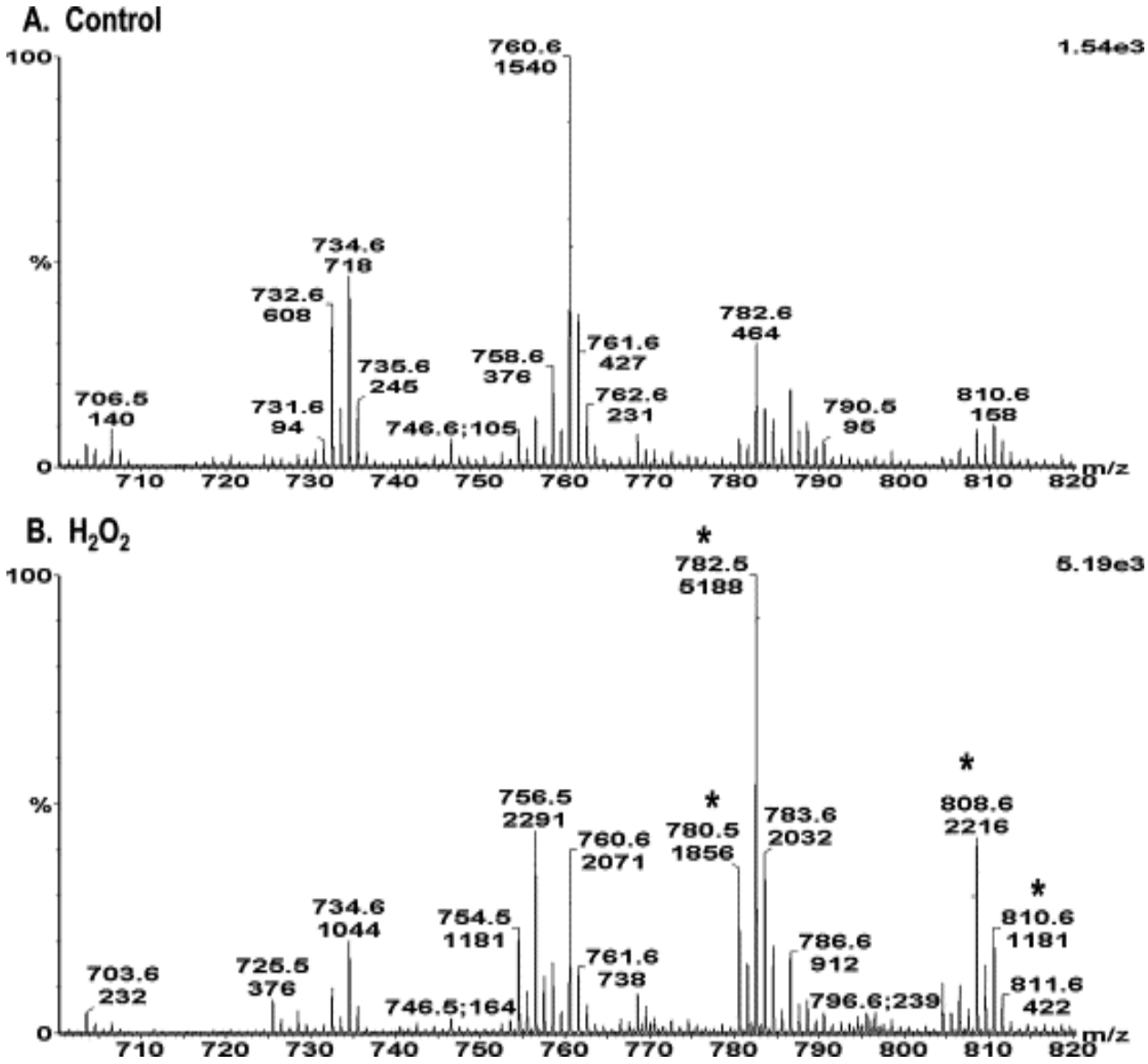

Figure 3.1. Positive-ion ESI-MS spectra of oxidant treated primary neocortical cultures for control cells (**A**) and 500μM H_2O_2 for 24 hours (**B**). Peaks marked with * indicate those that increased after treatment with oxidant. Taken from Peterson et al., 2008.

A previous study conducted in our laboratory (Peterson et al., 2008) showed that phospholipids containing polyunsaturated fatty acids (PUFA) showed a greater increase in response to treatment with oxidants compared to phospholipids with mono/di-unsaturated fatty acids. Treatment with H_2O_2 for 24 h increased the level of only two di-unsaturated containing species; 34:1 and 36:2 PtdCho. These phospholipids represent either 18:0-18:1 or 16:0-20:1 PtdCho (34:1) and 18:1-18:1 or 18:0-18:2 PtdCho (36:2). Treatment with TBHP for 24 h only slightly altered mono/di-unsaturated phospholipids. On the other hand, both H_2O_2 and TBHP significantly increased the levels of most PUFA-containing phospholipids compared to controls, with H_2O_2 inducing greater changes than TBHP. The greatest changes occurred in phospholipids with *m/z* values of 754, 780, 782, 804, 808 and 810. From this group, four species represent phospholipids containing AA at the *sn-2* position (780, 782, 808, and 810). This indicated that treatment of neurons with oxidants increases the levels of AA-containing phospholipids after 24 h. ESI-MS-MS-MS confirmed that the phospholipids corresponding to *m/z* 780, 782, 808, and 810 contain PtdCho and AA. ESI-MS-MS of peaks at the *m/z* of 808 resulted in fragmentation products of 749.7 and 625.7. These values correspond to the loss of a choline group (-59) and a phosphocholine head group (-183). Further fragmentation (ESI-MS-MS-MS) of the 625.7 peak resulted in the appearance of a major peak at *m/z* 303, which corresponds to arachidonic acid (AA). Similar results were obtained from analysis of peaks at *m/z* 780, 782, and 810, demonstrating that all these phospholipids contained phosphocholine head groups and AA at the *sn-2* position. These four AA-containing species were identified as 16:1-20:4 (*m/z* 780), 16:0-20:4 (*m/z* 782), 18:1-20:4 (*m/z* 808), and 18:0-20:4 (*m/z* 810) PtdCho (Peterson et al., 2008).

