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.

a. P. Liaison, 2002; B. W. Writer and J. E. Schillerstrom, 2009). 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 sever 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_2s 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 ecosanoids 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 PLA_2

PLA_2 contains 4 subfamilies, secretory PLA_2 ($sPLA_2$), $cPLA_2$, Ca^2+-independent PLA_2 ($iPLA_2$) and platelet activating factor acetyl hydrolase (PAF-AH)/oxidized lipid lipoprotein associated (Lp)PLA_2 (M. Murakami and I. Kudo, 2002; R. H. Schaloske and E. A. Dennis, 2006). $sPLA_2$s are synthesized intracellularly and secreted and act extracellularly. They include 10 isoforms all of which have low molecular mass (14-18KD). Submilliomolar to millimolar concentrations of Ca^2+ are required for effective hydrolysis of $sPLA_2$s on its substrate phospholipid. $sPLA_2$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). $iPLA_2$s are intracellular enzymes responsible for maintenance of membrane phospholipids. Their molecular weights are 28-91KD. They comprise 7 isoforms. Hydrolysis effects of $iPLA_2$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). $cPLA_2$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 $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $cPLA_2\delta$, $cPLA_2\varepsilon$ 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 α/β 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, p_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-α and IFN-γ 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 and15 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$_2$α and cPLA$_2$β 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$_2$γ 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$_2$β and cPLA$_2$γ have 30% homology to cPLA$_2$α and the active site residues of cPLA$_2$α are conserved in these paralogs. Catalytic residues of cPLA$_2$α, are highly conserved in both cPLA$_2$β and cPLA$_2$γ 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$_2$β and cPLA$_2$γ. This suggests that the phosphorylation activation pathway of cPLA$_2$α may not be operating for the cPLA$_2$β and cPLA$_2$γ (R. T. Pickard et al., 1999). Although multiple potential PKC phosphorylation sites have been found in cPLA$_2$γ, whether they are involved in cPLA$_2$γ regulation is still unknown. In addition, unlike cPLA$_2$α, both cPLA$_2$β and cPLA$_2$γ have little specificity for the sn-2 fatty acid (D. E. Tucker et al., 2005). cPLA$_2$-β prefers to hydrolyze fatty acids at the sn-1 position, and cPLA$_2$-γ 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$_2$δ, cPLA$_2$ε and cPLA$_2$ζ are similar to cPLA$_2$β. 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$_2$δ, cPLA$_2$ε and cPLA$_2$ζ are transiently expressed in the mammalian cells. However, cPLA$_2$ζ preferred phosphatidylethanolamine to phosphatidylcholine (T. Ohto et al., 2005)

and cPLA$_2$δ, preferentially releases linoleic acid rather than AA (A. A. Farooqui et al., 2006).

cPLA$_2$ distribution in the mammalian central nervous system

cPLA$_2$α can be found in the brain of rat and human. RT-PCR analysis showed that cPLA$_2$α mRNA can be detected in the normal rat brain. cPLA$_2$α 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$_2$α 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$_2$α is expressed in the monkey brain (G. R. Weerasinghe et al., 2006). Immunohistochemistry study showed that cPLA$_2$α 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$_2$α 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$_2$α can be found in the cultured cortical neurons and astrocytes (J. Luo et al., 1998). cPLA$_2$α protein was not detected in oligodendrocytes in brain by immunohistochemistry (Lautens et al 1998), however, cPLA$_2$α 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 $cPLA_2\alpha$ is presented in the soma and dendrites of cerebellum Purkinje cells. $cPLA_2\beta$ is located in the granule cells of the cerebellum (Y. Shirai and M. Ito, 2004). Northern analysis showed that $cPLA_2\beta$ and $cPLA_2\gamma$ 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 $cPLA_2\beta$ mRNA has the highest expression level in the human brain cerebellum. $cPLA_2\gamma$ 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).

$cPLA_2$ in traumatic brain injury

A great amount of evidence indicates the potential role of $cPLA_2$ 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 PLA_2 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 PLA_2 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 PLA_2 activation and subsequent production of prostaglandin E_2, 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 $cPLA_2$ and 4-HNE, a product of peroxidation and marker of oxidative stress. These increases can be reversed by PLA_2 inhibitor, mepacrine (X. R. Lu et al., 2001a). Therefore, $cPLA_2$ may serve as a key molecule in mediating secondary TBI. If this is true, $cPLA_2$ may serve as a novel target for therapeutic interventions following TBI. However, to date, it is not clear what role that PLA_2 plays in mediating secondary TBI. The $cPLA_2$ expression pattern after TBI remains to be determined. Furthermore, if $cPLA_2$ expression level increases following TBI, investigating the cPLA2 expression time course is essential for determining the optimal time for inhibiting $cPLA_2$ expression increase after TBI. Therefore, in the present study, we investigated the temporal profile and spatial changes of $cPLA_2$ expression after TBI and identified its cellular sources in a TBI model in adult rats to explore the role of $cPLA_2$ in the secondary injury after TBI.

2. MATERIALS AND METHODS

Chemicals, equipment and materials

Chemicials: 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 bacitracinzinc 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_2HPO4 (#S0876), NaH_2PO4 (#S282), NaCl (#S9625); Xylene (#1330207); KMnO4 (#7722647), Acetic acid/glacial acetic acid (#64197), H_2O_2 (#216763), Cresyl violet acetate (#10510540), Pheylmethanesulfany 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-cPLA$_2$ antibody (#SC438) and mouse anti-cPLA$_2$ 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., Mellville, NY; Nikon eclipse TE 2000-E confocal microscope from Nikon instruments, Mellville, NY; LI-COR Odyssey system from Li-Cor Biosciences; Nitrocellulose membranes (#BA85) from Whatman, Dassel, Germany; Gel mount (#M01) from Biomeda 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 bacitracinzinc 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. Rat

