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Induction of secretory phospholipase A_2 in reactive astrocytes in response to transient focal cerebral ischemia in the rat brain

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Abstract

Although mRNA expression of group IIA secretory phospholipase A_2 (sPLA$_2$-IIA) has been implicated in responses to injury in the CNS, information on protein expression remains unclear. In this study, we investigated temporal and spatial expression of sPLA$_2$-IIA mRNA and immunoreactivity in transient focal cerebral ischemia induced in rats by occlusion of the middle cerebral artery. Northern blot analysis showed a biphasic increase in sPLA$_2$-IIA mRNA expression following 60-min of ischemia–reperfusion: an early phase at 30 min and a second increase at a late phase ranging from 12 h to 14 days. *In situ* hybridization localized the early-phase increase in sPLA$_2$-IIA mRNA to the affected ischemic cortex and the late-phase increase to the penumbral area. Besides sPLA$_2$-IIA mRNA, glial fibrillary acidic protein (GFAP) and cyclo-oxygenase-2 mRNAs, but not cytosolic PLA$_2$, also showed an increase in the penumbral area at 3 days after ischemia–reperfusion. Immunohistochemistry of sPLA$_2$-IIA indicated positive cells in the penumbral area similar to the GFAP-positive astrocytes but different from the isolectin B4-positive microglial cells. Confocal microscopy further confirmed immunoreactivity of sPLA$_2$-IIA in reactive astrocytes but not in microglial cells. Taken together, these results demonstrate for the first time an up-regulation of the inflammatory sPLA$_2$-IIA in reactive astrocytes in response to cerebral ischemia–reperfusion.

Keywords: astrocytes, cerebral ischemia, group IIA secretory phospholipase A_2, immunohistochemistry, *in situ* hybridization, microglial cells.

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Early studies by Bazan (1970) recognized the rapid release of arachidonic acid in brain owing to cerebral ischemia and other excitatory insults. This observation has since triggered considerable interest in delineating the mechanism(s) underlying the arachidonic acid release phenomenon. Studies have focused on phospholipase A_2 (PLA$_2$), a family of enzymes ubiquitous in mammalian cells (Murakami *et al*. 1997; Murakami and Kudo 2002). These enzymes are known to play an important role in maintenance of membrane phospholipids as well as production of lipid mediators, which regulate cellular activity. Increase in mRNA expression of PLA$_2$ has been implicated in a number of pathological conditions, including neuronal damage in response to cerebral ischemia (Farooqui *et al*. 1997; Cummings *et al*. 2000; Arai *et al*. 2001; Sun *et al*. 2004). However, the presence of multiple isoforms of PLA$_2$ in different cellular systems in the brain (Molloy *et al*. 1998) has made elucidation of the involvement of specific PLA$_2$ subtypes in neurodegenerative diseases a difficult and challenging endeavor (Sun *et al*. 2004).

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Abbreviations used: COX, cyclo-oxygenase; cPLA$_2$, cytosolic phospholipase A_2; DAPI, 4′,6-Diamidine-2′-phenylindole; DEPC, diethylpyrocarbonate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; MCA, middle cerebral artery; NSE, neuron-specific enolase; PBS, phosphate-buffered saline; PLA$_2$, phospholipase A_2; SDS, sodium dodecyl sulfate; sPLA$_2$, secretory PLA$_2$; sPLA$_2$-IIA, group IIA secretory phospholipase A_2.

Secretory PLA_2 ($sPLA_2$) consists of a family of enzymes of low molecular weight (Murakami and Kudo 2002). Among these, the group IIA $sPLA_2$ ($sPLA_2$-IIA) has been studied extensively because of its involvement in inflammatory processes (Fuentes *et al*. 2002). Up-regulation of $sPLA_2$-IIA mRNA expression has been reported in rat brain after transient global ischemia (Lauritzen *et al*. 1994) and an increase in $sPLA_2$ activity was observed after photochemically induced focal cerebral ischemia (Yagami *et al*. 2002). Although cerebral ischemia is associated with increased production of cytokines, chemokines, adhesion molecules and lipid mediators (Iadecola and Alexander 2001), it is not clear whether $sPLA_2$-IIA is one of the proteins induced as a result of the insult. In this study, a rat focal cerebral ischemia–reperfusion model induced by middle cerebral artery (MCA) occlusion was used to investigate the temporal and spatial response of $sPLA_2$-IIA mRNA and immunoreactivity. It is well recognized that cyclo-oxygenases (COXs) are important downstream enzymes for prostanoid synthesis. Therefore, an *in situ* hybridization study examining expression of cytosolic PLA_2 ($cPLA_2$) and COX-2 mRNA was also included in this study. We demonstrated a biphasic increase in $sPLA_2$-IIA mRNA after ischemia–reperfusion, and $sPLA_2$-IIA immunoreactivity associated with reactive astrocytes and not with microglial cells.

