1. INTRODUCTION

Traumatic brain injury

Traumatic brain injury (TBI), an acquired brain injury, results from a brain damage caused by sudden trauma. Two mechanisms of TBI have been identified: a primary injury and a secondary injury following it. In the United State, each year approximately 1.4 million people experience a TBI, approximately 1 million of them are treated in hospital emergency rooms, and approximately 230,000 of them are hospitalized for TBI and survive, approximately 50,000 of them die from head injury (M. R. Hoane et al., 2007). TBI highly occurs among male adolescents and young adults ages 15 to 24, and among elderly people more than 75 years old. Children aged 5 and younger are also at high risk of TBI (1998).

A TBI can cause problems with arousal, consciousness, awareness, alertness, and responsiveness. Sometimes, TBI is followed by complications. Complications of TBI include immediate seizures, hydrocephalus or post-traumatic ventricular enlargement, vascular injuries, cranial nerve injuries, cerebrospinal fluid (CSF) leaks, infections, bed sores, pain, multiple organ system failure in unconscious patients, and polytrauma. Disabilities may also be induced by TBI depending upon the severity and location of the injury, and general health and the age of the patients. Disabilities resulting from a TBI include problems with cognition (memory, thinking, and reasoning), sensory processing (sight, hearing, taste, smell and touch), communication (expression and understanding), and behavior or mental health (D. W. Marion, 1991; O. o. C.
Furthermore, TBI can be followed by other long-term problems such as Parkinson's disease and other motor problems, Alzheimer's disease, dementia pugilistica, and post-traumatic dementia (D. W. Marion, 1991).

Approximately half of patients who suffered severe TBI will need surgery to remove or repair hematomas or contusions. However, until now there are no effective medical treatments for TBI. In order to develop the effective potential therapy for TBI, understanding the mechanisms that underly it is critical. Previous studies have shown that cytosolic phospholipase A$_2$ (cPLA$_2$) mediated secondary spinal cord injury (N. K. Liu et al., 2006) and several studies indicate that cPLA$_2$ are implicated in traumatic brain injury (A. A. Farooqui et al., 2006).

Phospholipase A$_2$ family

PLA$_2$ is a lipolytic enzyme which hydrolyzes the acyl bond at sn-2 position of glycerophospholipids to release lysophospholipids and free fatty acids. These products are precursors of platelet activating factor (PAF) and eicosanoids respectively, which contribute to inflammation and tissue damage (A. A. Farooqui et al., 1997b; M. Murakami et al., 1997; I. Kudo and M. Murakami, 2002). In physiological conditions, PLA$_2$s are involved in multiple cellular responses, such as phospholipid digestion and metabolism, host defense, and signal transduction. In pathological conditions, increased PLA$_2$ activity will result in excessive production of free fatty acid such as arachidonic acid (T. K. McIntosh et al.) which results in production of proinflammatory mediators such as eicosanoids and PAF.
and lead to the loss of membrane integrity, inflammation, oxidative stress and neuronal injury (N. G. Bazan et al., 1995; J. W. Phillis and M. H. O'Regan, 2004; A. A. Farooqui et al., 2006).

Classification, isoform, structure and properties of \( \text{PLA}_2 \)

\( \text{PLA}_2 \) contains 4 subfamilies, secretory \( \text{PLA}_2 \) (s\( \text{PLA}_2 \)), c\( \text{PLA}_2 \), Ca\(^{2+}\)-independent \( \text{PLA}_2 \) (i\( \text{PLA}_2 \)) and platelet activating factor acetyl hydrolase (PAF-AH)/oxidized lipid lipoprotein associated (Lp)\( \text{PLA}_2 \) (M. Murakami and I. Kudo, 2002; R. H. Schaloske and E. A. Dennis, 2006). s\( \text{PLA}_2 \)s are synthesized intracellularly and secreted and act extracellularly. They include 10 isoforms all of which have low molecular mass (14-18KD). Submillimolar to millimolar concentrations of Ca\(^{2+}\) are required for effective hydrolysis of s\( \text{PLA}_2 \)s on its substrate phospholipid. s\( \text{PLA}_2 \)s do not show any fatty acid selectivity (R. J. Mayer and L. A. Marshall, 1993; E. A. Dennis, 1994; M. Murakami et al., 1997; R. H. Schaloske and E. A. Dennis, 2006). i\( \text{PLA}_2 \)s are intracellular enzymes responsible for maintenance of membrane phospholipids. Their molecular weights are 28-91KD. They comprise 7 isoforms. Hydrolysis effects of i\( \text{PLA}_2 \)s on substrate are Ca\(^{2+}\)-independent. PAF-AH family consists of 4 enzymes which do not require Ca\(^{2+}\) for activation and exhibit unusual substrate specificity to PAF and/or oxidized phospholipids (M. Murakami et al., 1997; M. Murakami and I. Kudo, 2002; R. H. Schaloske and E. A. Dennis, 2006). c\( \text{PLA}_2 \)s consist of 6 isoforms and have high molecular mass (85-110KD). They selectively hydrolyze phospholipids which contain AA and their effective hydrolysis effects require submicromolar
concentrations of Ca\(^{2+}\) (I. Kudo and M. Murakami, 2002; M. Murakami et al., 1997; R. H. Schaloske and E. A. Dennis, 2006). Because cPLA\(_2\) is one of the most important PLA\(_2\) isozymes implicated as an effector in receptor-mediated release of AA and exhibited strong preference for deacylation of AA, which can serve as ROS source and act as a precursor of several proinflammatory mediators (C. Kim et al., 2008), over other fatty acids (M. Hernandez et al., 2000; A. A. Farooqui et al., 2006), the present study focused on the role of cPLA\(_2\) in secondary brain injury. cPLA\(_2\)s consist of cPLA\(_2\)\(\alpha\), cPLA\(_2\)\(\beta\), cPLA\(_2\)\(\gamma\), cPLA\(_2\)\(\delta\), cPLA\(_2\)\(\epsilon\) and cPLA\(_2\)\(\zeta\), which are grouped into the group IVA, IVB, IVC, IVD, IVE and IVF respectively. cPLA\(_2\)\(\alpha\) is the most extensively studied cPLA\(_2\) isoforms. The gene of cPLA\(_2\)\(\alpha\) can be found on the human and mouse chromosome 1. cPLA\(_2\)\(\alpha\) contains a Ca\(^{2+}\) binding site (C2 domain), which locates at N-terminal and confers Ca\(^{2+}\)-binding ability. Catalytic and multiple phosphorylation sites of cPLA\(_2\)\(\alpha\) are found in C-terminal. The catalytic domain is composed of a core \(\alpha/\beta\) hydrolase region. Unlike other cPLA\(_2\) isoforms, the catalytic domain of cPLA\(_2\)\(\alpha\) contains a cap region. This cap region posses a lid region. Conformational change of the lid region has been shown in the presence of substrate. The lid region prevents the modeling of a phospholipid substrate in the active site. (J. E. Burke et al., 2008). cPLA\(_2\)\(\alpha\) activity is regulated by translocation and phosphorylation. Intracellular Ca\(^{2+}\) can bind to cPLA\(_2\)\(\alpha\) C2 domain and then induce translocation of cPLA\(_2\)\(\alpha\) catalytic domain from cytosol to the nuclear, Golgi, endoplasmic reticulum (D. E. Tucker et al., 2005). This translocation allows Ca\(^{2+}\)-dependent phospholipid-binding domain at cPLA\(_2\)\(\alpha\) N-terminal reaches the
membrane where its substrate such as phospholipids and downstream enzymes such as cyclooxygenases and lipoxygenases, are located (J. D. Clark et al., 1995). Furthermore, sustained phosphorylation of both Ser-505 and Ser-727 of cPLA$_2$$\alpha$ by mitogen-activating protein kinase (MAPK) and protein kinase C (PKC) are also required for the cPLA$_2$$\alpha$ activation (J. D. Clark et al., 1995; T. Hirabayashi and T. Shimizu, 2000; T. Hirabayashi et al., 2004). Ca$^{2+}$/calmodulin kinase II can also bind to and phosphorylate cPLA$_2$$\alpha$ on Ser515 and subsequently activate it in a MAPK-independent manner (C. Song et al., 1999; A. Stewart et al., 2002). Full cPLA$_2$ activation requires protein kinase phosphorylation at three cPLA$_2$ phosphorylation sites, Ser505/515/727 (J. W. Phillis and M. H. O'Regan, 2004; M. Chalimoniuk et al., 2009). In addition, in the neural membranes, cPLA$_2$ activity and AA release are linked to glutamate, dopamine, serotonin, $\rho_2$-purinergic, muscarinic, cytokine and growth factor receptors by different coupling mechanisms; some of these receptors involve G protein (A. A. Farooqui et al., 2006). cPLA$_2$$\alpha$ expression can be induced by several proinflammatory cytokines such as IL-1, TNF-$\alpha$ and IFN-$\gamma$ and growth factor such as EGF, M-CSF and stem cell factor (J. D. Clark et al., 1995; M. Murakami et al., 1997). cPLA$_2$$\alpha$ expression can also be reduced by glucocorticoids (M. Murakami et al., 1997).