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

Name	Concentration	1L
Na_2HPO_4	80 mM	11.5 g
NaH_2HPO_4	20 mM	2.96 g
NaCl	100 mM	5.84 g

pH was adjusted to 7.5 by HCl and dilute to 1 L with DD H_2O

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 H_20 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 coversliped 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

Name	Concentration	0.5%
Cresyl Violet acetate	0.016 mM	1.5 g
Absolute ethanol	80%	240 ml
DD H_2O	20%	60 ml

Table 3. Working eosin solution

Name	Concentration	Volume
Stock eosin	24.9%	200 ml
95% ethanol	74.7%	600 ml
Glacial acetic acid	4%	4 ml

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 H_2O 3 times for 5 min each, and incubated with 0.06% KMnO4 for 8 min followed by DD H_2O washing 2 times for 5min each. Sections were incubated with 0.0004% Fluoro-Jade B for

20 min followed by DD H_2O washing 3 times for 5 min each. The sections were allowed to dry and then placed in xylene for 1 min. Slides are coversliped with DPX mountant.

Immunohistochemistry

To confirm the $cPLA_2$ 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 warned at the room temperature followed by PBS washing 2 times for 5 min each. Secondly, sections were incubated with 0.2% H_2O_2 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-$cPLA_2$ 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 coversliped 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

Name	Concentration	500 ml
Tris HCl (pH 7.5)	100 mM	6.005 g
NaCl	300 mM	8.776 g
NP-40	2%	10 ml
SDS	0.2%	1 g
DD H_2O	92%	460 ml
Sodium deoxycholate	1%	5 g
Adjust pH to 7.5 with HCl and then add DD H_2O to 500 ml, store at 4 °C		

Table 5. 1x lysis buffer

Name	Concentration	8 ml
2x lysis buffer	50%	4 ml
PMSF	100 µg/ml	46 µl
Proteinase inhibitor cocktail set III	10 µl/ml	80 µl
Phosphotase inhibitor cocktail set II	10 µl/ml	80 µl
0.5 M EDTA	2 µl/ml	16 µl
DD H_2O		Add DD H_2O to 8 ml

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 H_2O (DD H_2O). 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 H_2O before storing in -80°C freezer.

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

	Standard Protein (0.8mg/ml)	DD H_2O	Dye
Blank	0 µl	20 µl	1 ml
0.2 mg/ml standard protein	5 µl	15 µl	1 ml
0.4 mg/ml standard protein	10 µl	10 µl	1 ml
0.6 mg/ml standard protein	15 µl	5 µl	1 ml
0.8 mg/ml standard protein	20 µl		1 ml
Sample	18 µl	2 µl	1 ml

Western blotting

In order to detect the $cPLA_2$ 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 H_2O. I waited for 30 min and then sucked up the DD H_2O 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-$cPLA_2$ 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

Name	7% separating gel	Stacking gel
Acrylamide/bis (40%)	4.7 ml	1.3 ml
DD H_2O	10 ml	6.1 ml
1.5M Tris-HCl (pH 8.8)	5 ml	0 ml
0.5M Tris-HCl (pH 6.8)	0 ml	2.5 ml
10% SDS	200 µl	100 µl
10% AP	200 µl	5 µl
TEMED	10 µl	10 µl

Table 8. Running buffer (pH 8.3)

Name	Concentration	1 L
Tris base	25 mM	9 g
Glycine	192 mM	43.2 g
SDS	0.1%	3 g
DD H_2O		Add DD H_2O to 1 L

Table 9. Transfer buffer

Name	Concentration	1 L
Tris base	25 mM	9 g
Glycine	192 mM	43.2 g
Methanol	20%	200 ml
DD H_2O		Add DD H_2O to 1 L

Statistical analysis

Data were presented as mean $\pm$ 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 $\pm$ 1.7 mm^3) and 2.0 mm and 2.5 mm impact depth produced a moderate (6.5 $\pm$ 3.0 mm^3) and a severe (15.1 $\pm$ 5.9 mm^3) cortical injury, respectively (Figure 1 A).. 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).