Materials and methods

Stroke model

The focal cerebral ischemia–reperfusion model in rats has been described previously (Chen *et al*. 1986; Lin *et al*. 1993). Briefly, male Long–Evans rats weighing 250–300 g were anesthetized with chloral hydrate (360 mg/kg bodyweight, i.p.). The trunk of the right MCA above the rhinal fissure was identified under a stereomicroscope and ligated with a 10–0 suture. Interruption of blood flow distal to ligation was confirmed under the microscope. Both common carotid arteries were then occluded using non-traumatic aneurysm clips. After 60 min ischemia, the aneurysm clips and the suture were removed, and restoration of blood flow in all three arteries was verified under the dissecting microscope. While under anesthesia, the rectal temperature was monitored and maintained at 37.0 ± 0.5°C using a homeothermic blanket (Harvard Apparatus, Holliston, MA, USA). In this model, ischemia for 60 min consistently resulted in a large infarct confined to the right MCA cortex. No morphological or biochemical evidence of ischemic brain injury was noted in the left MCA cortex. Regional cerebral blood flow in this model has been studied using labeled isopropyliodoamphetamine-iodoantipyrine (An *et al*. 1993; Lin *et al*. 2002), although the procedure was not used in this particular experiment. In previous studies, the right MCA cortex that sustained severe ischemia showed reduction of blood flow by 88–92%. Only very mild reduction was observed outside the right MCA cortex.

After the ischemic insult (60 min), rats were kept in an air-ventilated incubator at 24.0 ± 0.5°C for up to 2 weeks, and were provided with water and lab chow *ad libitum* until completion of the experiments. At the end of the 60-min MCA occlusion, 0, 30, 60 and 90 min, 4 and 12 h, 1, 3, 7 and 14 days after reperfusion, rats were killed by decapitation under anesthesia, and the brains were quickly removed to collect the cerebral cortex. The ischemic right and uninjured left MCA cortices were separated and immediately frozen in liquid nitrogen. Brain samples were stored at − 70°C until further processing. Sham controls were killed 24 h after sham operation. Other rats were killed by transcardial perfusion with normal saline under anesthesia, followed by cold 4% paraformaldehyde. The brains were removed and cryoprotected with 30% sucrose at 4°C overnight. All procedures were approved by the institutional Animal Studies Committee, and were in accordance with the PHS Guide for the Care and Use of Laboratory Animals, USDA Regulations, and the Guidelines of the AVMA Panel on Euthanasia.

RNA isolation and RT–PCR

Total RNA was isolated from frozen cerebral cortex as described previously (Lin *et al*. 2000). Total RNA (1 μg) was mixed with 200 units of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Rtase; Clontech, Palo Alto, CA, USA) in a buffer containing a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 units Rnase inhibitor, 1 μM poly-dT oligomer, and 0.5 mM of each dNTP in a final volume of 20 μL. The reaction mixture was incubated at 42°C for 1 h and then 94°C for 5 min to inactivate the enzyme. A total of 80 μL DEPC-treated water was added to the reaction mixture before storage at − 70°C.

Five microliters of the RT reaction solution was used in the PCR reaction. PCR was carried out in a final volume of 50 μL containing 200 μM each of dATP, dCTP, dGTP, and dTTP, 5 pmol of each primer, 1.25 units Taq polymerase (BRL, Fayetteville, GA, USA), 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$ and 50 mM KCl. The mixture was incubated in a thermal cycler for 35 cycles using the following profile: 94°C for 7 min, repeat cycles of 94°C for 45 s; 55°C for 45 s; and 72°C for 90 s. Samples were then incubated at 72°C for 7 min and cooled to 4°C (GeneAmp 2400, Perkin Elmer, Foster City, CA, USA). Primer sequences for $sPLA_2$-IIA are: forward 5′-TGACTCATGACTGTTGTTACAACC-3′; and reverse: 5′-TCTCAGGACCCTCTTAGGTACTA-3′ which amplified a 493-bp fragment from rat $sPLA_2$-IIA cDNA (Ishizaki *et al*. 1989; Li *et al*. 1999). PCR products were run on 2% agarose gel for DNA fragment size verification, then eluted and subcloned (Invitrogen, Grand Island, NY, USA) to confirm sequence identity and served as a probe to detect $sPLA_2$-IIA mRNA in northern blots and *in situ* hybridization analysis.

Northern blot analysis

Northern blot analysis has been described previously (Lin *et al*. 2000). Briefly, RNA samples (15 μg/lane) from the entire cortex were applied to 1.2% agarose gel in the presence of 2.2 M formaldehyde. After electrophoresis, gels were transblotted on to Nytran™ membranes (Gene Screen Plus; DuPont, Boston, MA, USA). Membranes were prehybridized at 60°C in a solution containing 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 10% dextran sulfate and 100 μg/mL sheared salmon sperm DNA. RT–PCR-amplified cDNA probes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were labeled with [^{32}P]dCTP using the random-primer labeling method (Amersham,

Arlington Heights, IL, USA). Radioactive probes (1×10^6 cpm/mL) were added directly to the prehybridization solution. Following overnight hybridization at 60°C, membranes were washed twice in 2 × sodium citrate buffer (SSC) at room temperature for 5 min each, followed by two 30-min washes at 60°C in 2 × SSC/1% SDS and two 30-min washes at 60°C in 0.1 × SSC. Membranes were then exposed to Kodak X-Omat/XB-1 films (Fisher Scientific, St Louis, MO, USA). The radioactive bands were quantified by a densitometer.