The genes of cPLA$_2$$\beta$, cPLA$_2$$\delta$, cPLA$_2$$\varepsilon$ and cPLA$_2$$\zeta$ form a gene cluster on human and mouse chromosome 2 and 15 respectively. cPLA$_2$$\beta$ contains Ca$^{2+}$-dependent C2 domain (C. Song et al., 1999; J. G. Pilitsis et al., 2003) and posses low intrinsic activity (K. K. Lucas and E. A. Dennis, 2004). The gene for cPLA$_2$$\gamma$ is
located on human chromosome 19 and mouse chromosome 7 respectively. It is distinct from cPLA$_2$$\alpha$ and cPLA$_2$$\beta$ in that it lacks the C2 domain and has Ca$^{2+}$-independent activity (K. W. Underwood et al., 1998; M. Murakami and I. Kudo, 2002; R. H. Schaloske and E. A. Dennis, 2006). Furthermore, cPLA$_2$$\gamma$ binds to membrane constitutively. Its membrane association may be regulated by putative acylation sites and a C-terminal prenylation site (D. E. Tucker et al., 2005). Both cPLA$_2$$\beta$ and cPLA$_2$$\gamma$ have 30% homology to cPLA$_2$$\alpha$ and the active site residues of cPLA$_2$$\alpha$ are conserved in these paralogs. Catalytic residues of cPLA$_2$$\alpha$, are highly conserved in both cPLA$_2$$\beta$ and cPLA$_2$$\gamma$ and also appear to contribute to catalytic activity of them (R. T. Pickard et al., 1999). However a MAPK motif is not found in cPLA$_2$$\beta$ and cPLA$_2$$\gamma$. This suggests that the phosphorylation activation pathway of cPLA$_2$$\alpha$ may not be operating for the cPLA$_2$$\beta$ and cPLA$_2$$\gamma$ (R. T. Pickard et al., 1999). Although multiple potential PKC phosphorylation sites have been found in cPLA$_2$$\gamma$, whether they are involved in cPLA$_2$$\gamma$ regulation is still unknown. In addition, unlike cPLA$_2$$\alpha$, both cPLA$_2$$\beta$ and cPLA$_2$$\gamma$ have little specificity for the sn-2 fatty acid (D. E. Tucker et al., 2005). cPLA$_2$$\beta$ prefers to hydrolyze fatty acids at the sn-1 position, and cPLA$_2$$\gamma$ efficiently hydrolyzes fatty acid at the sn-1 and also sn-2 positions of the glycerol moiety (C. Song et al., 1999). Primary structure of cPLA$_2$$\delta$, cPLA$_2$$\epsilon$ and cPLA$_2$,$\zeta$ are similar to cPLA$_2$$\beta$. They all posses C2 domain at N-terminal and catalytic domain (Y. Kita et al., 2006). Ca$^{2+}$-dependent phospholipase activity was found when cPLA$_2$$\delta$, cPLA$_2$$\epsilon$ and cPLA$_2$$\zeta$ are transiently expressed in the mammalian cells. However, cPLA$_2$$\zeta$ preferred phosphatidylethanolamine to phosphatidylcholine (T. Ohto et al., 2005).
and cPLA$_2$$\delta$, preferentially releases linoleic acid rather than AA (A. A. Farooqui et al., 2006).

cPLA$_2$ distribution in the mammalian central nervous system

 cPLA$_2$$\alpha$ can be found in the brain of rat and human. RT-PCR analysis showed that cPLA$_2$$\alpha$ mRNA can be detected in the normal rat brain. cPLA$_2$$\alpha$ mRNA level is the highest in the brainstem, hippocampus, striatum and midbrain and is relatively low in the cerebellum (G. Y. Molloy et al., 1998). Northern blot and in situ hybridization studies showed that cPLA$_2$$\alpha$ mRNA is located in most regions of rat brain, especially in the gray matter region, for example, the cerebral cortex, hippocampus, amygdala, thalamic and hypothalamic nuclei and cerebellum (K. Kishimoto et al., 1999). Western blot analysis also demonstrated that cPLA$_2$$\alpha$ is expressed in the monkey brain (G. R. Weerasinghe et al., 2006). Immunohistochemistry study showed that cPLA$_2$$\alpha$ is found in neurons of the normal rat brain, neurons and astrocytes of kainate-lesioned rat hippocampus (T. L. Sandhya et al., 1998). Immunohistochemistry studies also revealed that cPLA$_2$$\alpha$ is primarily present in the astrocytes of the gray matter but not in the white matter of the human brain (D. T. Stephenson et al., 1994). Furthermore, cPLA$_2$$\alpha$ can be found in the cultured cortical neurons and astrocytes (J. Luo et al., 1998). cPLA$_2$$\alpha$ protein was not detected in oligodendrocytes in brain by immunohistochemistry (Lautens et al 1998), however, cPLA$_2$$\alpha$ mRNA was found in the oligodendrocytes in the brain by in situ hybridization (Kishimoto et al 1999; Lautens et al 1998). In situ hybridization and immunostaining analysis
demonstrated that cPLA2α is presented in the soma and dendrites of cerebellum Purkinje cells. cPLA2β is located in the granule cells of the cerebellum (Y. Shirai and M. Ito, 2004). Northern analysis showed that cPLA2β and cPLA2γ mRNA can also be found in the human brain tissues (R. T. Pickard et al., 1999; C. Song et al., 1999). Northern blot study revealed that in the cPLA2β mRNA has the highest expression level in the human brain cerebellum. cPLA2γ mRNA is expressed most strongly in the human heart and skeletal muscle (K. W. Underwood et al., 1998; R. T. Pickard et al., 1999; C. Song et al., 1999).