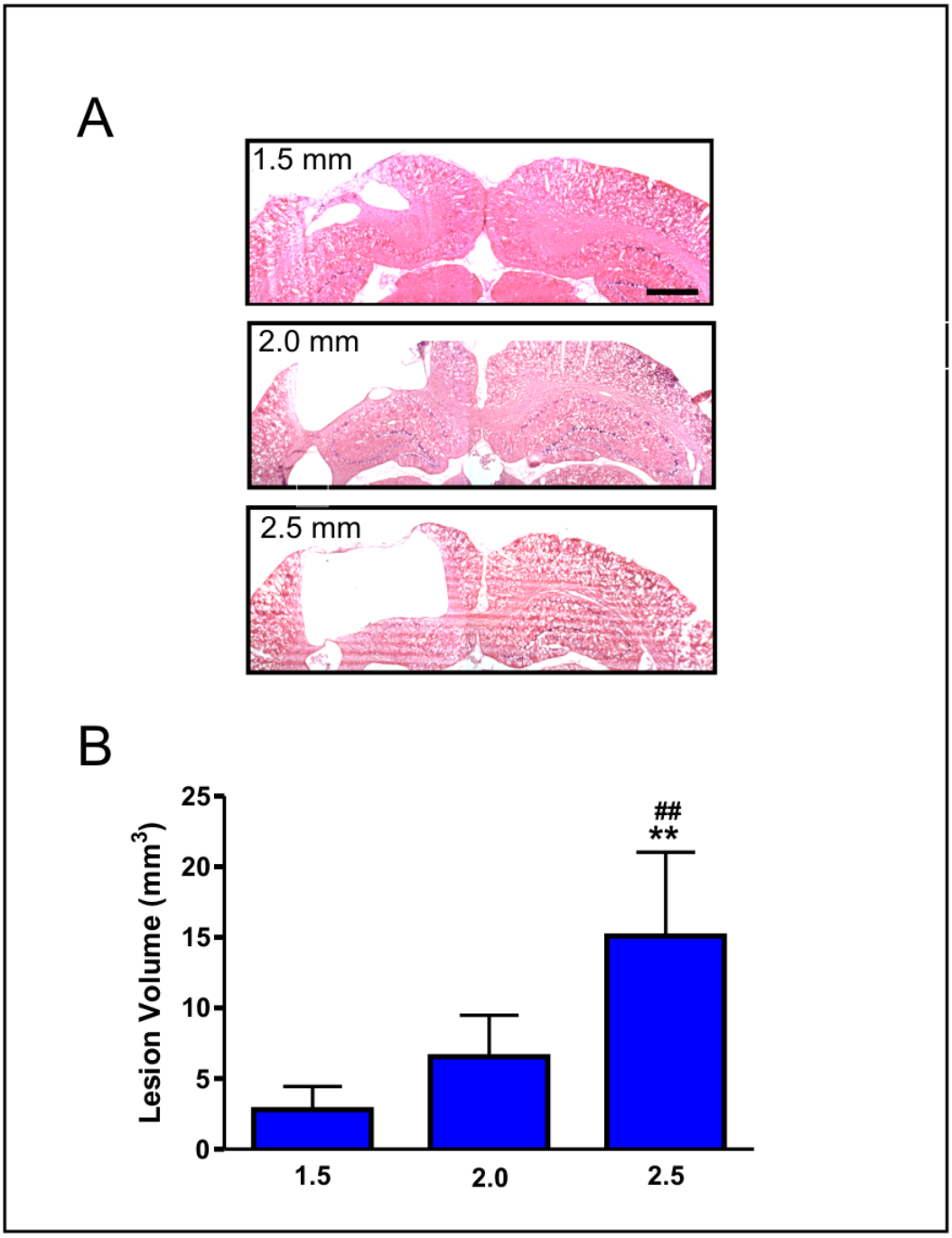

Figure 1. Histological analysis of EMI device-induced injury. (A) Histological images of cresyl violet-stained coronal sections of rats received 1.5, 2.0, 2.5 mm impact produced by EMI device. Brain tissue damage increased in a graded manner as the impact depth increased. (B) Lesion volume of ipsilateral 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).

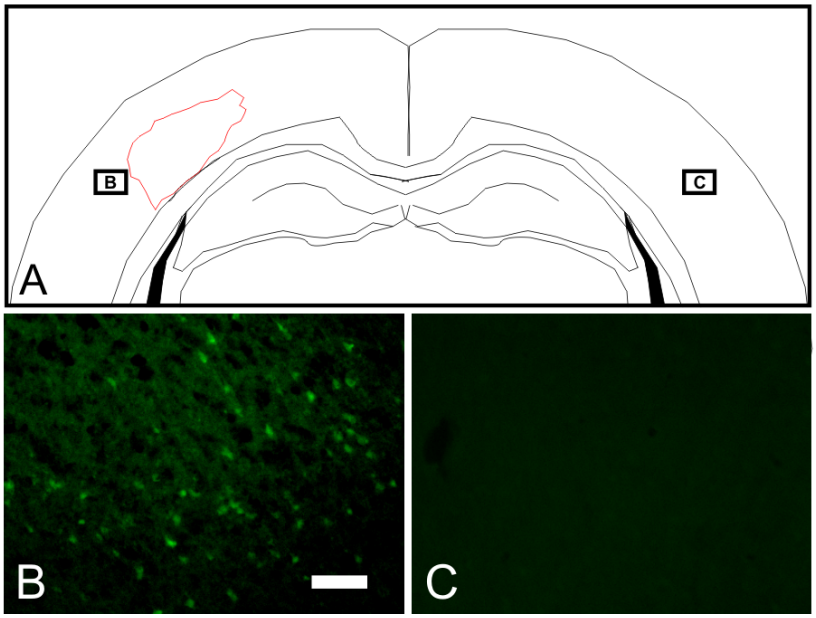

Figure 2. Neurodegeneration detected by Fluoro-Jade B staining at 3 days after 2.0 mm TBI. (A) A montage photomicrograph indicates a lesion of the cortex on the left side. (B, C) Photomicrographs of Fluoro-Jade B stained sections taken from cortical areas around the lesion epicenter (B) or contralateral to it (C). Many Fluoro-Jade B-positive labeled neurons were found on the injury side of the cortex (B), but not on the contralateral side (C). Bars = 1 mm (A); 50 μm (B, C).

$cPLA_2$ 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 expressional changes were detected in the rat hippocampus up to 14 days following TBI (Figure 3B).