In situ hybridization

In situ hybridization to detect the regional distribution of mRNA signals has been described previously (Lin *et al*. 1996). In brief, brain slices were frozen-sectioned at 25 μm thickness and mounted on poly-L-lysine-coated slides. Brain sections were subjected to 0.001% proteinase K digestion at 37°C for 30 min, then immersed in 0.1 mM triethanolamine with 0.25% acetic anhydride at room temperature for 10 min, and subsequently dehydrated in 50%, 70%, 95% and 100% ethanol (3 min each). Hybridization was carried out in a solution containing 12.5 mM formamide, 10% dextran sulfate, 0.3 mM NaCl, 1 × Denhardt's solution, 10 mM Tris-HCl (pH 8.0), 500 μg/mL sheared salmon sperm DNA, 100 μg/mL tRNA, 20 mM dithiothreitol and 10^7 cpm/mL of probe at 55°C. RT–PCR-amplified cDNA probes were labeled with [^{33}P]dCTP using the random primer labeling method (Amersham). Following overnight hybridization, slides were then washed sequentially in 2 ×, 1 ×, 0.2 × and 0.1 × SSC at 55°C for 30 min each, followed by dehydration in 50%, 70%, 95% and 100% ethanol for 3 min each. Brain sections were exposed to Kodak BioMax-MR-1 film. In control experiments, sections were incubated with a 100-fold excess of unlabeled probe or pretreated with Rnase A (100 μg/mL, 37°C, 30 min). These experiments resulted in no or negligible signal.

In situ hybridization was also carried out with neuron-specific enolase (NSE) (representing neurons), glial fibrillary acidic protein (GFAP) (representing astrocytes), $cPLA_2$ and COX-2 using oligonucleotide probes on ischemic brain sections 3 days after a 60-min occlusion (Farwell *et al*. 1998; Simonyi *et al*. 2002). The sequences were: GFAP: 5′-TTGGTGTCCAGGCTGGTTTCTCGGATCTGGAGGTTGGAGAAGGTC-3′; $cPLA_2$: 5′-ACACGCCTCCGTCCCCAGCAACTCTGAGTAGCAGTCAGTCGCTTGTGC-3′; COX-2: 5′-ATGGCATCGATGTCATGGTAGAGGGCTTTTCAACTCTGCAG-3′; and NSE: 5′-TAAGTAACGCTGTTTGTCCCCATCCCTTAGTTCCAGGGCCTCATAGAT-3′.

Histochemical and immunohistochemical staining of brain sections

Three days after ischemia, rats were transcardially perfused with heparinized saline (30 mL) and then fixed with 100 mL 4% paraformaldehyde in 0.05 M phosphate-buffered saline (PBS) (pH 7.4). The brains were then post-fixed in the same fixative for 5 days. Brain tissues were embedded in paraffin and coronal sections 6 μm thick were cut at the dorsal hippocampal area with a microtome. Sections were mounted on microscope slides to be used for staining.

To detect neuronal damage in penumbral and infarct areas, brain sections were stained with cresyl violet as described by Wang *et al*. (2002). Immunohistochemical staining of GFAP, a marker for astrocytes, was performed on paraffin-embedded sections according to protocol described by Wang *et al*. (2002). Briefly, brain sections were deparaffinized in xylene, and hydrated in graded series of ethanol and distilled water. Sections were placed in 0.3% H_2O_2 for 30 min and then in 20% normal goat serum in PBS for 30 min. Sections were incubated overnight at 4°C with rabbit anti-human GFAP antibody (Sigma, St Louis, MO, USA) diluted 1 : 100 with PNT (PBS containing 1% normal goat serum and 0.3% Triton-X 100) and then incubated with goat anti-rabbit IgG peroxidase conjugate (1 : 200; Sigma) in PNT for 2 h at room temperature. These sections were then post-stained with 0.05% cresyl violet for 10 min. Microglial cells were detected by staining brain sections with peroxidase-labeled isolectin B4 (L5391; Sigma) according to the protocol described by Streit (1990). Briefly, deparaffinized sections were placed in PBS containing 0.1 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ and 0.1% Triton X-100 for 30 min. Sections were incubated overnight at 4°C with peroxidase-labeled isolectin B4 diluted to 20 μg/mL in PBS containing the divalent ions and Triton-X 100. After washing with PBS, the peroxidase reaction was carried out by incubating the slides with freshly prepared 0.5 mg/mL diaminobenzidine (Sigma) and 0.03% H_2O_2 in 50 mM Tris-HCl (pH 7.6) for 5 min. After dehydration in graded ethanol, sections were mounted with Permount (Fisher Scientific Inc., Hampton, NH, USA).