cPLA2 in traumatic brain injury

A great amount of evidence indicates the potential role of cPLA2 in TBI. TBI can induce the secondary injury by multiple processes including inflammation, free radical production, ischemia, and excitatory amino acid (EAA) release (E. D. Hall and J. M. Braughler, 1986; W. Young, 1993; Y. Hatanaka et al., 1996; E. Park et al., 2004), which have been shown to result in PLA2 activation (T. K. McIntosh et al., 1998; J. W. Phillis and M. H. O'Regan, 2004; A. A. Farooqui et al., 2006). Excessive activation of PLA2 may induce membrane phospholipid degradation, accumulation of proinflammatory mediators and then result in lipid peroxidation and neuronal cell death (J. V. Bonventre, 1996; M. Farooque et al., 1996; A. A. Farooqui et al., 1999; A. A. Farooqui et al., 2006). A previous study has shown that a close brain injury can induce PLA2 activation and subsequent production of prostaglandin E2, a proinflammatory mediator, in rats (E. Shohami et al., 1989). Controlled cortical injury in rats is followed by membrane phospholipid
degeneration and free fatty acid release (H. S. Dhillon et al., 1994; P. Homayoun et al., 1997). Brain injury in human also results in increased free fatty acid in CSF (J. G. Pilitsis et al., 2003). In addition, fimbria-fornix transaction in rat brain increased level of cPLA₂ and 4-HNE, a product of peroxidation and marker of oxidative stress. These increases can be reversed by PLA₂ inhibitor, mepacrine (X. R. Lu et al., 2001a). Therefore, cPLA₂ may serve as a key molecule in mediating secondary TBI. If this is true, cPLA₂ may serve as a novel target for therapeutic interventions following TBI. However, to date, it is not clear what role that PLA₂ plays in mediating secondary TBI. The cPLA₂ expression pattern after TBI remains to be determined. Furthermore, if cPLA₂ expression level increases following TBI, investigating the cPLA₂ expression time course is essential for determining the optimal time for inhibiting cPLA₂ expression increase after TBI. Therefore, in the present study, we investigated the temporal profile and spatial changes of cPLA₂ expression after TBI and identified its cellular sources in a TBI model in adult rats to explore the role of cPLA₂ in the secondary injury after TBI.
2. MATERIALS AND METHODS

Chemicals, equipment and materials

Chemicals: ketamine/xylazine mixture from by Laboratory Animal Research Center of Indiana University and Purdue University at Indianapolis, Indianapolis, IN; Tears ointment (#NDC6791476002) from Webster, MA; Betadine (#NDC6761815117) from Purdue Products L.P., Stamford, CT; Neomycin and polymyxin B sulfates and bacitracin zinc ointment (#11747) from Pharmaderm Animal Health, Melville, NY; Paraformaldehyde (PFA, #19210) from Electron Microscopy Sciences, Hatfield, PA; Tissue freezing medium (#TFM5) from Triangle Biomedical Science, Inc., Durham, NC; Superfrost/Plus slides (#1255015) from Fisher Scientific, Pittsburgh, PA; DPX mountant (#360294H) from VWR international Ltd., Poole, Dorset; Filter paper (#1703932) from Bio-Rad, Hercules, CA; Sucrose (#S9378), Na$_2$HPO$_4$ (#S0876), NaH$_2$PO$_4$ (#S282), NaCl (#S9625); Xylene (#1330207); KMnO$_4$ (#7722647), Acetic acid/glacial acetic acid (#64197), H$_2$O$_2$ (#216763), Cresyl violet acetate (#10510540), Phenylnmethanesulfany fluoride (PMSF, #329986), 0.5 M EDTA (#139333), NaCl (#7647145), Sodium deoxycholate (#302954), Standard protein (#9048468), Tris-base (#77861), Glycine (#56406), Tris-HCl (#77861) and stock eosin (#17372871), all of them, from Sigma, St. Louis, MO; Sodium dodecyl sulfate (SDS, #151213) from Fisher Scientific, Fair Lawn, NJ; Absolute ethanol (#S73985) from Fisher Scientific, Rochester, NY; Fluoro-Jade B (#AG310) from Chemicon-Millipore, Billerica, MA; Proteinase inhibitor cocktail set III (#539134) and phosphotase inhibitor cocktail set II (#524625) from Calbiochem, La Jolla,
CA; Vectastain Elite ABC Kit (#PK6100), peroxidase substrate kit (#SK4100) from Vector Laboratories, Burlingame, CA; Phosphate buffer saline (PBS, #1666789001) from Roche, Mannheim, Germany; Amersham full-range rainbow molecular weight markers (#RPN800E) from GE healthcare, Piscataway, NJ; Li-blocking buffer (#92740000) from Mandel, Lincoln, Nebraska; Nonidet P-40 (NP, #19628) from USB, Cleveland, OH; Dye reagent (#50000006), acylamide/Bis (#1610146), 2-mercaptoethanol (#1610734) and laemmli sample buffer (#1610737) from Bio-Rad, Hercules, CA; Anti-NeuN (#MAB377) antibody from Chemicon, Temecula, CA; Anti-glial fibrillary acidic protein antibody (GFAP, #G3893) and anti-β-tubulin antibody (#T5076) from Sigma, St. Louis, MO; Rabbit anti-cPLA2 antibody (#SC438) and mouse anti-cPLA2 antibody (#SC454) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; Anti-CC1 antibody (#OP80) from Calbiochem, La Jolla, CA; Anti-ED1 antibody (#MCA341R) from AbD Serotech, Oxford, UK; Goat serum blocking (#S100) and secondary biotinylated goat anti-rabbit IgG antibody (#BA1000) from Vector Laboratories, Burlingame, CA; Fluorescein-conjugated goat anti-rabbit (#111095144) and rhodamine-conjugated goat anti-mouse (#115025003) from Jackson ImmunoResearch Laboratories, Inc.; Alexa Fluor 680 goat anti-rabbit antibody (#A21076) from Invitrogen, Eugene, Oregon; Infrared 800-conjugated goat anti-mouse antibody (#610132121) from Rockland Immunochemicals, Inc., Gilbertsville, PA.

