

Human Group IIA Secretory Phospholipase A₂ Induces Neuronal Cell Death via Apoptosis

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ABSTRACT

Expression of group IIA secretory phospholipase A₂ (sPLA₂-IIA) is documented in the cerebral cortex (CTX) after ischemia, suggesting that sPLA₂-IIA is associated with neurodegeneration. However, how sPLA₂-IIA is involved in the neurodegeneration remains obscure. To clarify the pathologic role of sPLA₂-IIA, we examined its neurotoxicity in rats that had the middle cerebral artery occluded and in primary cultures of cortical neurons. After occlusion, sPLA₂ activity was increased in the CTX. An sPLA₂ inhibitor, indoxam, significantly ameliorated not only the elevated activity of the sPLA₂ but also the neurodegeneration in the CTX. The neuroprotective effect of indoxam was observed even when it was administered after occlusion. In primary cultures, sPLA₂-IIA caused marked neuronal cell death. Morphologic and ultrastructural characteristics of neuronal cell death by sPLA₂-IIA were apoptotic, as evi-

denced by condensed chromatin and fragmented DNA. Before apoptosis, sPLA₂-IIA liberated arachidonic acid (AA) and generated prostaglandin D₂ (PGD₂), an AA metabolite, from neurons. Indoxam significantly suppressed not only AA release, but also PGD₂ generation. Indoxam prevented neurons from sPLA₂-IIA-induced neuronal cell death. The neuroprotective effect of indoxam was observed even when it was administered after sPLA₂-IIA treatment. Furthermore, a cyclooxygenase-2 inhibitor significantly prevented neurons from sPLA₂-IIA-induced PGD₂ generation and neuronal cell death. In conclusion, sPLA₂-IIA induces neuronal cell death via apoptosis, which might be associated with AA metabolites, especially PGD₂. Furthermore, sPLA₂ contributes to neurodegeneration in the ischemic brain, highlighting the therapeutic potential of sPLA₂-IIA inhibitors for stroke.

Stroke is caused by a critical alteration of blood flow to a region of the brain. An acute obstruction of an artery results in ischemia (i.e., insufficient blood flow to the tissue) (Schehr, 1996). The ischemic brain suffers a mismatch between its cellular energy demands and the ability of the vascular system to supply substrate, most importantly oxygen. Subsequently, neurologic malfunctions and neuronal cell death are caused by increased intracellular calcium, excessive extracellular glutamate, free radicals, and inflammation. At the beginning of the stroke, there is a definite gradation of injury, a central area or core, with low blood flow already showing signs of massive cell death and an outer area, the penumbra, that is still alive but will malfunction after several days. A rat with the middle cerebral artery (MCA) occluded has been established as an animal model for stroke (Umemura et al., 1995). MCA occlusion causes irreversible necrosis and infarc-

tion in the core (Hallenbeck, 1994). On the other hand, cell death is induced not only via necrosis but also via apoptosis, and cells remain viable for several hours in the penumbra (Li et al., 1995). Therefore, interventions designed to terminate the reversible proapoptotic state are expected to reduce the ischemic damage and lead to successful treatment of stroke.

Several inflammatory factors, including arachidonic acid (AA) and eicosanoids such as prostaglandin D₂ (PGD₂), are increased in the MCA-occluded rat brain (Gaudet et al., 1980). AA is liberated from cell membrane lipids by phospholipase A₂ (PLA₂), and PGs are metabolized from AA by cyclooxygenase (COX). PLA₂s are classified as secreted forms (sPLA₂), Ca²⁺-dependent forms, and Ca²⁺-independent forms. Human sPLA₂s are 14-kDa calcium-dependent enzymes and are classified into nine distinct types (Gelb et al., 2000). Among them, group IIA secretory PLA₂ (sPLA₂-IIA)

ABBREVIATIONS: MCA, middle cerebral artery; AA, arachidonic acid; PG, prostaglandin; PLA₂, phospholipase A₂; COX, cyclooxygenase; CTX, cerebral cortex; sPLA₂, secretory phospholipase A₂; sPLA₂-IIA, group IIA secretory phospholipase A₂; LPA, lysophosphatidic acid; OS₂, *Oxyuranus scutellatus* venom; TTC, triphenyltetrazolium; *p*-BPB, *p*-bromophenacyl bromide; MAP2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL, interleukin; TNF- α , tumor necrosis factor- α ; IFN, interferon; M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; LT, leukotriene; PIT, photochemically induced thrombosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; RIA, radioimmunoassay; ANOVA, analysis of variance.