In the present study, both oxidants were found to induce increases in AA-containing phospholipids in a time-dependent manner (**Figure 3.2.**). Treatment of cells with 500μM H_2O_2 or

100μM TBHP did not increase the level of any phospholipid studied between 2-8 h. However after 16 h exposure to 500μM H_2O_2, the levels of all four AA-containing phospholipids increased, and continued to do so at 24 h (**Figure 3.2.A**). Similarly, 100 μM TBHP induced increases in 16:1-20:4 after 16 h, while 16:0-20:4 and 18:1-20:4 were increased after 24 h (**Figure 3.2.B**).

The effects of $cPLA_2$ and $iPLA_2$ inhibition were also tested. Treatment of cells with 2.5μM BEL (selective inhibitor of $iPLA_2$) decreased H_2O_2-induced increases of all phospholipids analyzed, but did not prevent TBHP-induced increases (**Figure 3.2.C**). Treatment with MAFP (inhibitor of $cPLA_2$) had no effect on alterations in AA-containing phospholipids (**Figure 3.2.D.**). Additionally, treatment with BEL or MAFP alone did not alter lipid profiles.

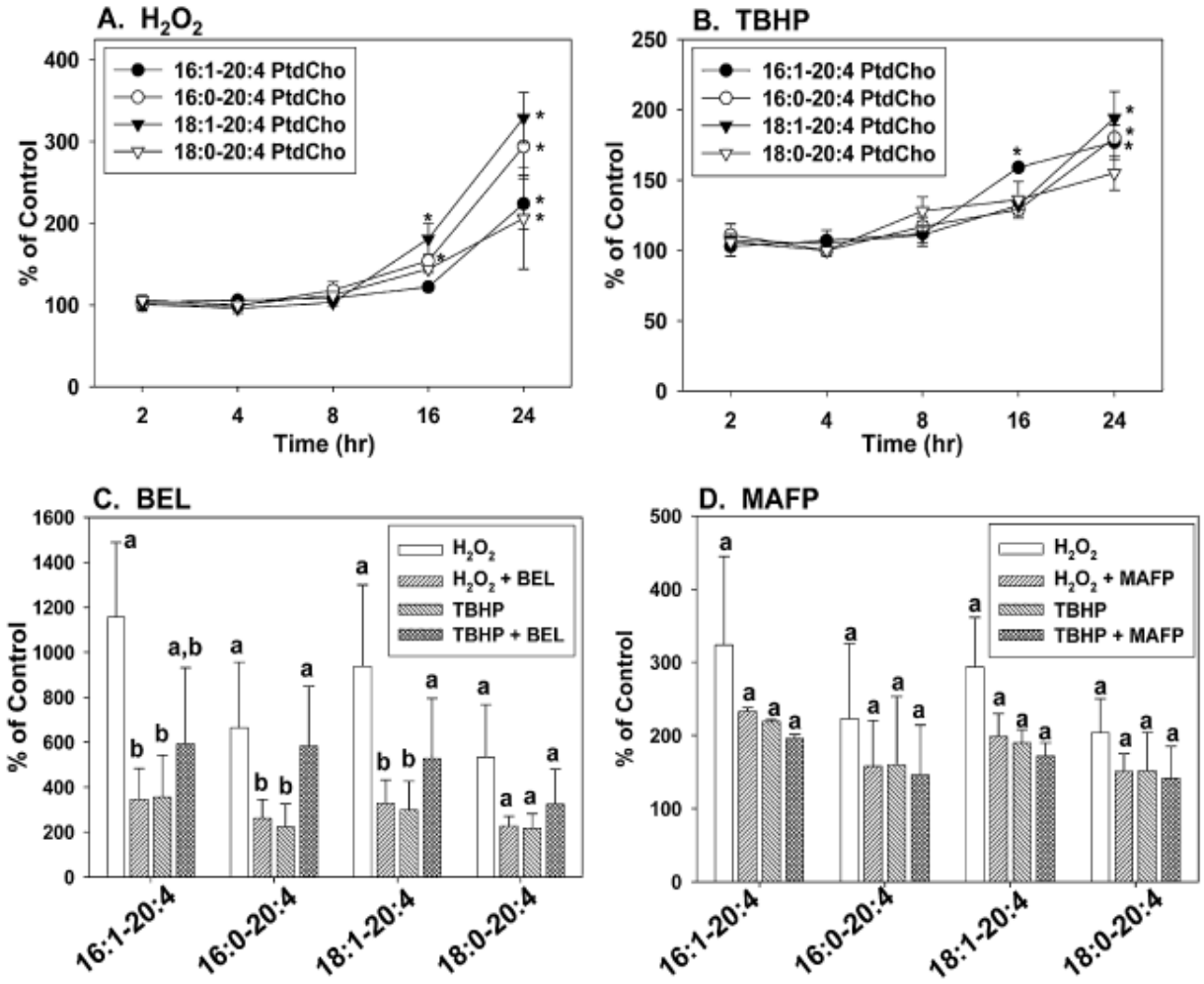

Figure 3.2. Effects of oxidants on AA-containing PtdCho species in primary neocortical cultures. Cultures were treated with 500μM H_2O_2 **(A)** or 100μM TBHP (**B**) in the presence and absence of 2.5μM BEL (**C**) or MAFP **(D)**. Cells in **A** and **B** were exposed to oxidatns for 2, 4, 8, 16, and 24 h while samples in **C** and **D** were exposed for 24 h only. Data are represented as the average ± the S.E.M of at least three (n=3) separate experiments. ***** in **A** and **B** indicates significant difference (P<0.05) from control. Means with different subscripts in C and D are significantly different from each other (P<0.05) within each group.