Equipment: Electromagnetic controlled cortical impact (EMI, #39463923) from Leica Microsystems; Biophotometer (#6131000071) from Eppendorf AG,
Hambrug, Germany; Olympus BX60 light microscope from Olympus America, Inc., Melville, NY; Nikon eclipse TE 2000-E confocal microscope from Nikon instruments, Melville, NY; LI-COR Odyssey system from Li-Cor Biosciences; Nitrocellulose membranes (#BA85) from Whatman, Dassel, Germany; Gel mount (#M01) from Biomedca Corp., Foster City, CA.

Animal and surgery

A total of 69 adult female Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 210-230 g, were used in this study. All surgical interventions and postoperative animal care were performed according to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, D.C., 1996) and Indiana University School of Medicine Institutional Animal Care and Use Committee. TBI was performed using electromagnetic controlled cortical impact, as described previously (D. L. Brody et al., 2007). On the surgery day, rats were firstly anesthetized by the ketamine/xylazine mixture. During anesthesia, the artificial tears ointment was applied to the rat eyes to protect their vision and their heads were shaved with an electric clipper. All tools and instruments were sterilized with 75% ethanol. The skin was treated with betadine. Rats were stabilized on the stereotaxic frame. A midline incision was made and soft tissue was reflected to expose the skull. A 6 mm craniotomy centered at 4.5 mm posterior to lambda and 4.5 mm laterally on the left side of the central suture was performed. The 3 mm-diameter impact tip was attached to a magnetically activated piston (www.myneurolab.com) set at an
angle of 20° and positioned sterotaxically in the center of craniotomy. The actuator was retracted and impact was produced after setting impact depth injury (1.5 mm, 2 mm or 2.5 mm) and impact speed (2.7 m/s) (P. V. Bayly et al., 2006). Sham control animals underwent a craniotomy without impact injury. After TBI, the musculature was closed with sutures, and the skin was closed with wound clips. Neomycin and polymyxin B sulfates and bacitracin zinc ointment was applied to the wound immediately after surgery. The animals were placed on AlphaDry bedding. Regular food, water, and water-softened rodent chow were also placed on the bedding.

Animal perfusion

For perfusion, rats were deeply anesthetized by the ketamine/xylazine mixture and placed on their back in the hood. Their forelimbs were spread. An 8 cm-length cut on the skin was made to expose their sternums end. The ends of their sternums were grasped with a hemostat. Diaphragms and ribs were cut laterally on both sides with a pair of sharp scissors. Diaphragms were reflected down to expose the rat hearts. A slit cut, which was large enough for cannula tip to penetrate, was made on the left ventricle with a scalpel. The cannula tip was inserted through the left ventricle into the ascending aorta until it was visible within the aorta. A hemostat was used to stabilize the cannula tip underneath it. Finally, the rats underwent transcardial perfusion with 100 ml PBS (see Table 1) followed by 300 ml of 4% PFA into the blood vessel via the cannula. The skin of the rat heads was removed with scissors and their brains were removed.
brains were post-fixed in 4% PFA for 2 days and equilibrated in 30% sucrose for 7 days at 4 °C.

Table 1. Phosphate buffer saline

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>1L</th>
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<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>80 mM</td>
<td>11.5 g</td>
</tr>
<tr>
<td>NaH₂HPO₄</td>
<td>20 mM</td>
<td>2.96 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>100 mM</td>
<td>5.84 g</td>
</tr>
</tbody>
</table>

pH was adjusted to 7.5 by HCl and dilute to 1 L with DD H₂O

Tissue fixation, embedding and cutting

After cytoprotection in 30% sucrose, brains were embedded in tissue freezing medium in -70 °C in absolute ethanol and dry ice mixtures. Rat brains were cut transversely and serially at 20 µm and then mounted on superfrost/Plus slides in five identical sets.

Cresyl violet-eosin staining

Neuronal viability was assessed by using cresyl violet-eosin staining (D. Stephenson et al., 1999). To perform cresyl violet-eosin staining, the sections were firstly warmed for 30 min at the room temperature. Secondly, they were stained with working cresyl violet solution (45ml stock solution and glacial acetic acid, see Table 2) for 7 min. Thirdly, they were dipped into DD H₂O for 30 times to remove excess cresyl violet staining. Fourthly, they were rinsed with 95% ethanol for 2 min. Fifthly, they were counterstained in the working eosin solution (See Table 3) for 30 s. Sixthly, they were rinsed with 95% ethanol for 2 min, 100%
ethanol for 1 min and 40 s. Seventhly, they were rinsed with 100% ethanol for 1 minute. Ninthly, they were rinsed with xylene 2 times for 2 min each. And finally, they are coverslipped with DPX mountant. For each section containing visible lesion tissue, the lesion tissue contours were traced and the lesion areas were measured using the Stereo Investigator Contour Tracing Tool (Stereo Investigator Users Guide, version 7, MicroBrightField, Inc). The lesion volume of each rat was measured by summing its lesion area in each section and multiplying space (20 µm) between sections.

Table 2. Stock cresyl echt solution

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>0.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cresyl Violet acetate</td>
<td>0.016 mM</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>80%</td>
<td>240 ml</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>20%</td>
<td>60 ml</td>
</tr>
</tbody>
</table>

Table 3. Working eosin solution

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock eosin</td>
<td>24.9%</td>
<td>200 ml</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>74.7%</td>
<td>600 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>4%</td>
<td>4 ml</td>
</tr>
</tbody>
</table>

Fluoro-Jade B staining

In order to detect the neuron response to the TBI, Fluoro-Jade B staining was performed according to the manufacture’s protocol (#AG310; Chemicon-Millipore, Billerica, MA). Sections were washed with DD H₂O 3 times for 5 min each, and incubated with 0.06% KMnO₄ for 8 min followed by DD H₂O washing 2 times for 5 min each. Sections were incubated with 0.0004% Fluoro-Jade B for
20 min followed by DD H₂O washing 3 times for 5 min each. The sections were allowed to dry and then placed in xylene for 1 min. Slides are coverslipped with DPX mountant.