gene expression is induced in the ischemic brain (Lauritzen et al., 1994). It is strictly localized to the hippocampus and the cerebral cortex (CTX), in which neurons are vulnerable to ischemia (Smith et al., 1984). A PLA₂ inhibitor reduces the infarct size in MCA-occluded rats (Estevez and Phillis, 1997). Thus, sPLA₂-IIA seems to play an important role in neurodegeneration after ischemia.

sPLA₂-IIA is stimulated upon degradation of sphingomyelin and produces lysophosphatidic acid (LPA) (Fourcade et al., 1995). Moreover, the enzyme releases PGD₂ from IgE-sensitized rat mast cells (Murakami et al., 1991). The perturbed membrane of cells undergoing apoptosis is also susceptible to the liberation of AA by sPLA₂-IIA (Atsumi et al., 1997). sPLA₂-IIA causes cell death only in the presence of phosphatidylethanolamine (Vadas, 1997). Although sPLA₂ purified from *Oxyuranus scutellatus* (taipan) venom (OS₂) is responsible for neurotoxicity (Lambeau et al., 1989), it has not yet been clarified whether mammalian sPLA₂-IIA is involved in neuronal cell death. There are two approaches to examining the effects of sPLA₂-IIA. One is transfection of target cells with sPLA₂-IIA; another is exogenous addition of sPLA₂-IIA to target cells. In the present study, we evaluated the toxicity of sPLA₂-IIA on neurons by the latter approach, because neurons are differentiated cells and cannot be transfected. We provide the first evidence that mammalian sPLA₂-IIA causes apoptosis in cortical neurons and might be associated with neurodegeneration in the ischemic brain.

Experimental Procedures

Materials

Indoxam and its precursor, a methyl ester of indoxam, were synthesized as reported previously (Hagishita et al., 1996). NS-398 was synthesized at our laboratories by a method reported previously (Inagaki et al., 2000). Recombinant human sPLA₂-IIA was provided by Lilly Research Laboratories (Indianapolis, IN) and rat sPLA₂-IIA was purified from rat spleens as reported by Ono et al. (1988). Rose bengal, triphenyltetrazolium chloride (TTC), *p*-bromophenacyl bromide (*p*-BPB), arabinosylcytosine C, poly(L-lysine), antimicrotubule-associated protein 2 (anti-MAP2), and anti-gial fibrillary acidic protein (anti-GFAP) were obtained from Sigma (St. Louis, MO). Anti-microglial antigen (OX-42) was purchased from BMA Biomedicals AG (Augst, Switzerland). An avidin-biotin complex with peroxidase kit was purchased from Vector Laboratories (Burlingame, CA). Dulbecco's modified Eagle's medium (DMEM), Leibovitz's L-15 medium, RPMI 1640 medium, trypsin, deoxyribonuclease I, fetal bovine serum (FBS), horse serum, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Interleukin (IL)-1 β , IL-2, IL-6, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , macrophage colony-stimulating factor (M-CSF), and granulocyte-M-CSF (GM-CSF) were obtained from Genzyme (Cambridge, MA). PGD₂, PGE₂, 9 α -11 β -PGF₂, PGL₂, Δ ¹²-PGJ₂, and leukotriene B₄ (LTB₄) were purchased from Cascade Biochem Ltd. (Berkshire, UK). U-46619, a stable agonist for thromboxane A₂ receptor, was synthesized in our laboratory (Arimura et al., 1998). An [³H]AA and PGD₂ [³H] assay system was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK).

Photochemically Induced Thrombotic Middle Cerebral Artery Occlusion in Rats

Wistar male rats weighing 240 to 260 g were used. The body temperature of the animals was maintained at 37.5°C with a heating pad during operation. Anesthesia was induced with 3% halothane in 30% oxygen and maintained with 1 to 1.5% halothane in 30% oxygen.