Immunohistochemical detection of $sPLA_2$ was performed on paraffin-embedded coronal sections. After deparaffinization and hydration in graded ethanol and distilled water, sections were placed in10 mM sodium citrate buffer (pH 6.0), heated at 95°C for 5 min and then cooled for 20 min to unmask the antigen. Sections were further incubated in 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity and permeabilized with 0.3% Triton-X 100 in PBS for 30 min. Normal donkey serum (1.5%) in PBS was used to as a pre-blocking agent for 1 h. Brain sections were then incubated with goat anti-mouse $sPLA_2$ polyclonal antibodies (sc-14472; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1 : 100 in PBS containing 1.5% normal donkey serum and incubated at 4°C overnight. Sections were incubated for 30 min with biotinylated secondary antibody at 1 : 200 in PBS containing 1.5% normal donkey serum and 30 min in PBS with diluted avidin and biotinylated peroxidase 20 μL/mL (sc-2023; Santa Cruz Biotechnology). After rinsing with PBS three times at room temperature, sections were exposed to peroxidase substrate for 3 min. As a negative control, brain sections were subjected to the same incubation protocol except with omission of primary $sPLA_2$ antibody. After dehydration in a graded series of ethanol solutions, sections were mounted with Permount (Fisher). Immunoreactivity of GFAP, isolectin-B4 and $sPLA_2$ was examined using a Nikon microscope with digital still camera DXM1200 (Nikon Corporation, Melville, NY, USA).

To identify expression of $sPLA_2$ in astrocytes and/or microglial cells, confocal laser microscopy was used. For double immunofluorescence staining of $sPLA_2$ and GFAP, brain sections were incubated overnight at 4°C with goat anti-mouse $sPLA_2$ polyclonal antibody (sc-14472; Santa Cruz) diluted at 1 : 100 and rabbit anti-human GFAP antibody diluted at 1 : 100. For secondary antibodies, sections were incubated at room temperature for 2 h with donkey anti-goat IgG labeled with Texas red for $sPLA_2$ (1 : 200; Santa Cruz) and goat anti-rabbit IgG (H + L) labeled with FITC for GFAP (1: 200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For localization of $sPLA_2$ in microglial cells,

slides were incubated overnight at 4°C with goat anti-mouse polyclonal $sPLA_2$ antibody at 1 : 100 dilution followed by donkey anti-goat IgG labeled with Texas red (1 : 200; Santa Cruz) and isolectin-B4 labeled FITC (12.5 μg/mL; Sigma) at room temperature for 2 h.

To demonstrate the presence of damaged nuclei, brain sections were stained with 0.1 μg/mL 4′,6-Diamidine-2′-phenylindole (DAPI) (Roche Molecular Biochemicals, Indianapolis, IN, USA) in PBS for 20 min at room temperature. After staining, slides were washed three times with PBS for 5 min and coverslips were applied with Crystal/Mount media (Fisher). Slides were stored in a dark location for microscopic examination.

Confocal microscopy was carried out in the Molecular Cytology Core (University of Missouri, MO, USA) using an Olympus IX-70 inverted microscope (Olympus America, Melville, NY, USA) with a Sensys digital camera and a Bio-Rad Radiance 2000 sharp laser scanning confocal microscope (Bio-Rad, Hercules, CA, USA). The images were obtained using sequential excitation of 488 and 568 nm laser lines under 515/30 nm (488 line) and 600/40 nm (568 line) emission filters, and pictures were captured through a water immersion objective. The confocal images were obtained with single optical sections along the median plane of the sample.

Statistical analysis

One-way ANOVA followed by post-hoc Fisher-protected *t*-test using the GB-STAT 5.0.4 program (Dynamic Microsystem, Silver Springs, MD, USA) was used to compare the temporal expression of $sPLA_2$-IIA mRNAs. $p < 0.05$ was considered significant.

Results

Expression of $sPLA_2$-IIA mRNA after ischemia–reperfusion

Northern blot analysis and RT–PCR were carried out with RNA extracted from the ischemic or non-ischemic (sham) right MCA cortex at different times after 60 min of ischemia and reperfusion. There were low levels of $sPLA_2$-IIA in the sham controls (Fig. 1). However, significant ($p < 0.05$) increases in $sPLA_2$-IIA mRNA could be observed during two time periods: a small increase at 30 min after 60 min ischemia and a late-phase increase between 12 and 72 h after reperfusion (Fig. 1). Figure 1 also shows a decline in $sPLA_2$-IIA mRNA levels after 72 h but a slight increase was observed after 14 days (Fig. 1).