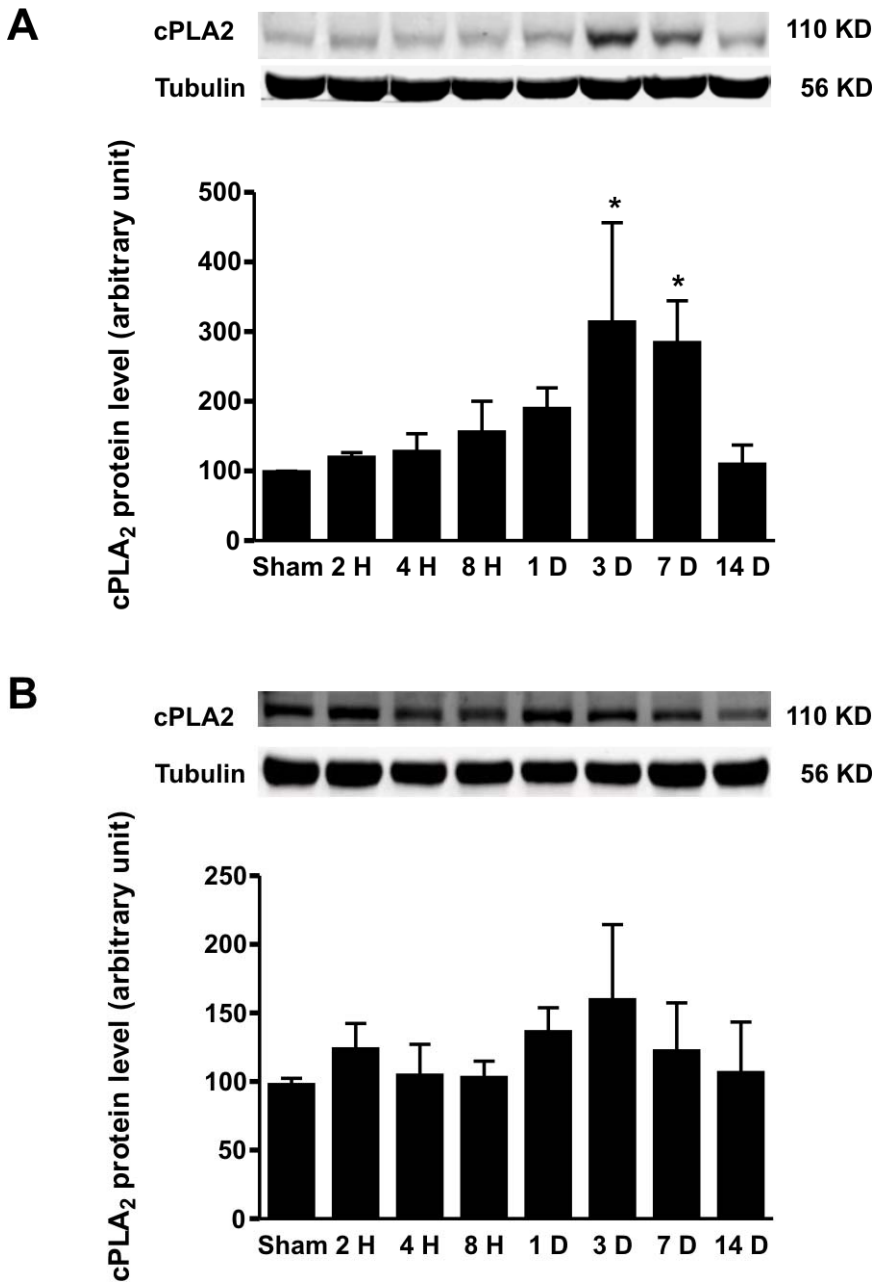

Figure 3. Temporal changes of $cPLA_2$ expression after TBI. (A) TBI induced a significant increase in cortical $cPLA_2$ expression which peaked at 3 to 7 days after TBI. The top panel shows a representative time course of cortex $cPLA_2$ expression following TBI. The bottom panel shows compiled cortex $cPLA_2$ 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 $cPLA_2$ expression following TBI. The top panel shows a representative time course of hippocampus $cPLA_2$ expression following TBI. The bottom panel shows compiled hippocampus $cPLA_2$ expression following TBI of each group in a bar graph. n= 6 rats in each group.

$cPLA_2$ immunoreactivity was localized in neurons and glial cells after TBI

To further confirm the results of Western blotting and determine the spatial distribution of $cPLA_2$ after TBI, ABC immunohistochemistry was performed on the brain sections for the $cPLA_2$ at 7 days after TBI. $cPLA_2$ immunoreactivity was weak in the contralateral side of rat cortex (Figure 4A and C). In contrast, $cPLA_2$ immunoreactivity was increased in the lesioned cortex (Figure 4A and B). $cPLA_2$ labeling was increased closer to the lesion epicenter. However, no apparent difference of $cPLA_2$ immunoreactivity was found in the rat hippocampus ipsilateral to the injury (Figure 4 A, D), compared with the contralateral hippocampus (Figure 4A, E).

To investigate the source of $cPLA_2$ in cortex, we performed double-labeling immunofluorescence to localize specific cell types that express $cPLA_2$. $cPLA_2$ immunoreactivity was detected in neurons which were labeled by a neuronal marker, NeuN (Figure 5A-C). $cPLA_2$ 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.

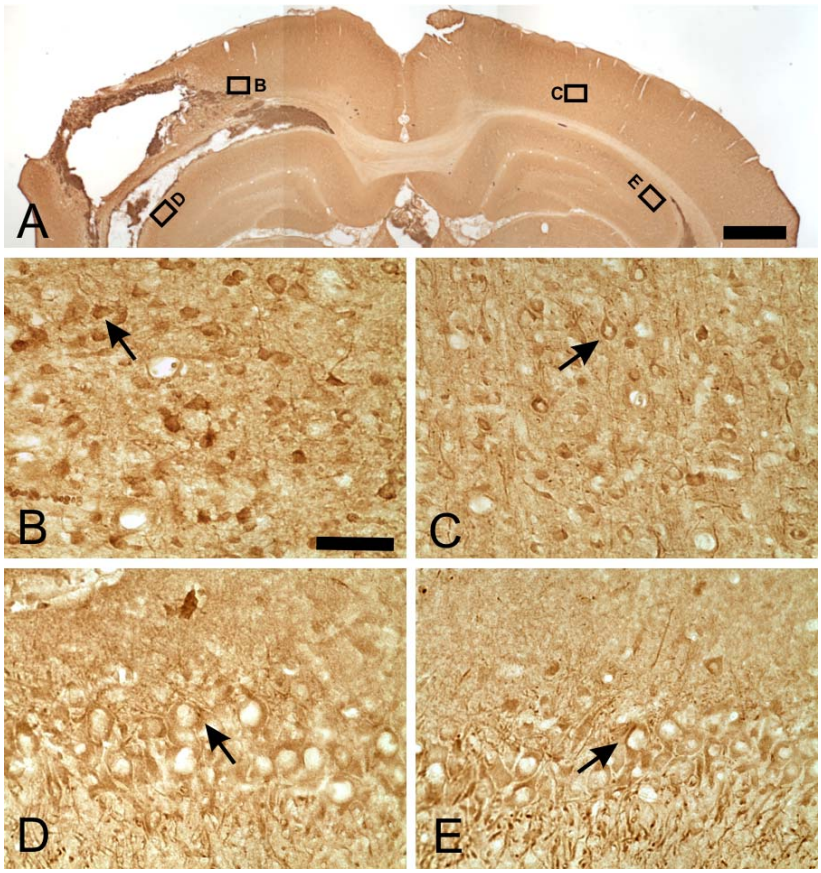

Figure 4. Distribution of $cPLA_2$ immunoreactivity (IR) in the cortex and the hippocampus at 7 days after moderate TBI. (A) A weak signal of $cPLA_2$ IR was detected in the contralateral hemisphere. TBI induced a marked increase in $cPLA_2$ IR surrounding the injury epicenter. There was no apparent 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).