Occlusion of the photochemically induced thrombotic (PIT) MCA was performed according to the method of Umemura et al. (1995). A catheter for administration of rose bengal was placed in the femoral vein. A subtemporal craniotomy was performed with a dental drill under an operating microscope to open a 3-mm circular bone window, through which photoirradiation with green light (wavelength, 540 nm) was achieved with a xenon lamp. The head of an optic fiber with a 3-mm diameter was placed on the window in the skull base, and rose bengal (20 mg/kg) was injected intravenously. Photoirradiation of the main trunk of the left MCA was performed for 10 min. The incisions were closed after confirmation of thrombotic occlusion. Twenty-four hours after the completion of the irradiation, the cerebrum was removed under pentobarbital (50 mg/kg, i.p.) anesthesia. The cerebrum was coronally sectioned at 1-mm thickness from the frontal lobe with a microslicer, and then consecutive slices were stained with TTC. Photographs of the slices were taken. The infarction volumes of cerebral cortex and striatum were determined by integration of the surfaces of sections and the distances between them.

Measurements of sPLA₂-IIA Activity of Brain Homogenates after PIT-MCA Occlusion

Animals were anesthetized with pentobarbital (50 mg/kg i.p.) at fixed intervals after the thrombotic MCA occlusion. The brain was removed, and coronary sections were made at the level of the optic chiasm and the caudal edge of the mamillary body by a surgical blade and then divided into left and right portions by a midline incision. Each portion of the brain was further dissected out to the cerebral cortex and the striatum. Four portions of the brain (right cortex, right striatum, left cortex, and left striatum) were weighed and homogenized with three times the weight of 7.7 mM EDTA containing 1.5 μ g/ml prostaglandin E₁. PLA₂ activity in the supernatant of the brain homogenates was measured in the presence or absence of 1 μ M indoxam with a phosphatidyl glycerol/sodium cholate mixed micelle assay (Tojo et al., 1993).

Tissue Cultures

Neuronal cell cultures were prepared from the cerebral cortices of day-19 Sprague-Dawley rat embryos as reported previously (Ueda et al., 1997). Cerebral cortices were dissociated in isotonic buffer with 4 mg/ml trypsin and 0.4 mg/ml deoxyribonuclease I. Cells were plated at a density of 2.5×10^5 cells/cm² on poly(L-lysine)-coated dishes in conditioning medium, Leibovitz's L-15 medium supplemented with 5% FBS and 5% horse serum at 37°C in 5% CO₂/9% O₂/86% N₂. On day 1 after plating, cultures were treated with 0.1 μ M arabinosylcytosine C. On day 4, cortical cultures were immunostained with anti-MAP2 specific for neurons (titer, 1:500), anti-GFAP for astrocytes (titer, 1:100), and anti-microglial antigen (titer, 1:100). Immunostained neurons, astrocytes, and microglial cells were detected with an avidin-biotin complex with peroxidase kit. The present cultures contained neurons at least 95%.

Other non-neuronal cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO₂. Human astrocytes were obtained from Clonetics (San Diego, CA) and were cultured at a density of 3.5×10^3 cells/cm² on 48-well plates in CCMD 190 medium (Clonetics, San Diego, CA) supplemented with 5% FBS, 50 μ g/ml gentamicin, 50 ng/ml amphotericin, 20 ng/ml human epidermal growth factor, 25 μ g/ml insulin, 25 ng/ml progesterone, and 50 μ g/ml transferrin. Rat astrocytes were prepared from cortical cultures of day-19 Sprague-Dawley rat embryos as reported (Lazarini et al., 1996). Astrocytes were cultured at a density of 3.5×10^3 cells/cm² on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Cardiac myocytes were prepared from day-1 or -2 Sprague-Dawley rats as described previously (Hayasaka et al., 1996). Myocytes were cultured at a density of 2×10^5 cells/cm² on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Human