In situ hybridization using coronal sections was carried out to further examine the regional distribution of $sPLA_2$-IIA mRNA at different times after 60 min of ischemia. The increase in $sPLA_2$-IIA mRNA observed at 30 min after 60 min ischemia was found mainly in the ischemic right MCA cortex (Fig. 2). However, the increase in $sPLA_2$-IIA mRNA at 1 and 3 days after 60 min ischemia was observed mainly in the penumbral area (Fig. 2). There was no obvious increase in mRNA in the right MCA cortex in similar coronal sections from brains obtained 3 days after sham operation (data not shown).

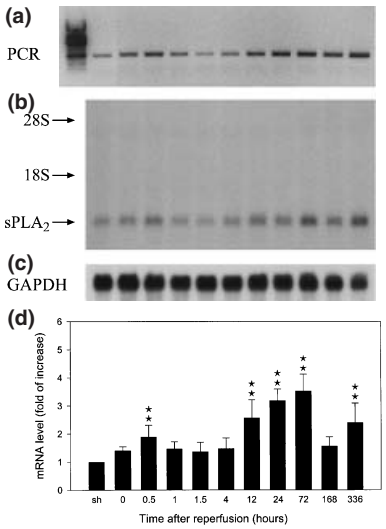

Fig. 1 Time course of $sPLA_2$-IIA mRNA in the ischemic cortex after 60 min of ischemia. 0 represents the end of the 60-min period of ischemia (MCA occlusion). (a) Representative RT–PCR analysis. (b) Typical northern blot analysis showing expression of mRNA for $sPLA_2$-IIA. (c) The same blots were stripped and rehybridized with GAPDH to serve as controls. (d) Radioactive bands were quantified and normalized with those derived from GAPDH mRNA. Sham-operated controls (Sh) were arbitrarily defined as 1. Data are expressed as mean ± SD ($n = 4$ for each time point). *$p < 0.05$, **0.01 versus sham-operated controls (one-way ANOVA followed by post-hoc Fisher-protected *t*-test).

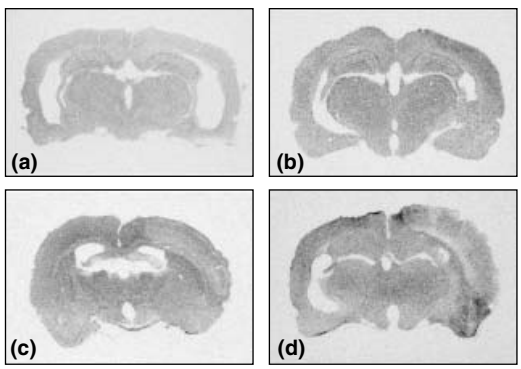

Fig. 2 Photomicrographs from *in situ* hybridization study depicting regional distribution of $sPLA_2$-IIA mRNA in sham control (a), 30 min (b), 1 day (c) and 3 days (d) after MCA occlusion for 60 min. Coronal brain sections (25 μm) were hybridized with ^{33}P-labeled probes. Similar results were observed in two other experiments.

Expression of NSE, GFAP, COX-2 and $cPLA_2$ mRNA

In situ hybridization was carried out to examine mRNA expression of $cPLA_2$ and COX-2 in brain sections obtained 3 days after ischemia for 60 min. Consecutive sections were also probed for NSE and GFAP mRNAs, which represented

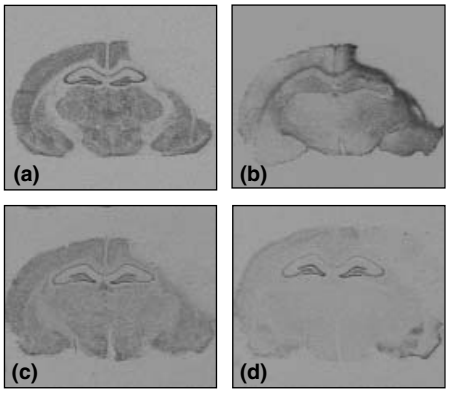

Fig. 3 Photomicrographs from *in situ* hybridization depicting regional expression of mRNA for (a) NSE (b) GFAP (c) $cPLA_2$ and (d) COX-2 in coronal sections of brain obtained 3 days after ischemia lasting 60 min.

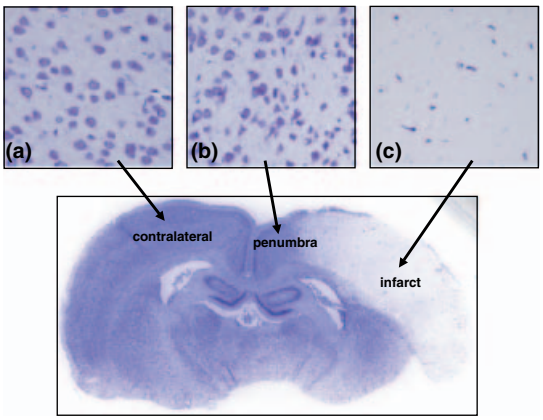

Fig. 4 Cresyl violet staining of brain coronal sections showing neurons in the contralateral cortex (a), penumbral area (b) and infarct area (c). (Original magnification × 400).