The effects of oxidants and PLA_2 inhibitors on fatty acid release were also studied. The characteristic negative-ion ESI-MS spectra of lipid extracts from control and oxidant treated cells is shown in **Figure 3.3.**

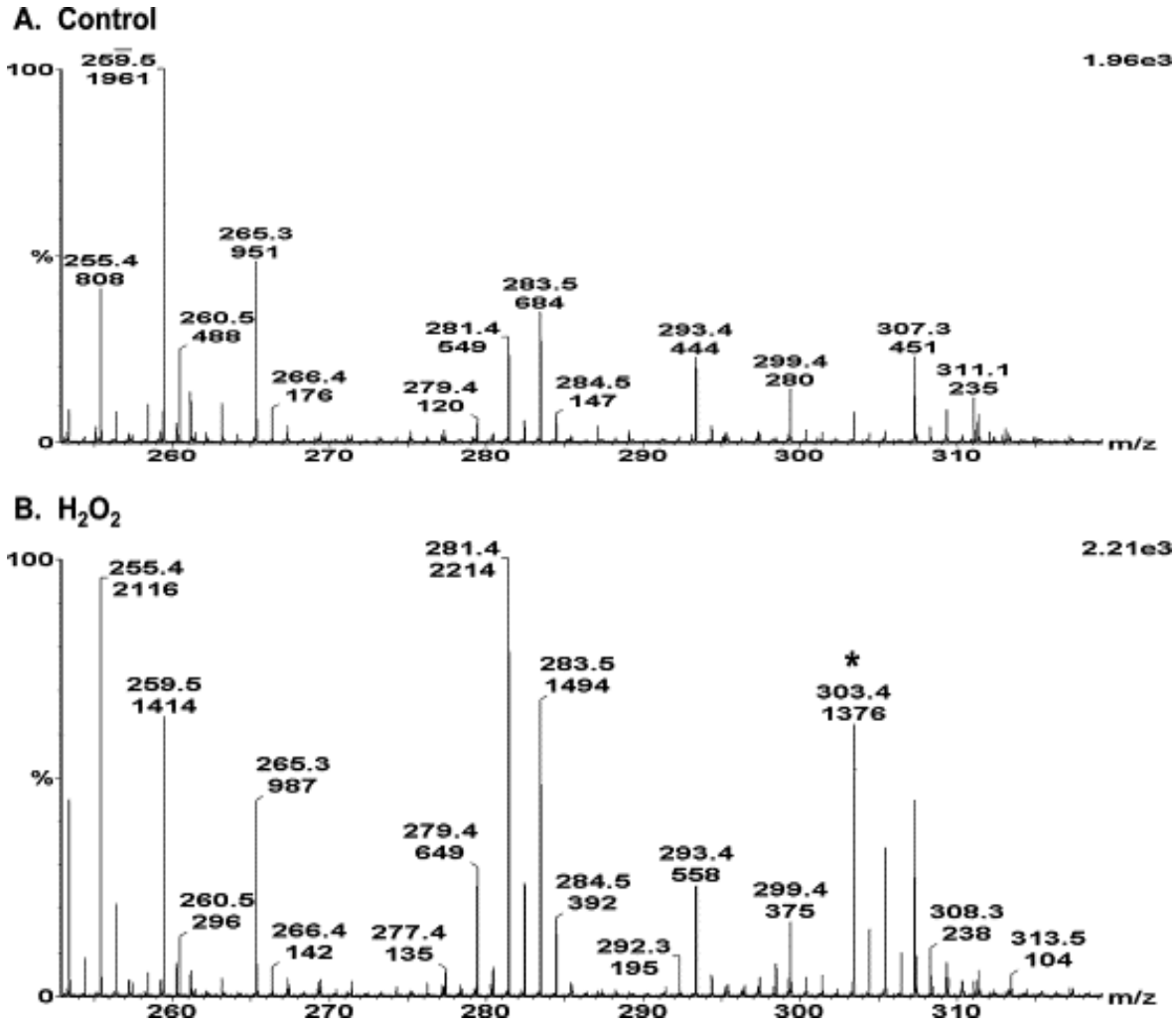

Figure 3.3. Negative-ion ESI-MS spectra of oxidant treated primary neocortical cultures. Comparison of the mass spectra of phospholipids for control cells (**A**) and cells treated with 500μM H_2O_2 for 24 h (**B**). The peak corresponding to the *m/z* value for arachidonic acid is marked with an *. Taken from Peterson et al., 2008.

Previously, our lab analyzed the effects of H_2O_2 and TBHP treatment on eight different fatty acids**.** As expected, AA showed the greatest alteration in response to oxidant treatment (Peterson et al., 2008). This result was confirmed in the present study. In addition, time-dependent studies indicated that H_2O_2 treatment increased AA levels threefold after 4 h of exposure, with no further increases taking place. Similarly, TBHP increased AA levels after 4 h followed by stabilization of AA levels up to 24 h (**Figure 3.4.A**.**)** Pre-treatment with BEL did not affect AA increases after 24 h (**Figure 3.4.B**), while MAFP reduced oxidant-induced increases in AA at the 24 h time point (**Figure 3.4.C**).

In addition to increases in AA levels, oxidant exposure also increased the abundance of oxidized AA products. These changes were first detected at 16 h, and neither BEL nor MAFP reduced oxidant-induced increases in these species.