hepatocytes were obtained from the Applied Cell Biology Research Institute (Kirkland, WA) and were cultured at a density of 5×10^4 cells/cm² on 48-well plates in CS-C medium (Applied Cell Biology Research Institute) supplemented with 10% FBS. Human mesangial cells were obtained from Clonetics and were cultured at a density of 3.5×10^3 cells/cm² on 48-well plates in CCMD 180 medium (Clonetics) supplemented with 5% FBS, 50 μ g/ml gentamicin, and 50 ng/ml amphotericin. Rat mesangial cells were prepared from 4-week-old Slc-Wistar rats (Japan SLC Inc, Shizuoka, Japan) as described previously (Okuda et al., 1986). Mesangial cells were cultured at a density of 3.5×10^3 cells/cm² on 48-well plates in RPMI medium supplemented with 20% FBS, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Human bronchial smooth-muscle cells were obtained from Clonetics and were cultured at a density of 3.5×10^3 cells/cm² on 48-well plates in Molecular, Developmental, and Cellular Biology medium supplemented with 5% FBS, 50 μ g/ml gentamicin, 50 ng/ml amphotericin, 0.5 ng/ml human epidermal growth factor, 5 μ g/ml insulin, and 2 ng/ml human fibroblast growth factor. Porcine basilar arterial smooth muscle cells were purchased from Takara (Shiga, Japan). Smooth-muscle cells were cultured at a density of 3.5×10^3 cells/cm² on 48-well plates in DMEM supplemented with 10% FBS, 10 mM HEPES, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Human brain microvascular endothelial cells were obtained from Applied Cell Biology Research Institute and were cultured at a density of 5×10^4 cells/cm² on 48-well plates in CS-C medium. Human aorta endothelial cells were obtained from Clonetics and were cultured at a density of 5×10^4 cells/cm² on 48-well plates in modified Czapek Dox broth medium supplemented with 2% FBS, 50 μ g/ml gentamicin, 50 ng/ml amphotericin, 10 ng/ml human epidermal growth factor, 12 mg/ml bovine brain extract, and 1 ng/ml hydrocortisone. Human lung fibroblasts were obtained from Clonetics and were cultured at a density of 5×10^4 cells/cm² on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Human dermal fibroblasts were obtained from Clonetics and were cultured at a density of 5×10^4 cells/cm² on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μ g/ml streptomycin.

Analysis of Neuronal Survival

Neurons (2.5×10^5 cells/cm²) were treated with sPLA₂-IIA in the presence or absence of indoxam at 37°C. Two different methods were employed for assessment of neurotoxicity of sPLA₂-IIA, as previously reported (Ueda et al., 1996). First, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) reduction assay reflecting mitochondrial succinate dehydrogenase activity was employed. Second, residual cells were counted according to morphologic criteria; neurons with intact neurites and a smooth, round soma were considered viable, whereas those with degenerated neurites and an irregular soma were considered nonviable.

Fluoromicroscopic Analysis

Assessment of condensation of chromatin was performed as described previously (Ueda et al., 1996). Neurons (2.5×10^5 cells/cm²) were treated with 1 μ M sPLA₂-IIA in the presence or absence of 10 μ M indoxam at 37°C for 48 h. The culture medium was exchanged with PBS containing 10 mM Hoechst 33258 fluorescent dye. Cells were incubated for 10 min at 37°C in the dark and washed with PBS. Stained nuclei were categorized as follows: 1) nuclei with homogeneously stained chromatin; 2) nuclei with condensed chromatin and with crescent-shaped areas of condensed chromatin often located near the periphery of the nucleus; and 3) fragmented nuclei, more than two condensed micronuclei within the area of a neuron.

In Situ Labeling of Nuclear DNA Fragments

Neurons (2.5×10^5 cells/cm²) were treated with 1 μ M sPLA₂-IIA in the presence or absence of 10 μ M indoxam at 37°C for 48 h. Cortical cell cultures were stained by the terminal deoxynucleotidyl

transferase-mediated dUTP nick-end labeling (TUNEL) technique (Garrieli et al., 1997). Apoptotic cells could be distinguished morphologically from necrotic cells by the presence of condensed brown nuclei.

Transmission Electron Microscopy

Neurons (2.5×10^5 cells/cm²) were treated with 1 μ M sPLA₂-IIA in the presence or absence of 10 μ M indoxam at 37°C for 24 h. Cells were fixed in situ with 2.5% glutaraldehyde in phosphate buffer for 2 h at 4°C and postfixed with 1% osmium tetroxide at 4°C. To increase contrast, cells were double-fixed with saturated thiocarbonylhydrazide-osmium. Samples were dehydrated in a graded series of ethanols and embedded in Epon 812. Ultrathin sections cut on a Reichert ultramicrotome were stained with uranyl acetate and lead citrate and were examined with a JEM 1200EX electron microscope (JEOL, Tokyo, Japan).