genes specific for neurons and astrocytes respectively. Focal ischemia resulted in the formation of a large infarct in the ischemic right MCA cortex. Examination of the NSE mRNA pattern indicated loss of neurons in the infarct area (Fig. 3a), whereas GFAP mRNA markedly increased in the penumbral area (Fig. 3b). Although mRNA for $cPLA_2$ appeared to be present in most brain regions, it was especially abundant in the hippocampal region and the dentate gyrus (Fig. 3c). However, there was no obvious changes in $cPLA_2$ mRNA depression in the penumbral area. Expression of COX-2 mRNA seemed to be particularly intense in the hippocampal CA3 area and in the dentate gyrus (Fig. 3d). At 3 days after 60 min of ischemia, an increase in COX-2 mRNA expression was observed in the piriform cortex and in the penumbral area (Fig. 3d).

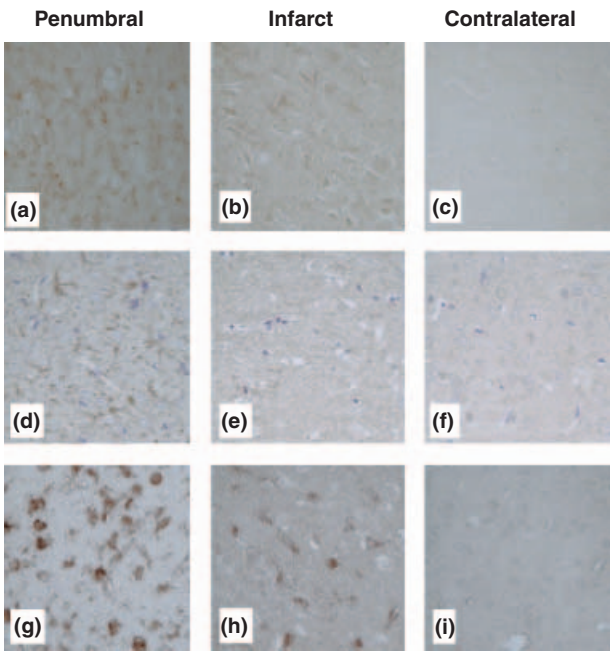

Fig. 5 (a–c) Immunohistochemical staining of $sPLA_2$-IIA in the penumbral, infarct and contralateral cortex regions of rat coronal sections at 3 days after 60 min of ischemia. (d–f) Immunostaining of GFAP (astrocytes) followed by post-staining with cresyl violet (neurons). (g–i) Isolectin B4-positive microglial cells. (Original magnification × 400).

Histochemistry and immunohistochemistry of neurons, glia and $sPLA_2$-IIA in infarct area, penumbral area and contralateral cortex of rat brain sections

Cresyl violet was used to detect neurons in different brain regions at 3 days after the 60-min ischemic insult. As shown in Fig. 4(a), neurons in the contralateral cortex were mostly round with small nuclei. In the penumbral area (Fig. 4b), there was a mixture of round and angular neurons, whereas there were almost no round neurons in the infarct area (Fig. 4c).

A polyclonal antibody (Santa Cruz sc-14472) was used to examine the immunoreactivity of $sPLA_2$-IIA in brain sections obtained at 3 days after 60 min ischemia–reperfusion. Parallel brain sections were immunostained with GFAP and isolectin B4 to detect astrocytes and microglial cells in the penumbral, infarct and contralateral areas. $sPLA_2$-IIA immunoreactivity was found mainly in the penumbral area and only background activity was present in the contralateral cortex (Figs 5a–c). This expression profile was similar to that seen in sections immunostained for GFAP-positive astrocytes (Figs 5d–f). On the other hand, isolectin B4-positive microglial cells were found in both the penumbral area and the infarct area, but not in the contralateral cortex (Figs 5g–i).

Although early expression of $sPLA_2$-IIA mRNA was noted in the ipsilateral cortex at 30 min after the 60-min ischemic

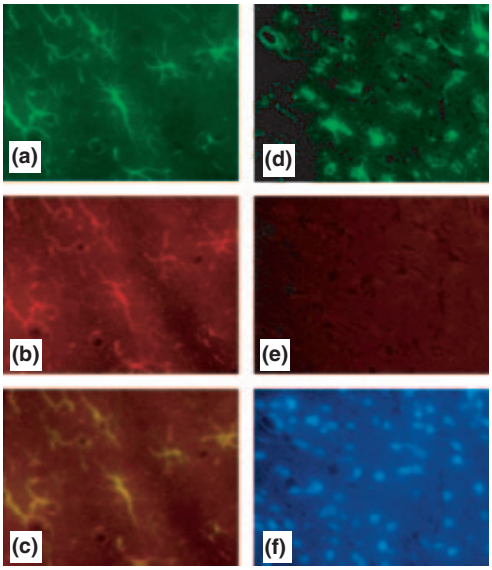

Fig. 6 Confocal microscopic images documenting the localization of $sPLA_2$-IIA immunoreactivity in GFAP-positive astrocytes but not in isolectin B4-positive microglial cells in parietal cortex 3 days after ischemia for 60 min. High-power photomicrographs showing GFAP-astrocytes (a; green) and $sPLA_2$-IIA (b; red) co-localized (c; yellow) in the penumbral area. Isolectin B4-positive microglial cells are present in the infarct area (d) but these cells do not show positive immunoreactivity for $sPLA_2$-IIA (e). DAPI-stained brain sections (f) indicate numerous degenerating nuclei in the infarct area. (Original magnification: a–c, ×1000; d–f, ×400).