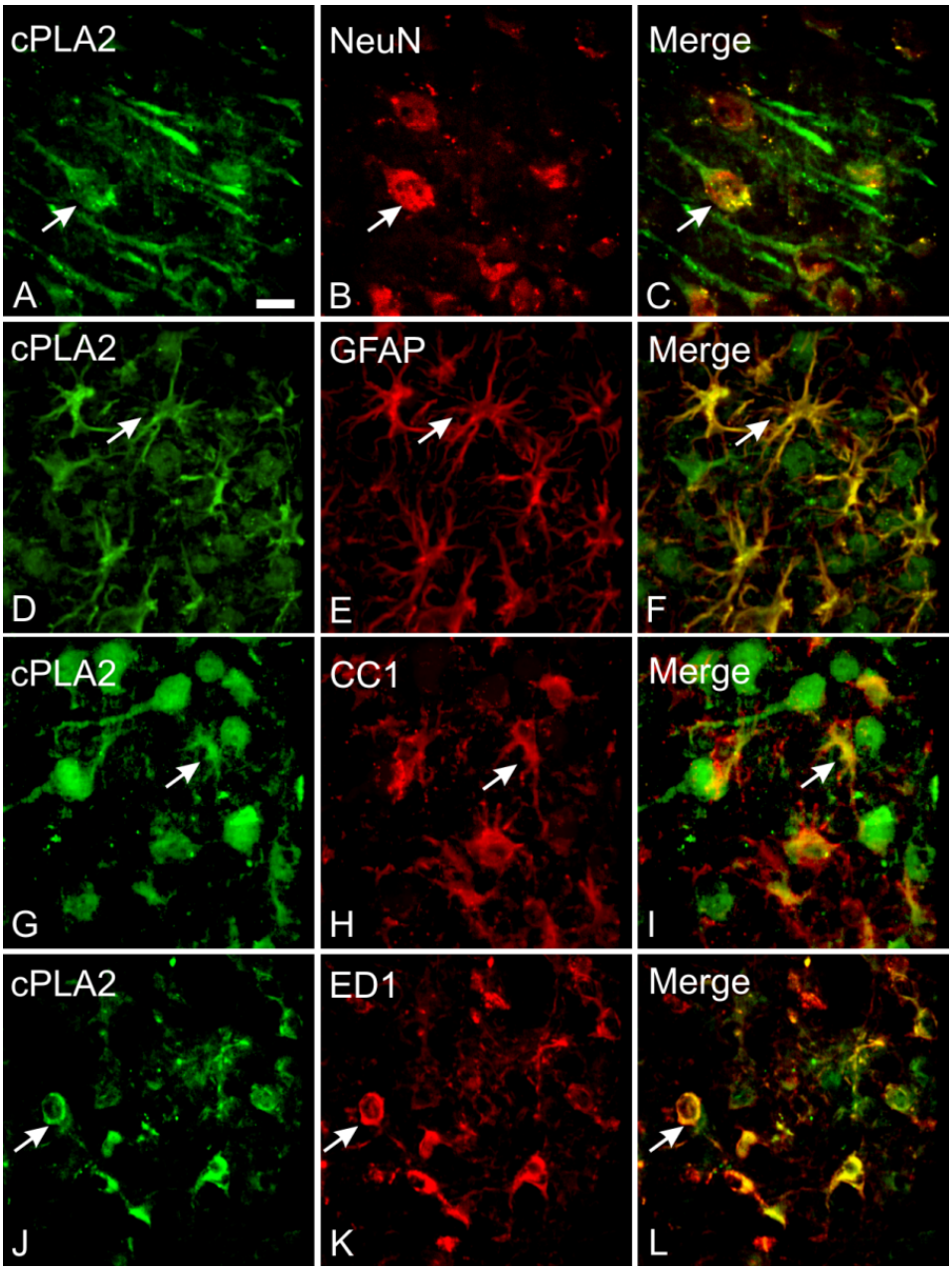

Figure 5. Cellular localization of $cPLA_2$ expression is shown in the rat brain at 7 days after moderate TBI. (A-C) $cPLA_2$ IR (A, arrow) was detected in neurons with NeuN IR (B, arrow), which is demonstrated by the merged image (C, arrow). (D-F) $cPLA_2$ IR (D, arrow) was also localized in astrocytes 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 $cPLA_2$ expression pattern in both the rat cortex and hippocampus after moderate TBI. We found that $cPLA_2$ 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 $cPLA_2$ expression between ipsilateral and contralateral hippocampus after TBI. These results are in agreement with other reports showing increased expression of $cPLA_2$ 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_2 activation (H. S. Dhillon et al., 1994; P. Homayoun et al., 1997).

Our immunohistochemistry analysis further demonstrated that $cPLA_2$ 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 $cPLA_2$ 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 $F_{2\alpha}$, prostaglandin D_2, leukotrienes, and thromboxane B_2 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 $cPLA_2$ immunoreactivity in rat hippocampus neurons and astrocytes (T. L. Sandhya et al., 1998). Such a $cPLA_2$ 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 $cPLA_2$ and 4-HNE increase can be inhibited by the PLA_2 inhibitor, mepacrine (X. R. Lu et al., 2001b; W. Y. Ong et al., 2003). Furthermore, a $cPLA_2$ 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 Ca^2+ increase, stimulates PLA_2 activation and eventually causes neuronal death (J. Klein, 2000).