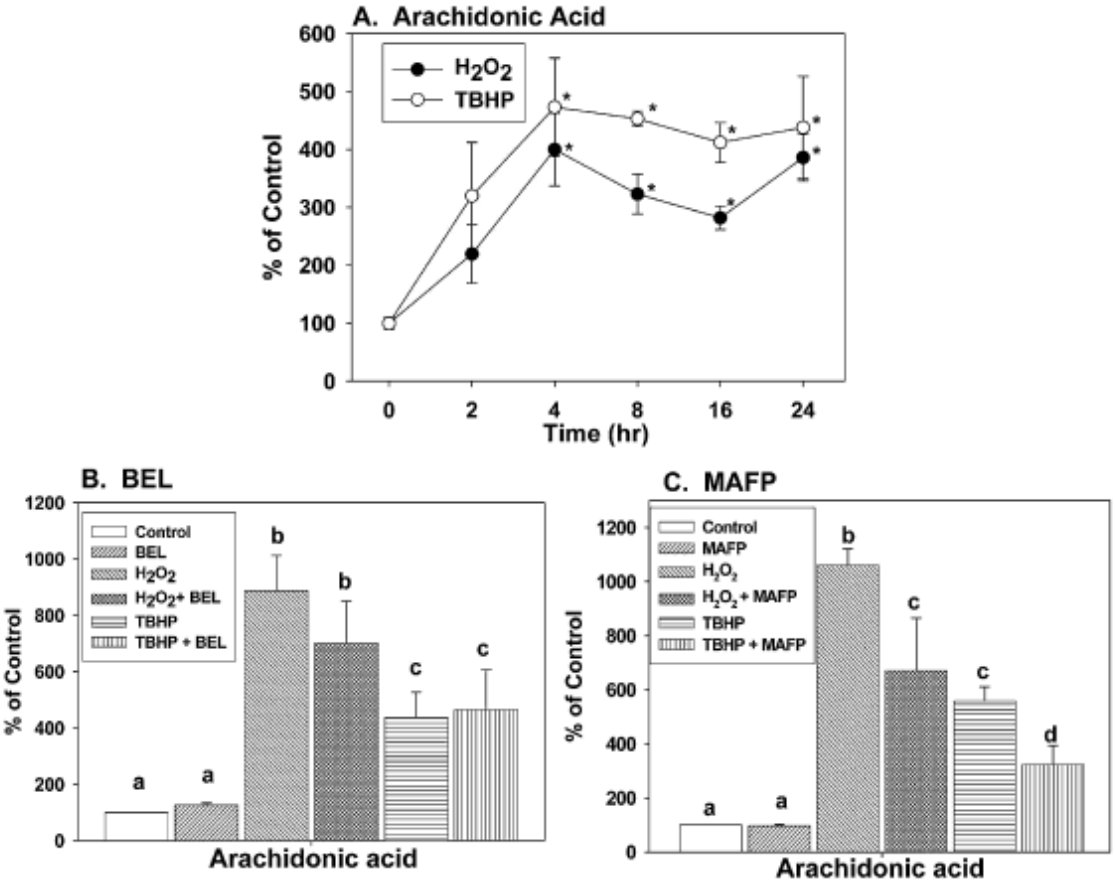

Figure 3.4. Effects of oxidants on AA levels in primary neocortical cultures. Cultures were treated with 500μM H_2O_2 or 100μM TBHP for 0, 2, 4, 8, 16 and 24 h (**A**) in the presence and absence of 2.5μM BEL (**C**) or MAFP **(D**). Data are represented as the average ± the S.E.M of at least three (n=3) separate experiments. ***** in **A** and **B** indicates significant difference (P<0.05) from control. Means with different subscripts in C and D are significantly different from each other (P<0.05).

3.2. Alterations in phospholipids in rat brain after cocaine withdrawal

In the second part of this project, rats were subjected to acute cocaine withdrawal over a period of 16 days (**Figure 2.1.**). Following this treatment, rats were sacrificed and brain tissues harvested. Total phospholipids were extracted using the Bligh-Dyer method and analyzed by ESI-MS. As previously mentioned, three specific regions of the brain were studied. Positive-ion ESI-MS spectra of the three regions from control tissue are shown in **Figure 3.5.**

Initial analysis was focused on 25 phospholipids species. Regional differences in the expression of glycerophospholipids in rat brain are shown in **Figure 3.6**. In general, the expression of most phospholipids were similar between the three regions, with subtle differences. Specifically, **t**he phospholipid profile of the hippocampus and frontal lobe were similar to each other while the phospholipid profile of the cerebellum was different from both the frontal lobe and hippocampus. It is important to note that all three regions contained the same phospholipids but showed different levels of expression of specific phospholipids. For example, the cerebellum expressed lower levels of the 32:0 and 36:4 PtdCho, but higher levels of 34:2, 36:3 and 36:1 PtdCho. Furthermore, the cerebellum expressed higher levels of most sphingomyelin species, including 20:0, 22:1, 24:1 and 26:4 sphingomyelin with both the hippocampus and frontal lobe showing limited expression.