Measurement of Released [³H]AA and Generated PGD₂

[³H]AA. Neurons (2.5×10^5 cells/cm²) and myocytes (2.5×10^5 cells/cm²) were incubated for 24 h in culture medium containing 1 μ Ci/ml [³H]AA, washed twice with culture medium, and treated with sPLA₂-IIA in the presence or absence of 10 μ M indoxam at 37°C. The radioactivity of [³H]AA into each supernatant was measured.

PGD₂. Neurons (2.5×10^5 cells/cm²) were treated with sPLA₂-IIA in the presence or absence of 10 μ M indoxam at 37°C. At the times indicated in Figs. 9 to 11, PGD₂ was extracted according to the method described in a previous report (Powell, 1980). Supernatants of culture medium (1 ml) were mixed homogeneously with cold ethanol (4 ml). The mixture was centrifuged at 1500g at 4°C for 10 min for removal of the particulate matter. Supernatants were diluted with an appropriate volume of distilled water to yield a final concentration of 10% ethanol, and the pH was adjusted to 3.5 to 4.0. Samples were loaded onto reversed-phase (C18) Sep-Pack cartridges, which had been prepared by washing with ethanol followed by distilled water. Samples were washed onto the Sep-Pak with 15 ml of 10% aqueous ethanol, followed by 15 ml of petroleum ether. Samples were extracted with 5 ml of methyl formate. The methyl formate effluents were pooled and evaporated with a heating module and dissolved in RIA buffer (50 mM phosphate buffer, pH 7.3, with 0.1% gelatin, and 0.1% azide). The samples were stored frozen until RIA analysis for PGD₂, PGE₂, and LTB₄. These eicosanoids were measured with their respective RIA kits (duplicate/sample). The cross-reactivity of PGJ₂ with the PGD₂ RIA kit is 7%, whereas that of other PGs, such as PGA₂, PGE₁, PGE₂, PGF_{1 α} , PGF_{2 α} , 6-keto PGE₁, 6-keto PGF_{1 α} , and TXB₂, is less than 1%.

Results

Alterations in sPLA₂-IIA Activity in Rat Brains after PIT-MCA Occlusion. The levels of free fatty acids, mainly AA, increased rapidly in the brain after ischemia (Gaudet et al., 1980). Indoxam, which contains an indolizine nucleus, is a specific and potent inhibitor of sPLA₂, acting on its catalytic site (Yokota et al., 1999). sPLA₂ activity was examined by use of homogenates of rat brains after PIT-MCA occlusion (Fig. 1A). The occlusion was performed on the left side. In the ipsilateral cortex (left side), PLA₂ activity was increased rapidly and reached a maximum within 8 h after ischemia. In the contralateral cortex (right side), little change in PLA₂ activity was observed under the same conditions. On the other hand, PLA₂ activity was about 2-fold higher in the ipsilateral cerebral cortex than in the contralateral one. In the striatum, there was little significant difference in sPLA₂ activity within 24 h after the cessation of the photoirradiation. Indoxam intensely suppressed the occlusion-induced increase in sPLA₂ activity, indicating that sPLA₂ activity

was increased significantly in the neurogenerated region of the ischemic brain.

Cerebral Infarction after PIT-MCA Occlusion. Figure 2 is a typical photograph of a cerebral infarct stained with TTC 24 h after occlusion of the MCA in rats treated with vehicle (A) or the precursor compound of indoxam (B). The prodrug is an orally bioavailable methyl ester of indoxam administered p.o. and metabolized to indoxam in the blood. In rats treated with vehicle, the left dorsolateral frontotemporal cortex and the dorsolateral portion of the striatum were consistently infarcted (Figs. 1B and 2A). On the other hand, the infarction in the left dorsolateral cortex was attenuated significantly in rats pretreated with the prodrug at 1 h before occlusion, whereas the prodrug had no effect on the infarction of the striatum (Figs. 1B and 2B). Furthermore, the infarc-

tion was also ameliorated by treatment with indoxam 2 h after occlusion (Fig. 1B). Treatment with the sPLA₂-IIA inhibitor, even after occlusion, significantly protected neurons from ischemia-induced cell death.