Immunohistochemistry

To confirm the cPLA₂ expression level change after traumatic brain injury, ABC immunohistochemistry was performed according to the protocol as described previously (N. K. Liu et al., 2006). Firstly, sections were warmed at the room temperature followed by PBS washing 2 times for 5 min each. Secondly, sections were incubated with 0.2% H₂O₂ for 30 min followed by PBS washing 3 times for 5 min each. Thirdly, sections were incubated with 10% goat serum blocking for 1 hour. Fourthly, sections were incubated with rabbit anti-cPLA₂ antibody (1:50) for 60 hours at 4 °C followed by PBS-0.03%Triton (PBS-T) washing 3 times for 10 min each. In the negative control, the primary antibody was omitted. Fifthly, sections were incubated with secondary biotinylated goat anti-rabbit IgG antibody (1:400) for 40 min. Sixthly, sections were incubated with AB solution from Vectastain Elite ABC Kit (A:B:PBS-T=1:1:200) followed by PBS-T washing 3 times for 10 min each. Seventhly, sections were incubated with DAB from peroxidase substrate kit for 6 min followed by PBS-T washing 3 times for 10 min each. Seventhly, sections were then sequentially dehydrated in 50%, 70%, 95%, 100% ethanol for 2 min followed by xylene rinses 2 times for 2 min each. Sections were examined by using an Olympus BX60 light microscope.
Immunofluorescences double labeling

To examine the localization of cPLA$_2$ in specific cell types, immunofluorescence double labeling was performed according to the protocol as described previously (N. K. Liu et al., 2006). Brain sections were firstly warmed at the room temperature for 1 hour. Secondly, sections were washed by PBS-T 2 times for 15 min each. Thirdly, sections were incubated with 10% goat serum blocking for 30 min. Fourthly, sections were incubated with rabbit polyclonal anti-cPLA$_2$ (1:50) and anti-NeuN (1:100), anti-GFAP (1:200) or anti-CC1(1:50)/ anti-ED1 (1:50) for 60 hours at 4 °C respectively followed by PBS washing 5 times for 5 min each. In the negative control, primary antibodies were omitted. Fifthly, sections were incubated with the mixtures of fluorescein-conjugated goat anti-rabbit (1:100) and rhodamine-conjugated goat anti-mouse (1:100) antibodies for 1 hour at room temperature followed by PBS washing 5 times for 5 min each. The slides were coverslipped with gel mount and then they were examined by using a Nikon eclipse TE 2000-E confocal microscope.

Protein extraction and centrifugation

To extract protein of rat cortex and hippocampus for Western blot, 2x lysis buffer (see Table 4) was prepared and pH of this buffer was adjusted to 7.5 by HCl. Rats received TBI or sham-operated rats were given a lethal dose of ketamine/xylazine mixture and their hearts were exposed as described in the perfusion procedure. To allow PBS to flow into the blood vessel until the liver turned to white. After rat brains were exposed with a rongeur, they were removed.
The entire ipsilateral hippocampus and the cortex (1 cm diameter) that contained the lesion epicenter were dissected out and homogenized in 0.4 ml 1x lysis buffer (see Table 5) respectively. Homogenates were placed on ice for 30 sec and then were centrifuged at 10,000xg at 4 °C for 10 min. Supernatant were centrifuged at 10,000xg at 4 °C for 10 min. Centrifuged protein samples were saved in the -80 °C.

Table 4. 2x lysis buffer

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (pH 7.5)</td>
<td>100 mM</td>
<td>6.005 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>300 mM</td>
<td>8.776 g</td>
</tr>
<tr>
<td>NP-40</td>
<td>2%</td>
<td>10 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2%</td>
<td>1 g</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>92%</td>
<td>460 ml</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>1%</td>
<td>5 g</td>
</tr>
</tbody>
</table>

Adjust pH to 7.5 with HCl and then add DD H₂O to 500 ml, store at 4 °C

Table 5. 1x lysis buffer

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>8 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x lysis buffer</td>
<td>50%</td>
<td>4 ml</td>
</tr>
<tr>
<td>PMSF</td>
<td>100 µg/ml</td>
<td>46 µl</td>
</tr>
<tr>
<td>Proteinase inhibitor cocktail set III</td>
<td>10 µl/ml</td>
<td>80 µl</td>
</tr>
<tr>
<td>Phosphotase inhibitor cocktail set II</td>
<td>10 µl/ml</td>
<td>80 µl</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>2 µl/ml</td>
<td>16 µl</td>
</tr>
<tr>
<td>DD H₂O</td>
<td>Add DD H₂O to 8 ml</td>
<td></td>
</tr>
</tbody>
</table>

Protein concentration determination

Protein concentration was determined by Bio-Rad protein assay (Bio-Rad laboratories), a dye binding assay in which color changes of dye occurs in response to various concentration of proteins. Dye was prepared by mixing 1 part
dye reagent concentrate with 4 part distilled, deionized H₂O (DD H₂O). Filter these mixtures to remove the particulates. Prepared standards containing a range of 0.2 mg/ml to 0.8 mg/ml standard protein then incubate them with 1 ml dye for 5 min respectively (See Table 6). Blank and sample solutions were prepared (See Table 6). Blank, standard and sample solutions were then incubated with 1 ml dye for 5 min (See Table 6). The spectrophotometer was zeroed with the blank, and the absorbance of standards and sample solutions at 595 nm was measured respectively. I then plotted concentration of standards and their optical density into a standard curve. The protein concentration of each sample was determined from the graph of protein concentration versus absorbance. Each cortical protein and hippocampal protein sample was diluted to the 8.55 mg/ml and 1.25 mg/ml, respectively with DD H₂O before storing in -80°C freezer.

Table 6. Standard protein set and protein sample for Western blot

<table>
<thead>
<tr>
<th></th>
<th>Standard Protein (0.8mg/ml)</th>
<th>DD H₂O</th>
<th>Dye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0 µl</td>
<td>20 µl</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.2 mg/ml standard</td>
<td>5 µl</td>
<td>15 µl</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.4 mg/ml standard</td>
<td>10 µl</td>
<td>10 µl</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.6 mg/ml standard</td>
<td>15 µl</td>
<td>5 µl</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.8 mg/ml standard</td>
<td>20 µl</td>
<td></td>
<td>1 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>18 µl</td>
<td>2 µl</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Western blotting

In order to detect the cPLA₂ expression level after TBI, Western blotting was performed as described previously (N. K. Liu et al., 2006). 7% Separating
gel (see Table 7) was loaded into gel plate followed by DD H₂O. I waited for 30 min and then sucked up the DD H₂O loaded on the separating gel surface. Stacking gel (see Table 7) was then loaded to the separating gel. A gel comb was immediately inserted into stacking gel for 30 min. Protein samples diluted in sample buffer (2-mercaptoethanol:laemmli sample buffer =1:19) were then heated at 95 °C for 10 min. Thereafter, they were quickly placed on ice. I removed the gel comb and loaded 3 μl rainbow molecular weight markers/107.1 μg cortical protein/ 56.25 μg hippocampal protein to each well. Proteins were electrophoresed at 180 volts in running buffer (see Table 8) for 1 hour and 45 min in the cold room. After electrophoresis, gels were washed in transfer buffer (see Table 9) for 10 min. Nitrocellulose Membranes, filter paper and sponges were then soaked in transfer buffer for 10 min. Proteins were transferred at 35 volts in the transfer buffer at 4 °C overnight. After transferring, membranes were washed in PBS (Roche, Mannheim, Germany) 3 times for 5 min each. Membranes were then incubated in blocking buffer (Li-blocking buffer: PBS=1:1) followed by PBS-T (PBS-0.1% Tween-20) washing 2 times for 5 min each. The membranes were incubated with mouse anti-cPLA₂ antibody (1:50) or anti-β-tubulin antibody (1:2000) respectively overnight at 4 °C and followed by PBS-T washing 4 times for 5 min each. The membranes were then incubated with Alexa Fluor 680 goat anti-rabbit antibody and Infrared 800-conjugated goat anti-mouse antibody for 1 hour followed by PBS-T washing 3 times for 5 min each. The membranes were finally washed by PBS 2 times for 5 min each. The blots were visualized by Odyssey system at 700 nm and 800 nm respectively.
Table 7. Gel for Western blot