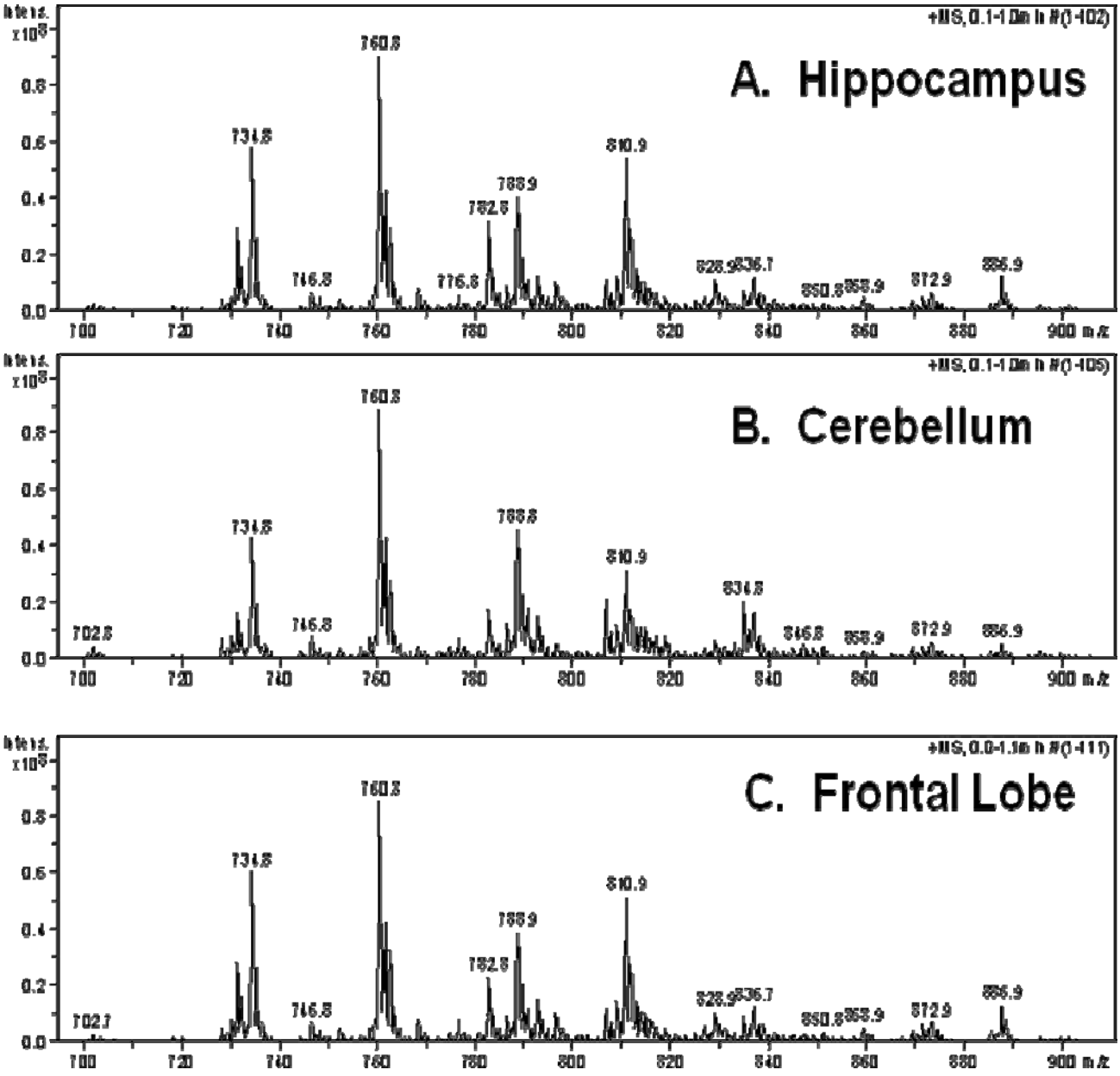

Figure 3.5. Positive-ion ESI-MS spectra of rat brain regions from control tissue; Hippocampus (**A**), Cerebellum (**B**), and Frontal Lobe (**C**). The peak represented by *m/z* 760.8 corresponds to a 34:1 PtdCho and is the most abundant peak in all samples.

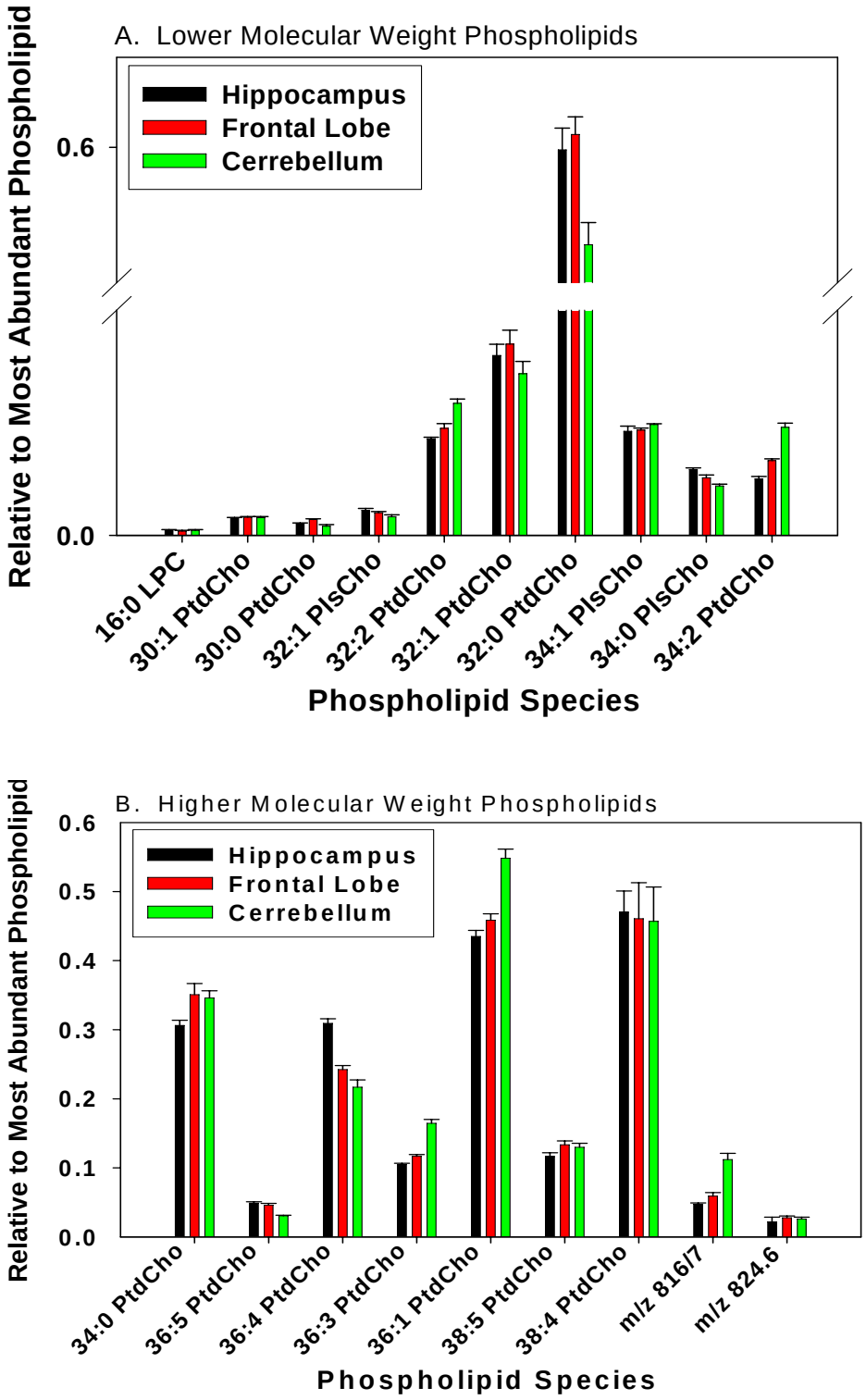

Figure 3.6. Differences in the expression of PtdCho in rat brain frontal lobes, hippocampus, and cerebellum. Tissues were isolated from untreated rats and lipids isolated using Bligh-Dyer extraction techniques and analyzed by ESI-MS. Data are represented as the mean $\pm$ the S.E.M. of at least 4 separate (n = 4) experiments

Of the different phospholipid species surveyed in the different regions of the rat brain, three were found to change in response to cocaine withdrawal. These were 16:0 lysophospholipid (*m/z* 496), 14:0-16:1 PtdCho (*m/z* 704.6) and 18:0-20:4 PtdCho (*m/z* 810.6). The differences in the expression of these lipids are shown in **Figures 3.7., 3.8.** and **3.9**. The level of 16:0 lysophospholipid was higher in the hippocampus after cocaine abstinence, but not after extinguishment (**Figure 3.7.)**. The level of 14:0-16:1 PtdCho was lower in the hippocampus after cocaine abstinence, but not after cocaine extinguishment (**Figure 3.8.**). Finally, the level of 18:0-20:4 PtdCho was lower in the hippocampus after both cocaine withdrawal and extinguishment (**Figure 3.9.**). No changes were detected in the frontal lobe or cerebellum for any of these three lipids. Also, withdrawal from cocaine did not alter the expression of sphingomyelins in any region analyzed.