The excessive $cPLA_2$ level in cortex following TBI may contribute to secondary TBI by multiple mechanisms. $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ 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, microglias, 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). Glycerophosphlipid 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-α, 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 oligonuleotides can inhibit AA release from neurons and also reduce Aβ-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 $cPLA_2$ may contribute to axon demylination by inducing oligodendrocyte death or by directly hydrolyzing phosphatidylcholine, one of major phospholipids. When $cPLA_2$ 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 PLA_2 expression correlates well with the rate of demylination 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 $cPLA_2$ 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 $cPLA_2$ (T. L. Sandhya et al., 1998; D. Stephenson et al., 1999). The increased $cPLA_2$ 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 $cPLA_2$ expression level was significantly increased after TBI and peaked at 3 to 7 days post-TBI. $cPLA_2$ was present in neurons, oligodendrocytes, astrocytes and macrophages. The increased $cPLA_2$ and lesion was found mainly at the lesion site ipsilateral to the injury. These findings suggest that abnormal expression of $cPLA_2$ 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

(1998) Rehabilitation of persons with traumatic brain injury. NIH Consens Statement 16:1-41.

Abe K, Yuki S, Kogure K (1988) Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger. Stroke 19:480-485.

Arai K, Ikegaya Y, Nakatani Y, Kudo I, Nishiyama N, Matsuki N (2001) Phospholipase A2 mediates ischemic injury in the hippocampus: a regional difference of neuronal vulnerability. Eur J Neurosci 13:2319-2323.

Balsinde J, Balboa MA, Dennis EA (2000) Group IV cytosolic phospholipase A2 activation by diacylglycerol pyrophosphate in murine P388D1 macrophages. Ann N Y Acad Sci 905:11-15.

Balsinde J, Perez R, Balboa MA (2006) Calcium-independent phospholipase A2 and apoptosis. Biochim Biophys Acta 1761:1344-1350.

Baran H, Heldt R, Hertting G (1987) Increased prostaglandin formation in rat brain following systemic application of kainic acid. Brain Res 404:107-112.

Bayly PV, Dikranian KT, Black EE, Young C, Qin YQ, Labruyere J, Olney JW (2006) Spatiotemporal evolution of apoptotic neurodegeneration following traumatic injury to the developing rat brain. Brain Res 1107:70-81.

Bazan NG, Rodriguez de Turco EB, Allan G (1995) Mediators of injury in neurotrauma: intracellular signal transduction and gene expression. J Neurotrauma 12:791-814.

Bonventre JV (1996) Roles of phospholipases A2 in brain cell and tissue injury associated with ischemia and excitotoxicity. J Lipid Mediat Cell Signal 14:15-23.

Brody DL, Mac Donald C, Kessens CC, Yuede C, Parsadanian M, Spinner M, Kim E, Schwetye KE, Holtzman DM, Bayly PV (2007) Electromagnetic controlled cortical impact device for precise, graded experimental traumatic brain injury. J Neurotrauma 24:657-673.

Bullock R, Zauner A, Woodward JJ, Myseros J, Choi SC, Ward JD, Marmarou A, Young HF (1998) Factors affecting excitatory amino acid release following severe human head injury. J Neurosurg 89:507-518.

Burke JE, Hsu YH, Deems RA, Li S, Woods VL, Jr., Dennis EA (2008) A phospholipid substrate molecule residing in the membrane surface mediates opening of the lid region in group IVA cytosolic phospholipase A2. J Biol Chem 283:31227-31236.

Chalimoniuk M, Stolecka A, Zieminska E, Stepien A, Langfort J, Strosznajder JB (2009) Involvement of multiple protein kinases in cPLA2 phosphorylation, arachidonic acid release, and cell death in in vivo and in vitro models of 1-methyl-4-phenylpyridinium-induced parkinsonism--the possible key role of PKG. J Neurochem 110:307-317.

Clark JD, Schievella AR, Nalefski EA, Lin LL (1995) Cytosolic phospholipase A2. J Lipid Mediat Cell Signal 12:83-117.

Clemens JA, Stephenson DT, Smalstig EB, Roberts EF, Johnstone EM, Sharp JD, Little SP, Kramer RM (1996) Reactive glia express cytosolic phospholipase A2 after transient global forebrain ischemia in the rat. Stroke 27:527-535.

Cummings BS, McHowat J, Schnellmann RG (2000) Phospholipase A(2)s in cell injury and death. J Pharmacol Exp Ther 294:793-799.

Dennis EA (1994) Diversity of group types, regulation, and function of phospholipase A2. J Biol Chem 269:13057-13060.

Dhillon HS, Donaldson D, Dempsey RJ, Prasad MR (1994) Regional levels of free fatty acids and Evans blue extravasation after experimental brain injury. J Neurotrauma 11:405-415.

Faden AI, Demediuk P, Panter SS, Vink R (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. Science 244:798-800.

Farooque M, Hillered L, Holtz A, Olsson Y (1996) Changes of extracellular levels of amino acids after graded compression trauma to the spinal cord: an experimental study in the rat using microdialysis. J Neurotrauma 13:537-548.

Farooqui AA, Yang HC, Horrocks L (1997a) Involvement of phospholipase A2 in neurodegeneration. Neurochem Int 30:517-522.

Farooqui AA, Ong WY, Horrocks LA (2004) Biochemical aspects of neurodegeneration in human brain: involvement of neural membrane phospholipids and phospholipases A2. Neurochem Res 29:1961-1977.

Farooqui AA, Ong WY, Horrocks LA (2006) Inhibitors of brain phospholipase A2 activity: their neuropharmacological effects and therapeutic importance for the treatment of neurologic disorders. Pharmacol Rev 58:591-620.

Farooqui AA, Yang HC, Rosenberger TA, Horrocks LA (1997b) Phospholipase A2 and its role in brain tissue. J Neurochem 69:889-901.