<table>
<thead>
<tr>
<th>Name</th>
<th>7% separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis (40%)</td>
<td>4.7 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>DD H$_2$O</td>
<td>10 ml</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH 8.8)</td>
<td>5 ml</td>
<td>0 ml</td>
</tr>
<tr>
<td>0.5M Tris-HCl (pH 6.8)</td>
<td>0 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>10% AP</td>
<td>200 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Table 8. Running buffer (pH 8.3)

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>25 mM</td>
<td>9 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>192 mM</td>
<td>43.2 g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
<td>3 g</td>
</tr>
<tr>
<td>DD H$_2$O</td>
<td>Add DD H$_2$O</td>
<td>1 L</td>
</tr>
</tbody>
</table>

Table 9. Transfer buffer

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>1 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>25 mM</td>
<td>9 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>192 mM</td>
<td>43.2 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>20%</td>
<td>200 ml</td>
</tr>
<tr>
<td>DD H$_2$O</td>
<td>Add DD H$_2$O</td>
<td>1 L</td>
</tr>
</tbody>
</table>

Statistical analysis

Data were presented as mean ± standard error of the mean values. One-way analysis of variance with post hoc Tukey t test was used to determine the statistical significance. A p value which is less than 0.05 was considered statistically significant.
3. RESULTS

Histological characteristics of the injury

In order to produce a moderate tissue damage in the rat brain, we first investigated the relationship between the impact depth and corresponding lesion volume. Three impact depths, 1.5, 2.0 and 2.5 mm, were chosen to produce TBI at a velocity of 2.7 m/sec. After cresyl violet staining, lesion volume in the rat brain was assessed at 1 month after injury. As the impact depth increased, the lesion volume was also increased (Figure 1). A 1.5 mm impact depth produced a mild cortical injury (2.8 ± 1.7 mm$^3$) and 2.0 mm and 2.5 mm impact depth produced a moderate (6.5 ± 3.0 mm$^3$) and a severe (15.1 ± 5.9 mm$^3$) cortical injury, respectively (Figure 1A). The lesion volume at 2.5 mm impact depth was much greater than that at 1.5 mm (p < 0.01) and 2.0 mm depth (p < 0.01) (Figure 1B). There was no apparent injury to the contralateral brain (Figure 1A). These results indicated that EMI can produce reliable graded injuries in the rat brain. Furthermore, the hippocampus of rats received 1.5 or 2.0 mm depth remained morphologically normal and those received 2.5 mm impact depth sustained mild tissue damage (Figure 1A). Fluoro-Jade B staining, which specifically stains the degenerating neurons, also showed that at the 2.0 mm impact depth there was moderate neuronal death in the ipsilateral cortex (Figure 2B) while there was no apparent neuronal death in the hippocampus and contralateral cortex at this injury level (Figure 2C).
Figure 1. Histological analysis of EMI device-induced injury. (A) Histological images of cresyl violet-stained coronal sections of rats received 1.5, 2.0, 2.5 mm impact produced by EMI device. Brain tissue damage increased in a graded manner as the impact depth increased. (B) Lesion volume of ipsilateral brain increase as the depth increased. **P < 0.01 versus 1.5 mm impact, ##P < 0.01 versus 2.0 mm impact. The numbers of rats in the 1.5 mm, 2.0 mm and 2.5 mm impact groups were 3, 6, and 9, respectively. Bar = 2 mm (A).
cPLA$_2$ expression was increased following TBI

cPLA$_2$ (molecular weight 110KD) can selectively hydrolyze phospholipids containing AA which can serve as ROS source and act as a precursor of several proinflammatory mediators (C. Kim et al., 2008). Our Western blot analysis showed that cPLA$_2$ was constitutively expressed in the rat cortex and hippocampus. After TBI, the cPLA$_2$ expression level in the rat cortex significantly increased and peaked at 3 (3.18-fold; p < 0.05) to 7 days (2.88-fold; p < 0.05), compared to the sham operated group, and then returned to the basal level at 14 days (Figure 3A). However, no significant expression changes were detected in the rat hippocampus up to 14 days following TBI (Figure 3B).
Figure 3. Temporal changes of cPLA₂ expression after TBI. (A) TBI induced a significant increase in cortical cPLA₂ expression which peaked at 3 to 7 days after TBI. The top panel shows a representative time course of cortex cPLA₂ expression following TBI. The bottom panel shows compiled cortex cPLA₂ expression following TBI of each group in a bar graph. n = 5 rats in each group. *p < 0.05 versus sham. (B) There was no significant difference in hippocampus cPLA₂ expression following TBI. The top panel shows a representative time course of hippocampus cPLA₂ expression following TBI. The bottom panel shows compiled hippocampus cPLA₂ expression following TBI of each group in a bar graph. n = 6 rats in each group.
cPLA2 immunoreactivity was localized in neurons and glial cells after TBI

To further confirm the results of Western blotting and determine the spatial distribution of cPLA2 after TBI, ABC immunohistochemistry was performed on the brain sections for the cPLA2 at 7 days after TBI. cPLA2 immunoreactivity was weak in the contralateral side of rat cortex (Figure 4A and C). In contrast, cPLA2 immunoreactivity was increased in the lesioned cortex (Figure 4A and B). cPLA2 labeling was increased closer to the lesion epicenter. However, no apparent difference of cPLA2 immunoreactivity was found in the rat hippocampus ipsilateral to the injury (Figure 4A, D), compared with the contralateral hippocampus (Figure 4A, E).