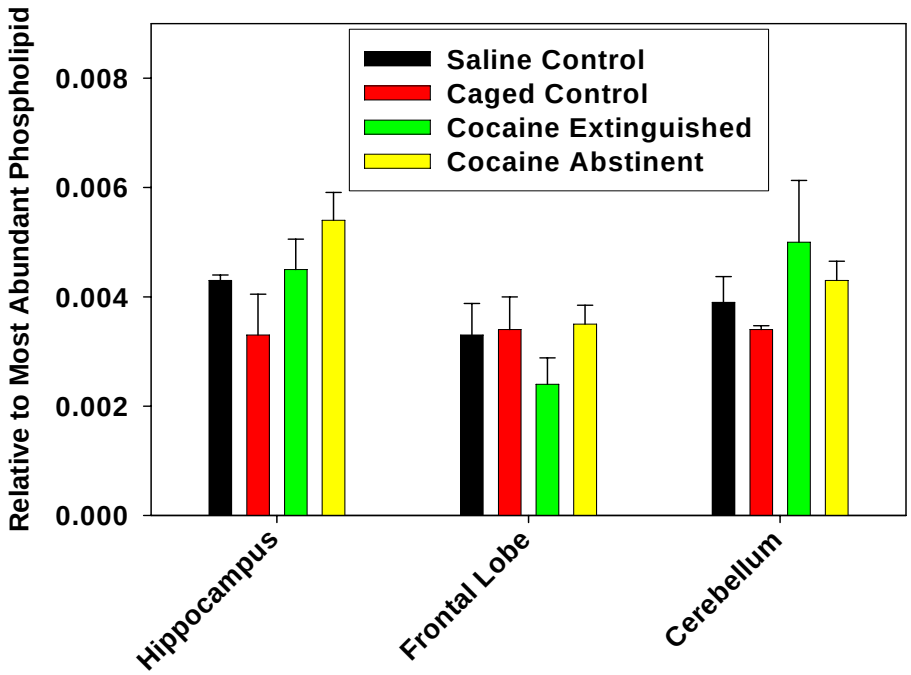

Figure 3.7. Region-specific differences in the expression of 16:0 lysophospholipid (*m/z* 496) in rat brain**.** Data are represented as the mean ± the S.E.M. of at least 2 separate (n=2) experiments.

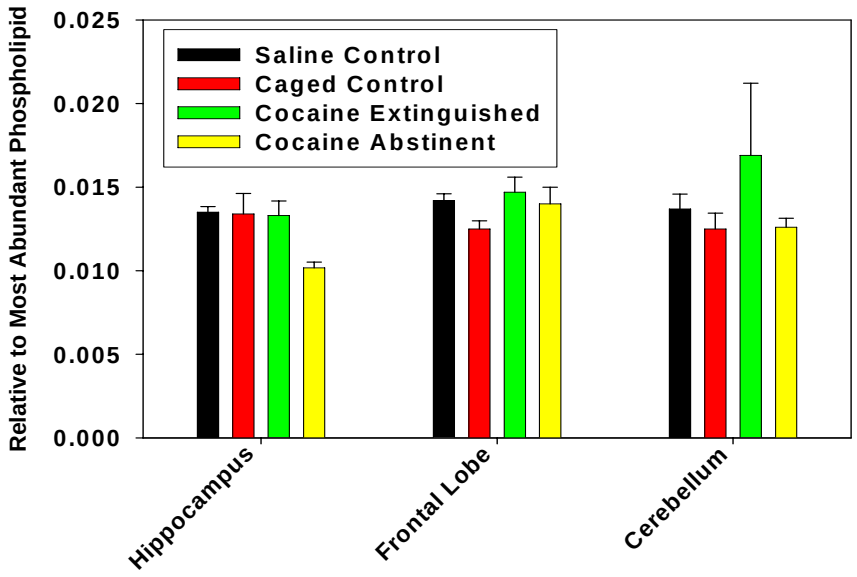

Figure 3.8. Region-specific differences in the expression of 14:0-16:1 PtdCho (*m/z* 704.6) in rat brain. Data are represented as the mean ± the S.E.M. of at least 2 separate (n=2) experiments.

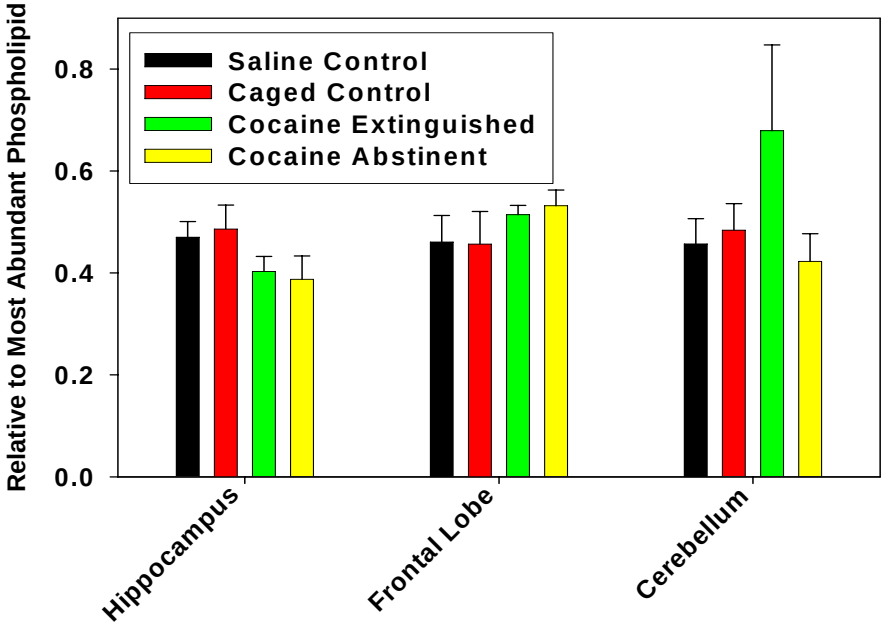

Figure 3.9. Region specific differences in the expression of 18:0-20:4 PtdCho (*m/z* 810.6) in rat brain. Data are represented as the mean ± the S.E.M. of at least 2 separate (n=2) experiments.