Farooqui AA, Litsky ML, Farooqui T, Horrocks LA (1999) Inhibitors of intracellular phospholipase A2 activity: their neurochemical effects and therapeutical importance for neurological disorders. Brain Res Bull 49:139-153.

Globus MY, Alonso O, Dietrich WD, Busto R, Ginsberg MD (1995) Glutamate release and free radical production following brain injury: effects of posttraumatic hypothermia. J Neurochem 65:1704-1711.

Greve MW, Zink BJ (2009) Pathophysiology of traumatic brain injury. Mt Sinai J Med 76:97-104.

Hall ED, Braughler JM (1986) Role of lipid peroxidation in post-traumatic spinal cord degeneration: a review. Cent Nerv Syst Trauma 3:281-294.

Hatanaka Y, Suzuki K, Kawasaki Y, Endo Y, Taniguchi N, Takei N (1996) A role of peroxides in Ca2+ ionophore-induced apoptosis in cultured rat cortical neurons. Biochem Biophys Res Commun 227:513-518.

Hernandez M, Nieto ML, Sanchez Crespo M (2000) Cytosolic phospholipase A2 and the distinct transcriptional programs of astrocytoma cells. Trends Neurosci 23:259-264.

Hirabayashi T, Shimizu T (2000) Localization and regulation of cytosolic phospholipase A(2). Biochim Biophys Acta 1488:124-138.

Hirabayashi T, Murayama T, Shimizu T (2004) Regulatory mechanism and physiological role of cytosolic phospholipase A2. Biol Pharm Bull 27:1168-1173.
Hoane MR, Pierce JL, Holland MA, Birky ND, Dang T, Vitek MP, McKenna SE (2007) The novel apolipoprotein E-based peptide COG1410 improves sensorimotor performance and reduces injury magnitude following cortical contusion injury. J Neurotrauma 24:1108-1118.
Homayoun P, Rodriguez de Turco EB, Parkins NE, Lane DC, Soblosky J, Carey ME, Bazan NG (1997) Delayed phospholipid degradation in rat brain after traumatic brain injury. J Neurochem 69:199-205.
Kim C, Kim JY, Kim JH (2008) Cytosolic phospholipase A(2), lipoxygenase metabolites, and reactive oxygen species. BMB Rep 41:555-559.
Kishimoto K, Matsumura K, Kataoka Y, Morii H, Watanabe Y (1999) Localization of cytosolic phospholipase A2 messenger RNA mainly in neurons in the rat brain. Neuroscience 92:1061-1077.
Kita Y, Ohto T, Uozumi N, Shimizu T (2006) Biochemical properties and pathophysiological roles of cytosolic phospholipase A2s. Biochim Biophys Acta 1761:1317-1322.
Klein J (2000) Membrane breakdown in acute and chronic neurodegeneration: focus on choline-containing phospholipids. J Neural Transm 107:1027-1063.
Kriem B, Sponne I, Fifre A, Malaplate-Armand C, Lozac'h-Pillot K, Koziel V, Yen-Potin FT, Bihain B, Oster T, Olivier JL, Pillot T (2005) Cytosolic phospholipase A2 mediates neuronal apoptosis induced by soluble oligomers of the amyloid-beta peptide. FASEB J 19:85-87.
Kudo I, Murakami M (2002) Phospholipase A2 enzymes. Prostaglandins Other Lipid Mediat 68-69:3-58.
Liaison OoCaP (2002) Traumatic brain injury: Hope through research: National institute of neurological disorders and stroke.
Liu NK, Zhang YP, Titsworth WL, Jiang X, Han S, Lu PH, Shields CB, Xu XM (2006) A novel role of phospholipase A2 in mediating spinal cord secondary injury. Ann Neurol 59:606-619.
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.
Lu XR, Ong WY, Halliwell B, Horrocks LA, Farooqui AA (2001b) Differential effects of calcium-dependent and calcium-independent phospholipase A(2) inhibitors on kainate-induced neuronal injury in rat hippocampal slices. Free Radic Biol Med 30:1263-1273.
Lucas KK, Dennis EA (2004) The ABC's of Group IV cytosolic phospholipase A2. Biochim Biophys Acta 1636:213-218.
Luo J, Lang JA, Miller MW (1998) Transforming growth factor beta1 regulates the expression of cyclooxygenase in cultured cortical astrocytes and neurons. J Neurochem 71:526-534.
Marion DW (1991) Complications of head injury and their therapy. Neurosurg Clin N Am 2:411-424.

Mayer RJ, Marshall LA (1993) New insights on mammalian phospholipase A2(s); comparison of arachidonoyl-selective and -nonselective enzymes. FASEB J 7:339-348.

McIntosh TK, Saatman KE, Raghupathi R, Graham DI, Smith DH, Lee VM, Trojanowski JQ (1998) The Dorothy Russell Memorial Lecture. The molecular and cellular sequelae of experimental traumatic brain injury: pathogenetic mechanisms. Neuropathol Appl Neurobiol 24:251-267.

Molloy GY, Rattray M, Williams RJ (1998) Genes encoding multiple forms of phospholipase A2 are expressed in rat brain. Neurosci Lett 258:139-142.

Murakami M, Kudo I (2002) Phospholipase A2. J Biochem 131:285-292.

Murakami M, Nakatani Y, Atsumi G, Inoue K, Kudo I (1997) Regulatory functions of phospholipase A2. Crit Rev Immunol 17:225-283.

Nakano S, Kogure K, Abe K, Yae T (1990) Ischemia-induced alterations in lipid metabolism of the gerbil cerebral cortex: I. Changes in free fatty acid liberation. J Neurochem 54:1911-1916.

Narita K, Kubota M, Nakane M, Kitahara S, Nakagomi T, Tamura A, Hisaki H, Shimasaki H, Ueta N (2000) Therapeutic time window in the penumbra during permanent focal ischemia in rats: changes of free fatty acids and glycerophospholipids. Neurol Res 22:393-400.