To investigate the source of cPLA2 in cortex, we performed double-labeling immunofluorescence to localize specific cell types that express cPLA2. cPLA2 immunoreactivity was detected in neurons which were labeled by a neuronal marker, NeuN (Figure 5A-C). cPLA2 immunoreactivity was also found in astrocytes, oligodendrocytes, macrophages, which were labeled by their specific markers GFAP (Figure 5D-F), CC1 (Figure 5G-I) and ED1 (Figure 5J-L), respectively.
Figure 4. Distribution of cPLA$_2$ immunoreactivity (IR) in the cortex and the hippocampus at 7 days after moderate TBI. (A) A weak signal of cPLA$_2$ IR was detected in the contralateral hemisphere. TBI induced a marked increase in cPLA$_2$ IR surrounding the injury epicenter. There was no apparent difference of cPLA$_2$ IR in the hippocampus between the ipsilateral and contralateral sides. (B-E) High magnification of demarcated areas in A shows that cPLA$_2$ IR was localized in neurons in both the cortex and hippocampus (arrow), and only increased in the cortical area ipsilateral to the injury. Bars = 1 mm (A); 50 µm (B-E).
Figure 5. Cellular localization of cPLA$_2$ expression is shown in the rat brain at 7 days after moderate TBI. (A-C) cPLA$_2$ IR (A, arrow) was detected in neurons with NeuN IR (B, arrow), which is demonstrated by the merged image (C, arrow). (D-F) cPLA$_2$ IR (D, arrow) was also localized in astrocytes with GFAP IR (E, arrow), which is shown in the merged image (F, arrow). (G-I) cPLA$_2$ IR (G, arrow) was also detected in oligodendrocytes with CC1 IR (H, arrow), which is shown in the merged image (I, arrow). (J-L) cPLA$_2$ IR (J, arrow) was also detected in macrophages with ED1 IR (K, arrow), which is shown in the merged image (L, arrow). Bar = 20 µm
4. DISCUSSION

The present study showed the relationship between the impact depths of EMI device with its injury severities on rat brains. As the impact depth increased from 1.5 to 2.5 mm, increased lesion size was detected in a severity dependent manner. These results further confirm the previous study (D. L. Brody et al., 2007) showing that EMI can produce reliable graded experimental brain injuries. The 2.0 mm impact depth used in this study caused moderate tissue lesion of the rat cortex but did not cause apparent tissue lesion in the hippocampus underlying it. The lesion did not cause apparent tissue lesion of the contralateral cortex and hippocampus. These results were consistent with the previous study (M. R. Hoane et al., 2007).

To our knowledge, this is the first study showing the cPLA\textsubscript{2} expression pattern in both the rat cortex and hippocampus after moderate TBI. We found that cPLA\textsubscript{2} expression was increased in the injured cortex after TBI and peaked at 3 to 7 days after TBI, as compared to that in the sham-operated control. There was no significant statistical difference of cPLA\textsubscript{2} expression between ipsilateral and contralateral hippocampus after TBI. These results are in agreement with other reports showing increased expression of cPLA\textsubscript{2} after brain transection injury (X. R. Lu et al., 2001a) and the release of free fatty acids and membrane phospholipid degradation in the cortex following TBI due to PLA\textsubscript{2} activation (H. S. Dhillon et al., 1994; P. Homayoun et al., 1997).

Our immunohistochemistry analysis further demonstrated that cPLA\textsubscript{2} expression was dramatically increased only near the site of injury in ipsilateral
cortex, compared with contralateral cortex. The immunofluorescence double labeling study further revealed that cPLA$_2$ is expressed in neurons, astrocytes, oligodendrocytes, and macrophages around the lesion epicenter. These results are consistent with other studies showing the cPLA$_2$ protein and mRNA are expressed in neurons, astrocytes and macrophages (T. L. Sandhya et al., 1998; D. Stephenson et al., 1999). Although a previous report indicated that cPLA$_2$ protein was not detected in oligodendrocytes in the normal rat brain by immunohistochemistry (Lautens et al., 1998), cPLA$_2$ mRNA was found in the oligodendrocytes in the brain by in situ hybridization (Kishimoto et al., 1999; Lautens et al., 1998). The discrepancy of cPLA2 protein localization in oligodendrocytes between the present study and previous study may be related to the increased cPLA$_2$ expression to a detectable level in oligodendrocytes after TBI.

No significant changes of cPLA$_2$ expression were detected in the hippocampus following TBI. These results are in agreement with our histological studies. The cresyl violet and Fluor-Jade B stainings showed that TBI (2.0 mm depth)-induced tissue damage and neuronal death were predominately restricted in the cortex, suggesting that severity of TBI in the present study was not enough to induce cPLA$_2$ expression in the hippocampus.

The present study shows that expression of cPLA$_2$ was significantly increased in the cortex after TBI, however, the mechanism(s) by which it increases remains unclear. Recent studies showed that cPLA$_2$ activity and/or expression was induced by ischemia, EAAs (T. L. Sandhya et al., 1998) and
several toxic factors such as inflammatory cytokines (Murakami et al., 1997; Beck et al., 2003) and free radicals, which have been demonstrated to increase after TBI (M. W. Greve and B. J. Zink, 2009).

Ischemia plays a key role in mediating the secondary injury after acute trauma (C. H. Tator, 1991; C. H. Tator and M. G. Fehlings, 1991). In an experimental ischemia brain model, significant increases in the level of free fatty acids, indirectly reflecting cPLA2 activation, were found (S. Yoshida et al., 1983; K. Abe et al., 1988; S. Nakano et al., 1990; K. Narita et al., 2000). Several studies have also showed that cPLA2 activity, cPLA2 mRNA and protein expression level were significantly increased following brain ischemia (J. A. Clemens et al., 1996; I. Saluja et al., 1997; D. Stephenson et al., 1999). A immunohistochemistry studies showed that cPLA2 immunoreactivity increases in astrocytes and activated microglia in hippocampus after ischemia. Hippocampus slices cultured under ischemic condition, oxygen and glucose deprivation (OGD), showed cPLA2 activity increased to 2-fold as compared with controls after 35 min of OGD. This increase remained at 24 h (K. Arai et al., 2001).

Enhanced extracellular EAA may also induce secondary TBI by stimulating cPLA2 activity. Extracellular EAAs such as glutamate and aspartate have been shown to increase after TBI (A. I. Faden et al., 1989; A. M. Palmer et al., 1993; M. Y. Globus et al., 1995; R. Bullock et al., 1998). Marked increases in prostaglandin F₂₀, prostaglandin D₂, leukotrienes, and thromboxane B₂ have been reported to occur in brain tissues after kainic acid (KA) injection (H. Baran et al., 1987). Immunohistochemistry study showed that intravenous injection of KA greatly
increased cPLA₂ immunoreactivity in rat hippocampus neurons and astrocytes (T. L. Sandhya et al., 1998). Such a cPLA₂ increase was associated with enhanced expression of 4-HNE, a product of lipid peroxidation and marker for oxygen free radical-induced membrane injury (X. R. Lu et al., 2001a). KA-induced cPLA₂ and 4-HNE increase can be inhibited by the PLA₂ inhibitor, mepacrine (X. R. Lu et al., 2001b; W. Y. Ong et al., 2003). Furthermore, a cPLA₂ specific inhibitor, AACOCF₃, can block KA-induced cPLA2 and 4-HNE increase (X. R. Lu et al., 2001b). It has been hypothesized that the glutamate release induces NMDA receptor activation which, in turn, results in intracellular Ca²⁺ increase, stimulates PLA₂ activation and eventually causes neuronal death (J. Klein, 2000).