Effects of sPLA₂-IIA on Cell Survival. We examined the effect of sPLA₂-IIA on cell survival, including neurons. Primary cultures of dissociated cortical neurons were exposed to sPLA₂-IIA, and neuronal cell death was quantified 48 h later (Fig. 3A). There was a close correlation between MTT-reducing activity and morphologic criteria. sPLA₂-IIA at doses less than 0.1 μ M did not reduce neuronal cell survival. In the absence of other cofactors or inflammatory factors, increasing concentrations of sPLA₂-IIA above 0.1 μ M resulted in a significant increase in neuronal cell death in a dose-dependent manner, with a half-maximal concentration of 1.1 μ M. On the other hand, sPLA₂-IIA had no effect on the survival of astrocytes (Table 1). sPLA₂-IIA triggered neuronal cell death after 24 h and killed most neurons within 72 h

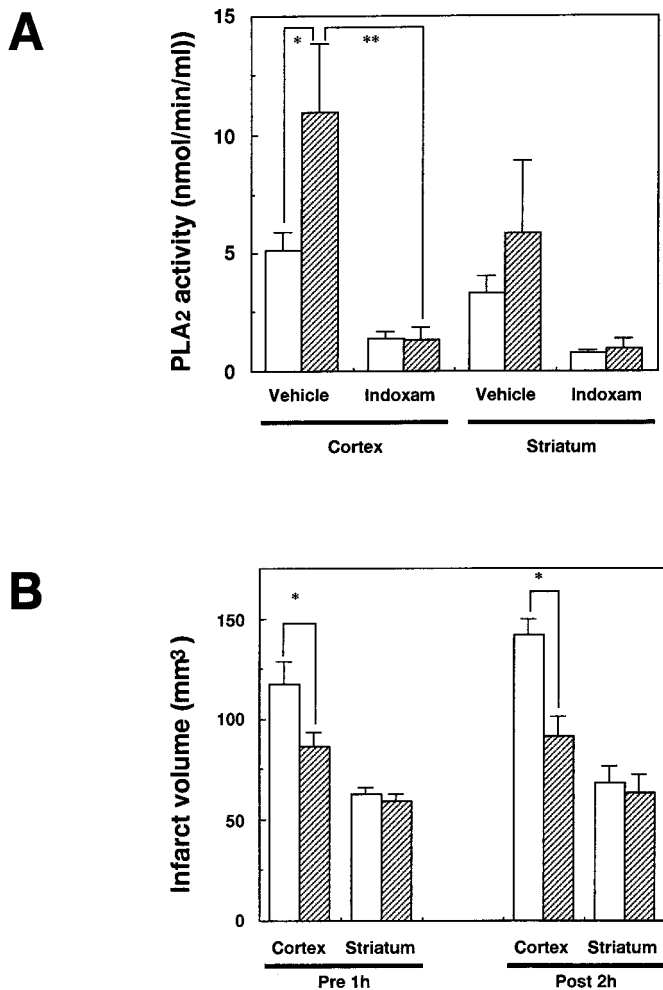


Fig. 1. Effect of sPLA₂-II inhibitor on sPLA₂ activity and infarct volume after PIT-MCA occlusion. Left MCA was occluded by PIT. A, sPLA₂ activity: homogenates of cerebral cortex and striatum were prepared for assay of sPLA₂ activity after MCA occlusion. sPLA₂ activity in the contralateral side (open bars) and the ipsilateral side (hatched bars) was measured in the absence or presence of 1 μ M indoxam with phosphatidyl glycerol/sodium cholate mixed micelle used as substrate. Data are expressed as means $\pm$ S.E.M. ($n = 5$). *, $P < 0.05$, compared with the right counterpart. **, $P < 0.01$, compared with vehicle-treated conditions by ANOVA followed by Dunnett's test. B, infarct volume: vehicle ($\square$) or methyl ester of indoxam (▨) was administered p.o. 1 h before or 2 h after MCA occlusion and then infused (0.5 mg/kg/h, i.v.) until 24 h after the MCA occlusion. Infarct volume in the ipsilateral side was measured. Data are expressed as means $\pm$ S.E.M. ($n = 6$). *, $P < 0.05$, compared with vehicle-treated conditions by ANOVA followed by Dunnett's test.