CHAPTER 4
DISCUSSION

Given the growing biological significance of lipids over the past few years, there has been a need for improvement and development of techniques for the comprehensive analysis of lipids (Carrasco-Pancorbo et al., 2009). Lipidomics, the systems-based study of all lipids and their functions within the cell is thus rapidly emerging as an active area of research (Navas-Iglesias et al., 2009). Briefly, the analysis of lipids involves three major steps; extraction, analytical separation, and identification & quantification. The advancement of mass spectrometry methods has virtually eliminated the need for analytical separation of lipids within extracts (Carrasco-Pancorbo et al., 2009). Specifically, ESI-MS, which offers several advantages over traditional methods, has emerged as the most used method for the analysis of phospholipids (Peterson and Cummings, 2006). In this study, we used ESI-MS to analyze alterations in phospholipids in neuronal tissue in response to two different stimuli.

First, the effects of the oxidants TBHP and H_2O_2 on the phospholipid profile of primary neocortical cells was investigated. The use of primary cultures was preferred over cell lines, which are often immortalized or cancerous in origin. ESI-MS allowed for the analysis of nearly 50 different phospholipid species and fatty acids. Previous studies done by our laboratory show that nearly all PUFA-containing phospholipids showed significant increases in abundance, while very few mono/di-unsaturated species were affected (Peterson et al., 2008). This finding is in agreement with previous studies (Cummings et al., 2004), and supports the hypothesis that PUFA-containing phospholipids are the more susceptible to oxidation. The most predominant

changes following oxidant exposure were found in six phospholid species. Using ESI-MS-MS-MS, four of these were identified as 16:0-, 16:1-, 18:0-, and 18:1- 20:4 PtdCho (all AA containing species) (Peterson et al., 2008). Several lines of evidence suggest that the changes in these phospholipids are part of the cells attempt to remodel the membrane, rather than global response to oxidant-induced injury. Firstly, the levels of these phospholipids increased after oxidant exposure as opposed to decreasing, which is what would normally happen if these changes were due to loss of membrane integrity. Inhibition of $iPLA_2$ using BEL prior to oxidant exposure reversed these changes, while having little effect on other PUFA-containing phospholipids. Furthermore, the concentration of oxidants used ensured that at least 30-40% viability is maintained after 24 hours. Finally, the levels of these phospholipids increased 12 h after initial increases in ROS formation and AA release. These findings support the hypothesis that upon exposure to oxidants, neuronal cells undergo remodeling of phospholipids and that this process is mediated by $iPLA_2$.

Time-dependent studies showed that increases in AA levels preceded increases in the four AA-containing phospholipids. This indicates that the cell may reincorporate some of the released AA back into the membrane leading to increases in AA-containing species. Inhibition of $cPLA_2$ using MAFP prior to oxidant treatment decreased AA levels, while BEL failed to have such an effect. This suggests that $cPLA_2$ and not $iPLA_2$ plays a role in oxidant-induced AA release. However the inability of $cPLA_2$ inhibition to decrease AA release to control levels implicates additional mechanisms such as direct attack by ROS in oxidant-induced AA release. While MAFP decreased oxidant-induced AA release, the levels of AA-containing phospholipids were unaffected. In contrast, BEL had no affect on AA release, but altered increases in AA-containing phospholipids, suggesting that $iPLA_2$ mediates increases in these phospholipids. This

makes sense given the ability of $iPLA_2$ to release lysophospholipid acceptors to which free fatty acids can be attached (Cummings, 2007).

Both TBHP and H_2O_2 treatment increased 16:0-, 16:1-, 18:0-, and 18:1- 20:4 PtdCho. BEL was able to inhibit H_2O_2-induced increases; however, TBHP-induced increases in these phospholipids were unaffected by BEL. This may be because TBHP is a more potent and lipophilic oxidant. Also, TBHP has previously been shown to inactivate $iPLA_2$ in renal cells (Cummings et al., 2004). Thus, prior inactivation of $iPLA_2$ by TBHP may explain the inability of BEL to inhibit oxidant-induced increases in phospholipids in the presence of TBHP. Both TBHP and H_2O_2 also induced increases in oxidized AA species. Since neither BEL nor MAFP reversed these increases, this process does not seem to be mediated by $iPLA_2$ or $cPLA_2$.

In conclusion, data from this study suggests that oxidative stress triggers the release of AA, a process mediated by $cPLA_2$. This is followed by increases in the levels of AA-containing phospholipids, a process mediated by $iPLA_2$. This suggests that the cell is actively reincorporating released AA into membranes. In order for this to happen, AA must be esterified to a lysophospholipid, likely generated by $iPLA_2$. We believe that AA is not reinserted into the same phospholipids from which it was released, as BEL did not alter AA levels itself. Based on these findings, we have proposed a mechanism for oxidant-induced increases in AA-containing phospholipids (**Figure 4.1.**). Oxidative stress induced increases specifically in AA-containing phospholipids, AA and oxidized-AA, despite the presence of several other phospholipids. This specificity may eventually be used as biomarkers of oxidant-induced injury.