insult, attempts to immunostain brain sections with $sPLA_2$-IIA antibody at 1 and 4 h after ischemia failed to detect positive cells in these brain sections (data not shown). An increase in immunoreactivity could be observed after 1 day although our experiment used the 3-day samples (data not shown).

Confocal microscopy was used to examine brain sections after double-immunostaining with $sPLA_2$-IIA and GFAP antibodies. This demonstrated localization of $sPLA_2$-IIA immunoreactivity in GFAP-positive astrocytes in the penumbral area (Figs 6a–c). On the other hand, despite clear demonstration of isolectin B4-positive microglial cells in the infarct area, these cells failed to show $sPLA_2$-IIA immunoreactivity (Figs 6d and e). DAPI staining revealed degenerating nuclei in the infarct area (Fig. 6f).

Discussion

Our study provided new information with regard to the increased response of $sPLA_2$-IIA mRNA and immunoreactivity owing to injury resulting from focal transient cerebral ischemia–reperfusion in the rat brain. After occlusion of the MCA for 60 min, RT–PCR and northern blot analysis indicated a biphasic increase in $sPLA_2$-IIA mRNA expression, first in the ischemic right MCA cortex at 30 min and then in the penumbral area between 12 h and 14 days. The increase in $sPLA_2$-IIA appeared to peak at 3 days and subsequently decrease although the physiological significance of the decrease to near-normal levels in 7 days followed by a small increase at 14 days is not known. The late phase increase in $sPLA_2$-IIA mRNA seemed to correlate well with the increase in GFAP mRNA in the penumbral area, suggesting a link between $sPLA_2$-IIA and reactive astrocytes. An immunohistochemical study using goat anti-mouse $sPLA_2$ polyclonal antibodies revealed positive immunoreactivity of $sPLA_2$-IIA in astrocytes in the penumbral area.

It is well recognized that reactive astrogliosis, as demonstrated by an increase in GFAP mRNA or protein expression, is an important marker of both global and focal cerebral ischemia (Cheung *et al*. 1999; Wang *et al*. 2002). Besides GFAP, reactive astrogliosis is also accompanied by an increase in mRNA expression of other cytoskeletal proteins, such as β-actin, suggesting considerable cytoskeletal rearrangement (Zhang *et al*. 1995). Reactive gliosis (astrocytes and microglial cells) is regarded an important process contributing to the inflammatory response in cerebral ischemia–reperfusion (Iadecola and Alexander 2001). Studies with cultured astrocytes have demonstrated the ability of astrocytes to respond to pro-inflammatory cytokines in the induction of genes including $sPLA_2$, COX-2 and inducible nitric oxide synthase (Oka and Arita 1991; Tong *et al*. 1995; Li *et al*. 1999; Morioka *et al*. 2002; Xu *et al*. 2003). Thus, the increase in $sPLA_2$-IIA and immunoreactivity observed in the penumbral area after ischemia is in agreement with the known features of astrogliosis.

The observation of an initial phase (30 min after occlusion of the MCA for 60 min) of increase in $sPLA_2$-IIA mRNA but not immunoreactivity in the ischemic cortex is intriguing. Previously, Lauritzen *et al*. (1994) reported a biphasic increase in $sPLA_2$-IIA mRNA in a global cerebral ischemia model, although the first phase was noted between 1 and 6 h and the second phase after 7 days. Because the early re-oxygenated period is marked by increased energy supply and cellular activities, including increase expression of immediate early genes (An *et al*. 1993; Lin *et al*. 1997), it is possible that the increase in $sPLA_2$-IIA mRNA observed during this period represents a response of astrocytes to the re-oxygenation process (Sun and Hsu 1996). However, with increasing time after ischemia–reperfusion, protein synthesis in neurons and astrocytes in the ischemic cortex may be halted owing to a second phase of depletion of energy and the onset of cell death pathways (Chen *et al*. 1993; Zhang *et al*. 1995; Sun and Hsu 1996).