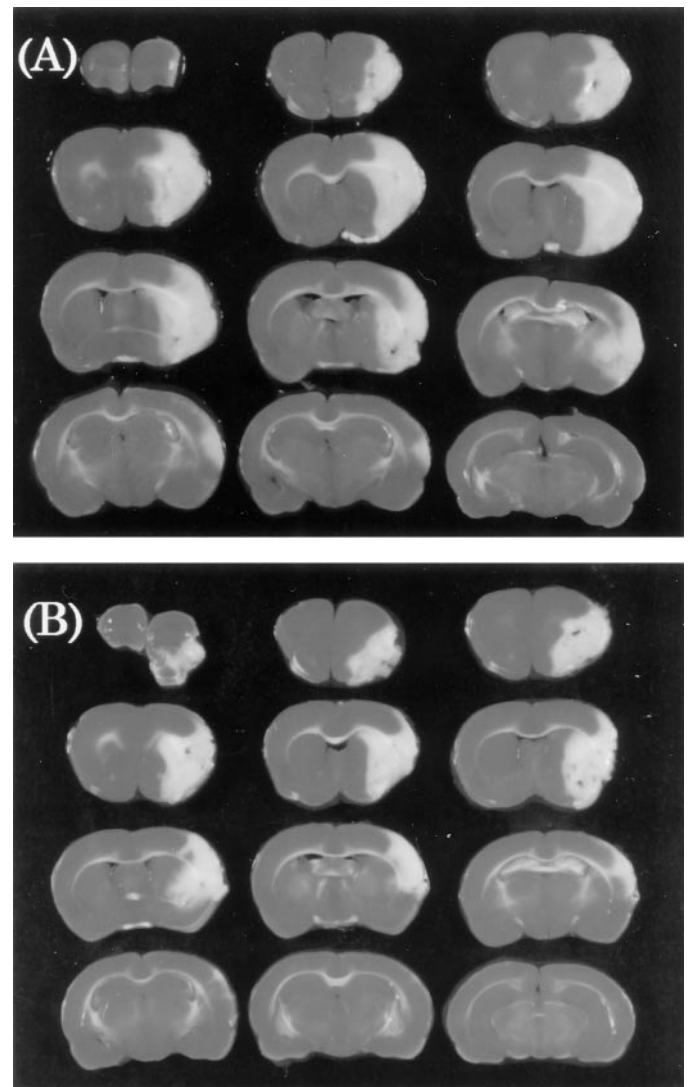


Fig. 2. Area of ischemic brain damage. Twenty-four hours after PIT-MCA, the cerebrum was coronary-sectioned in 1-mm-thick slices from 1.5 mm anterior to 4.5 mm posterior to the bregma; then nine consecutive slices were stained with triphenyltetrazolium chloride. A, the dorsolateral cortex and striatum were infarcted in the control group. B, indoxam was injected in bolus (3 mg/kg, i.v.) 5 min after the MCA occlusion and then infused (0.5 mg/kg/h, i.v.) until 24 h after the MCA occlusion.

(Fig. 3B). Thus, sPLA₂-IIA caused neuronal cell death, but only after a latent period.

Next, we examined the effects of sPLA₂-IIA on non-neuronal cell survival to ascertain whether sPLA₂-IIA induced the death of cells other than neurons (Table 1). sPLA₂-IIA caused complete neuronal cell death at 3 μ M within 48 h. However, it had no effect on the survival of astrocytes, myocytes, endothelial cells, or fibroblasts. On the other hand, it stimulated the growth of hepatocytes, mesangial cells, and smooth muscle cells. Thus, the toxicity of sPLA₂-IIA was specific for neurons among the various types of cells tested.

Effects of Inflammatory Factors on Neuronal Cell Survival. In the brain, various inflammatory cytokines, including IL-1 β , IL-2, IL-6, TNF- α , IFN- γ , M-CSF, and GM-CSF, are secreted by neurons, astrocytes, and microglial cells during inflammation. We evaluated the effects of these inflammatory cytokines on neuronal cell survival (Table 2). Rat

sPLA₂-IIA (ED₅₀ = 68 nM) induced neuronal cell death more effectively than did human sPLA₂-IIA (ED₅₀ = 1.1 μ M).