The excessive cPLA₂ level in cortex following TBI may contribute to secondary TBI by multiple mechanisms. cPLA₂ may directly induce secondary TBI by degenerating cell membranes. Phospholipids are the major constitutes and serves as the backbone of the cell membrane. cPLA2 is also involved in functional maintenance of protein, receptor, transporter, and ion channel of membrane through providing cell membrane with proper environment, fluidity, ion permeability. Stimulation of cPLA₂ activity may result in loss of membrane integrity by hydrolyzing membrane glycerophospholipids and subsequent changes in the membrane permeability, ion homeostasis, and behavior of cell membrane proteins including transporter and receptor (A. A. Farooqui et al., 1997b; J. Klein, 2000; A. A. Farooqui et al., 2004).

Increased cPLA₂ causes lysophospholipids and free fatty acid accumulation. High concentration of lysophospholipids acts as a detergents and
changes the membrane-bound enzyme activities (A. A. Farooqui et al., 1997b), membrane fluidity and permeability, and in turn, causes cell lysis (A. A. Farooqui et al., 1997a). In addition, AA, a free fatty acid, can cause changes in membrane permeability by regulating ion channels (A. A. Farooqui et al., 1997b). Inflammation is one of the mechanisms underlying cPLA$_2$ mediated TBI. cPLA$_2$ regulates the synthesis of multiple well-known proinflammatory mediators such as eicosanoids and PAF (A. A. Farooqui et al., 1997b; A. A. Farooqui et al., 1999). PAF can activate inflammatory cells such as leukocytes, microglia, and also induce inflammation at neuronal cell surface (A. A. Farooqui et al., 2006). Oxidation may also mediate cPLA$_2$-induced brain injury. AA serves as a source of ROS (M. Chalimoniuk et al.). Free radicals produced during oxidation can induce lipid peroxidation of neuronal membrane, oxidation of protein, RNA and DNA. A pathophysiological concentration of free fatty acid can induce oxidative damage in spinal cord cell culture (M. Toborek et al., 1999). Furthermore, a previous study showed that microinjection of PLA$_2$ into normal spinal cord can increase the expression of 4-HNE, a production of lipid peroxidation and also a marker of oxygen free radical-mediated membrane injury (N. K. Liu et al., 2006). Transection brain injury-induced expression of cPLA$_2$ and 4-HNE can be reduced by mepacrine, a PLA$_2$ inhibitor (X. R. Lu et al., 2001a). Increased excitatory neurotoxicity levels have been implicated with pathogenesis of neural injury and death in several neurological disorders. Injection of PLA$_2$ into the ischemic rat cerebral cortex induced a significant increase in EAA levels and application of mepacrine significantly decreased ischemic-induced release of EAA into cortical
superfusates. This suggested that PLA$_2$ can induce EAA release (M. H. O'Regan et al., 1995). Although the mechanism of PLA$_2$ induced EAA release remains unknown, it has been suggested that this is due to PLA$_2$ induced membrane integrity loss followed by neurotransmitter diffusion from intracellular to synaptic cleft (A. A. Farooqui et al., 1997b)

cPLA$_2$ may also play a role in the TBI induced apoptosis process. Apoptosis has been demonstrated to be a key mechanism of cell death in several neurological disorders including TBI (T. K. McIntosh et al., 1998; P. C. Waldmeier, 2003). Glycerophospholipid metabolism changes are accompanied by apoptosis or programmed cell death. Cells undergoing apoptosis release free fatty acids such as AA, which parallels the decrease in cell viability (M. M. Taketo and M. Sonoshita, 2002; J. Balsinde et al., 2006). This free fatty acids release suggested the involvement of PLA$_2$ in apoptosis. Moreover, previous studies found that apoptosis can be induced by TNF-$\alpha$, a proinflammatory cytokine, which can also activate cPLA$_2$ by caspase-3 (B. S. Cummings et al., 2000). Activated cPLA$_2$ induced AA release and also caspase-3 downstream which results in apoptosis (B. S. Cummings et al., 2000). In addition, selective inhibitor of cPLA$_2$ activity, MAFP, and cPLA$_2$ antisense oligonucleotides can inhibit AA release from neurons and also reduce A$_{\beta}$-induced neuronal apoptosis (B. Kriem et al., 2005)

Our study demonstrated the presence of cPLA$_2$ in neurons and oligodendrocytes around the lesion epicenter, suggesting that cPLA$_2$ may directly induce neuronal death and oligodendrocyte demyelination. Oligodendrocyte is responsible for myelination in the central nervous system. Since the TBI also
induces demyelination at the lesion epicenter (our observed data not shown), it is possible that cPLA₂ may contribute to axon demyelination by inducing oligodendrocyte death or by directly hydrolyzing phosphatidylcholine, one of major phospholipids. When cPLA₂ hydrolyzes phosphatidylcholine, lysophosphatidylcholine will be released and then serve as a detergent to cause myelin breakdown. This hypothesis was supported by the previous study which demonstrated that PLA₂ expression correlates well with the rate of demyelination associated with Wallerian degeneration in both peripheral nervous system and CNS. Because both neurons and oligodendrocyte are cell types which play an important role in CNS circuits and function, therapeutics targeting cPLA₂ expression or/and activity in these cells may effectively promote anatomical connection and functional recovery after TBI.

Our data was consistent with the previous study showing that astrocyte and macrophage markers colocalized with cPLA₂ (T. L. Sandhya et al., 1998; D. Stephenson et al., 1999). The increased cPLA₂ in astrocytes and macrophages may induce release of inflammatory mediators such as eicosanoids (prostaglandins, thromboxanes, leukotrienes and lipoxins) (T. L. Sandhya et al., 1998; K. F. Scott et al., 1999; J. Balsinde et al., 2000).

In summary, our study demonstrates that the cPLA₂ expression level was significantly increased after TBI and peaked at 3 to 7 days post-TBI. cPLA₂ was present in neurons, oligodendrocytes, astrocytes and macrophages. The increased cPLA₂ and lesion was found mainly at the lesion site ipsilateral to the injury. These findings suggest that abnormal expression of cPLA₂ may contribute
to the pathogenesis of TBI. However, more studies are needed to determine whether cPLA$_2$ can serve as a therapeutic target for the secondary TBI.
5. REFERENCES


Lu XR, Ong WY, Halliwell B (2001a) The phospholipase A2 inhibitor quinacrine prevents increased immunoreactivity to cytoplasmic phospholipase A2 (cPLA2) and hydroxynonenal (HNE) in neurons of the lateral septum following fimbria-fornix transection. Exp Brain Res 138:500-508.