O'Regan MH, Smith-Barbour M, Perkins LM, Phillis JW (1995) A possible role for phospholipases in the release of neurotransmitter amino acids from ischemic rat cerebral cortex. Neurosci Lett 185:191-194.

Ohto T, Uozumi N, Hirabayashi T, Shimizu T (2005) Identification of novel cytosolic phospholipase A(2)s, murine cPLA(2){delta}, {epsilon}, and {zeta}, which form a gene cluster with cPLA(2){beta}. J Biol Chem 280:24576-24583.

Ong WY, Lu XR, Ong BK, Horrocks LA, Farooqui AA, Lim SK (2003) Quinacrine abolishes increases in cytoplasmic phospholipase A2 mRNA levels in the rat hippocampus after kainate-induced neuronal injury. Exp Brain Res 148:521-524.

Palmer AM, Marion DW, Botscheller ML, Swedlow PE, Styren SD, DeKosky ST (1993) Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model. J Neurochem 61:2015-2024.

Park E, Velumian AA, Fehlings MG (2004) The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. J Neurotrauma 21:754-774.

Phillis JW, O'Regan MH (2004) A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders. Brain Res Brain Res Rev 44:13-47.

Pickard RT, Strifler BA, Kramer RM, Sharp JD (1999) Molecular cloning of two new human paralogs of 85-kDa cytosolic phospholipase A2. J Biol Chem 274:8823-8831.

Pilitsis JG, Coplin WM, O'Regan MH, Wellwood JM, Diaz FG, Fairfax MR, Michael DB, Phillis JW (2003) Measurement of free fatty acids in cerebrospinal fluid from patients with hemorrhagic and ischemic stroke. Brain Res 985:198-201.

Saluja I, Song D, O'Regan MH, Phillis JW (1997) Role of phospholipase A2 in the release of free fatty acids during ischemia-reperfusion in the rat cerebral cortex. Neurosci Lett 233:97-100.

Sandhya TL, Ong WY, Horrocks LA, Farooqui AA (1998) A light and electron microscopic study of cytoplasmic phospholipase A2 and cyclooxygenase-2 in the hippocampus after kainate lesions. Brain Res 788:223-231.

Schaloske RH, Dennis EA (2006) The phospholipase A2 superfamily and its group numbering system. Biochim Biophys Acta 1761:1246-1259.

Scott KF, Bryant KJ, Bidgood MJ (1999) Functional coupling and differential regulation of the phospholipase A2-cyclooxygenase pathways in inflammation. J Leukoc Biol 66:535-541.

Shirai Y, Ito M (2004) Specific differential expression of phospholipase A2 subtypes in rat cerebellum. J Neurocytol 33:297-307.

Shohami E, Shapira Y, Yadid G, Reisfeld N, Yedgar S (1989) Brain phospholipase A2 is activated after experimental closed head injury in the rat. J Neurochem 53:1541-1546.

Song C, Chang XJ, Bean KM, Proia MS, Knopf JL, Kriz RW (1999) Molecular characterization of cytosolic phospholipase A2-beta. J Biol Chem 274:17063-17067.

Stephenson D, Rash K, Smalstig B, Roberts E, Johnstone E, Sharp J, Panetta J, Little S, Kramer R, Clemens J (1999) Cytosolic phospholipase A2 is induced in reactive glia following different forms of neurodegeneration. Glia 27:110-128.

Stephenson DT, Manetta JV, White DL, Chiou XG, Cox L, Gitter B, May PC, Sharp JD, Kramer RM, Clemens JA (1994) Calcium-sensitive cytosolic phospholipase A2 (cPLA2) is expressed in human brain astrocytes. Brain Res 637:97-105.

Stewart A, Ghosh M, Spencer DM, Leslie CC (2002) Enzymatic properties of human cytosolic phospholipase A(2)gamma. J Biol Chem 277:29526-29536.

Taketo MM, Sonoshita M (2002) Phospolipase A2 and apoptosis. Biochim Biophys Acta 1585:72-76.

Tator CH (1991) Review of experimental spinal cord injury with emphasis on the local and systemic circulatory effects. Neurochirurgie 37:291-302.

Tator CH, Fehlings MG (1991) Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75:15-26.

Toborek M, Malecki A, Garrido R, Mattson MP, Hennig B, Young B (1999) Arachidonic acid-induced oxidative injury to cultured spinal cord neurons. J Neurochem 73:684-692.

Tucker DE, Stewart A, Nallan L, Bendale P, Ghomashchi F, Gelb MH, Leslie CC (2005) Group IVC cytosolic phospholipase A2gamma is farnesylated and palmitoylated in mammalian cells. J Lipid Res 46:2122-2133.

Underwood KW, Song C, Kriz RW, Chang XJ, Knopf JL, Lin LL (1998) A novel calcium-independent phospholipase A2, cPLA2-gamma, that is prenylated and contains homology to cPLA2. J Biol Chem 273:21926-21932.

Waldmeier PC (2003) Prospects for antiapoptotic drug therapy of neurodegenerative diseases. Prog Neuropsychopharmacol Biol Psychiatry 27:303-321.

Weerasinghe GR, Coon SL, Bhattacharjee AK, Harry GJ, Bosetti F (2006) Regional protein levels of cytosolic phospholipase A2 and cyclooxygenase-2 in Rhesus monkey brain as a function of age. Brain Res Bull 69:614-621.

Writer BW, Schillerstrom JE (2009) Psychopharmacological treatment for cognitive impairment in survivors of traumatic brain injury: a critical review. J Neuropsychiatry Clin Neurosci 21:362-370.

Yoshida S, Inoh S, Asano T, Sano K, Shimasaki H, Ueta N (1983) Brain free fatty acids, edema, and mortality in gerbils subjected to transient, bilateral ischemia, and effect of barbiturate anesthesia. J Neurochem 40:1278-1286.

Young W (1993) Secondary injury mechanisms in acute spinal cord injury. J Emerg Med 11 Suppl 1:13-22.