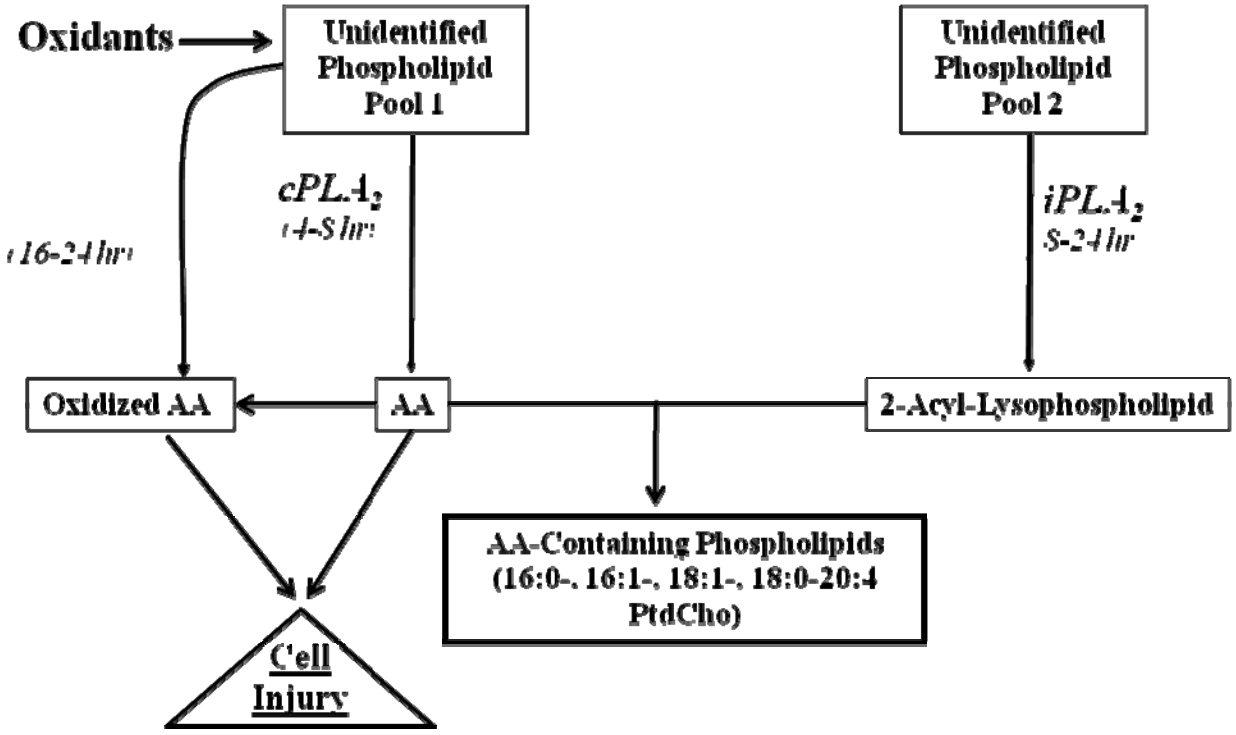

Figure 4.1. Proposed mechanism for oxidant-induced increases in AA-containing phospholipids. Oxidant exposure results in $cPLA_2$-mediated release of AA from unidentified phospholipid pool 1 (4-8 hr). This AA may be further oxidized to reactive species which appear after 16 h. Both un-oxidized and oxidized AA induce cell injury. Increases in AA are balanced by the release of acyl-lysophospholipids by $iPLA_2$ from unidentified phospholipid pool 2 (8-24 hr). The lysophospholipids and AA are re-acylated and esterified into a new phospholipid pool (AA-containing phospholipids). The release of AA appears to occur as early as 4 h, while increases in oxidized AA and AA-containing phospholipids occur after 16 h.

It is important to note that this study did not identify the phospholipids from which AA is released in the first place (phospholipid pool 1, **Figure 4.1.**). Furthermore, lipids whose levels decreased in correlation with increases in 16:0-, 16**:**1-, 18:0- and 18:1-20:4 PtdCho were not identified. We specifically focused on choline-containing species in this study. Phospholipids containing ethanolamine and serine were not tracked. It is highly likely that oxidative stress induces release of AA from PtdEth, PtdSer, and other phoshpolipids. Future studies using LC-MS or HP-TLC to separate lipids prior to injection are needed to address these issues.

In the second part of this study, we investigated the phospholipid alterations in three regions of the rat brain after cocaine withdrawal. In general, the phospholipid profiles of the hippocampus and frontal lobe were similar, but differed significantly from the cerebellum which displayed lower levels of 32:0- and 36:6-PtdCho, but higher levels of 34:2-, 36:3- and 36:1-PtdCho. Sphingomyelins, especially 20:0-, 22:1-, 24:1- and 26:4-sphingomyelins, were found to be most predominantly expressed in the cerebellum. The phospholipid profiles of the three regions studied differed mostly in the levels of expression of each phospholipid, not in the species of phospholipid expressed. To our knowledge, this was the first time that ESI-MS was used to report differences in phospholipid expression between different areas of the rat brain.

Rats were subjected to two cocaine withdrawal protocols, both of which have previously been shown to induce neuroadaptation. In the abstinence protocol, rats were confined to their home cages during withdrawal periods. In contrast, rats subjected to the extinguished protocol were injected with saline during withdrawal periods. The abstinence withdrawal protocol appeared to result in greater changes in phospholipid profiles compared to the extinguished protocol. Neither protocol induced changes in sphingomyelin expression in any brain region studied. Cocaine withdrawal induced region-specific alterations in the expression of glycrophospholipids. Importantly, all the withdrawal-induced alterations in phospholipids were confined to the hippocampus. Specifically, the abstinence protocol resulted in lower levels of 14:0-16:1 and 18:0-20:4 Ptdcho in the hippocampus. These decreases correlated to increases in the levels of 16:0- lysophospholipids in the hippocampus. This suggests the occurrence of active membrane remodeling, perhaps as part of the withdrawal-induced neuroadaptation.

Collectively, these data support the hypothesis that cocaine withdrawal can induce alterations in phospholipid expression in rat brain, specifically in the hippocampus. This finding

is especially important given the previously mentioned association between the hippocampus and LTP. The phospholipid alterations identified in this study could be part of the mechanism involved in inducing LTP, which is believed to be ultimately responsible for neuroadaptation. However, further studies are needed to determine if these changes are causing neuroadaptation or are just a response to neuroadaptation. The significance of the abstinence withdrawal protocol inducing greater changes than the extinguished protocol remains unclear. While the evidence presented suggests that cocaine withdrawal does indeed induce membrane remodeling, the exact mechanisms responsible for this remains to be identified. Specifically, investigating the involvement of $iPLA_2$ in mediating this process will help decipher the pathways involved. Furthermore, we limited our analysis in this study to alterations in PtdCho and sphingomyelin. As in the case of the first study, analysis of other phospholipid and fatty acid species along with time-dependent studies are needed to lend further credibility to these findings.

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