Extensive infiltration of microglial cells clustering around the border of the infarct area was observed. The association of microglial cells with dying neurons (and possibly astrocytes) in the infarct area is in agreement with the scavenging role of microglial cells for removal of cellular

debris. Activation of microglial cells is known to play a role in the immune response associated with a number of neurodegenerative diseases (Aloisi 2001). Surprisingly, our study failed to detect $sPLA_2$-IIA immunoreactivity in microglial cells. Because multiple subtypes of PLA_2 are present in brain (Molloy *et al*. 1998; Valentin and Lambeau 2000; Balboa *et al*. 2002), it is possible that microglial cells in rat harbor other $sPLA_2$ subtypes, e.g. $sPLA_2$ IIC and V, that do not cross-react with the $sPLA_2$-IIA antibodies. The presence of these other $sPLA_2$ subtypes in astrocytes and/or microglial cells may explain the discrepancy between measurement of $sPLA_2$ activity (using specific inhibitors) and immunohistochemical findings (Yagami *et al*. 2002). Studies to measure activity of PLA_2 subtypes in brain tissue are often limited by the specificity of inhibitors (Yang *et al*. 1999). Future studies using molecular tools are needed to better define the involvement of $sPLA_2$ subtypes in glial cell activation.

The rapid release of free fatty acids in response to cerebral ischemia has been well demonstrated by Bazan (1970). Our earlier study indicated a time-dependent increase in free fatty acids during the 60-min MCA ligation, and a second phase of free fatty acid increase at 16 and 24 h after reperfusion (Zhang and Sun 1995). Because $cPLA_2$ activation is intimately linked to receptor-mediated signaling pathways (Sun *et al*. 2004), it is possible that the increase in free fatty acid release during the early phase of ischemia is the result of $cPLA_2$ activation in both neurons and glial cells. Excessive release of ATP may stimulate $cPLA_2$ in astrocytes through up-regulation of signaling pathways mediated by P2Y purinergic receptors (Xu *et al*. 2002). There is also evidence for an increase in $cPLA_2$ mRNA expression in brain in response to global cerebral ischemia (Owada *et al*. 1994; Clemens *et al*. 1996; Stephenson *et al*. 1999). Kishimoto *et al*. (1999) reported high levels of $cPLA_2$ mRNA expression associated with neurons in the rat hippocampus. Although a similar pattern of $cPLA_2$ mRNA distribution was found in our study, we failed to detect obvious changes in the penumbral region at 3 days after 60 min of MCA occlusion. The transient focal cerebral ischemia resulting from MCA occlusion represents a rather specific model of insult to the cortex, and the present results provide evidence for clear differences in $cPLA_2$ mRNA response depending on the model of cerebral ischemia and nature of cellular damage in the brain.

As COX-2 is downstream of the PLA_2 pathways and the PLA_2–COX cascade is important in mediating production of prostanoids that elicit physiological responses associated with cerebral ischemic damage, it seemed important to examine COX expression in brain after MCA occlusion. PLA_2 and COX-2 inhibitors have been successful in ameliorating injury due to ischemia–reperfusion (Pilitsis *et al*. 2002; Yagami *et al*. 2002). In this study, *in situ* hybridization using brain samples obtained 3 days after 60 min MCA occlusion demonstrated constitutive expression of COX-2 mRNA in the hippocampal region, especially in the dentate gyrus. Similar to the $cPLA_2$ mRNA pattern, COX-2 mRNA in the hippocampal region was not altered as a result of MCA occlusion. On the other hand, in agreement with previous reports (Collaco-Moraes *et al*. 1996; Koistinaho *et al*. 1999), there was an increase in COX-2 mRNA expression in the penumbral area, albeit not as robust as that for GFAP mRNA. The present results agree with those observed in cultured astrocytes, showing that pro-inflammatory cytokines could cause the induction of COX-2 and $sPLA_2$, and that both events are important for the production of prostaglandins (Xu *et al*. 2003).

Demonstration of an increase in $sPLA_2$-IIA expression in reactive astrocytes is an initial step in the determination of the physiological role of this group of enzymes in ischemic injury. Previously, $sPLA_2$ was found in synaptic components and released together with excitation (Matsuzawa *et al*. 1996). $sPLA_2$ purified from taipan venom was shown to bind neural membranes with high affinity and elicit neurotoxicity (Lambeau *et al*. 1989). More recently, human $sPLA_2$-IIA was also shown to pontentiate Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels in neurons (Yagami *et al*. 2003). Indeed, there is strong evidence for $sPLA_2$-enhanced glutamate signaling leading to neuronal cell death (Kolko *et al*. 1996, 1999; DeCoster *et al*. 2002; Rodriguez de Turco *et al*. 2002). Consequently, it is possible that release of $sPLA_2$-IIA from reactive astrocytes during ischemia–reperfusion may constitute a source of injury to neurons. This notion is supported by the ability of indoxam, a specific $sPLA_2$ inhibitor, to ameliorate apoptotic neuronal cell death (Yagami *et al*. 2002).

Although an increase in PLA_2 expression and activity has been implicated in a number of neurodegenerative diseases (Sun *et al*. 2004), more studies are needed to identify the PLA_2 subtypes and their cellular involvement in these disease processes. Information on the cellular origin of $sPLA_2$-IIA in brain and mechanisms leading to its response to ischemic injury will be an important step towards development of new therapeutic approaches to ameliorating neuronal damage in neurodegenerative disease.

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