Next, we examined effects of other inflammatory cytokines expressed in the ischemic brain on neuronal cell survival. In *in vitro* applications (Certificate of Analysis; Genzyme), human IL-1 β , IL-2, IL-6, TNF- α , IFN- γ , M-CSF, and GM-CSF are active in the concentration ranges of 5 to 40 pg/ml, 1 to 100 ng/ml, 1 to 10 ng/ml, 0.1 to 10 ng/ml, 0.1 to 15 ng/ml, 125 to 250 ng/ml, and 10 to 20 ng/ml, respectively. At these optimal concentrations, these cytokines did not affect neuronal survival. Even at concentrations that were higher than optimal concentrations, they did not induce neuronal cell death (Table 2).

Effects of sPLA₂ Inhibitors on sPLA₂-IIA-Induced Neuronal Cell Death. To determine how the enzymatic activity of sPLA₂-IIA is involved in neuronal cell death, we examined the effects of sPLA₂ inhibitors on sPLA₂-IIA-induced neuronal cell death (Fig. 4). Indoxam prevented sPLA₂-IIA-induced neuronal cell death in a concentration-dependent manner (Fig. 4A). Similarly, *p*-BPB a putative irreversible sPLA₂ inhibitor, showed a concentration-dependent neuroprotective effect (Fig. 4A).

The delayed neuronal cell death induced by sPLA₂-IIA suggested that sPLA₂-IIA inhibitors might prevent the death of neurons after sPLA₂-IIA treatment. We examined the

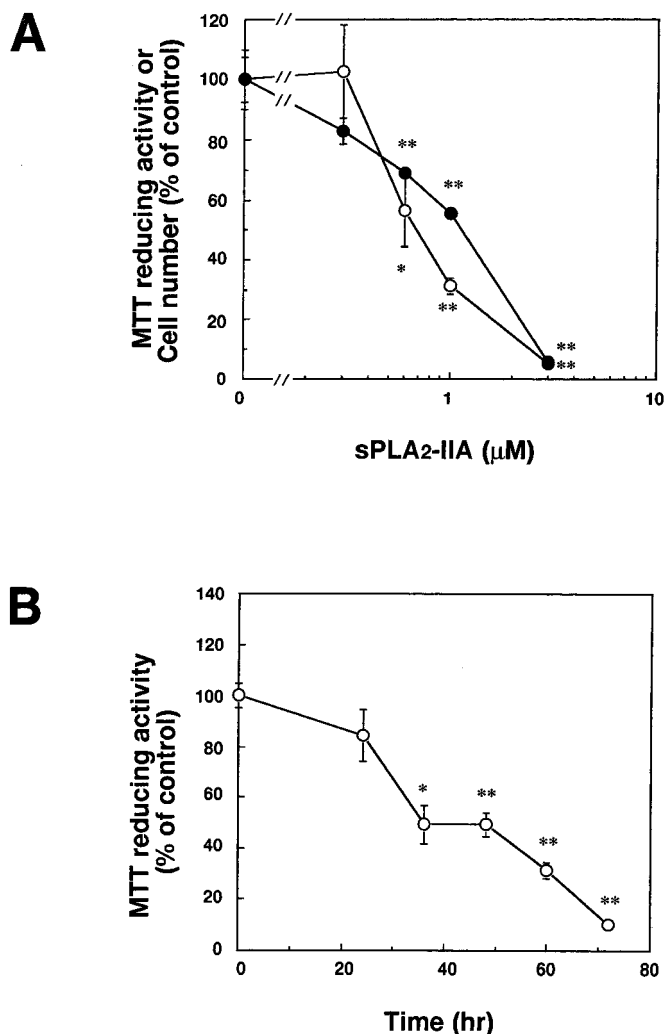


Fig. 3. Effect of sPLA₂-IIA on cell survival of cortical cultures. A, dose response: cortical cultures were treated with the indicated concentrations of sPLA₂-IIA. MTT reducing activity (○) and cell densities (●) were determined 48 h later. The cell density of control cultures was $2.7 \pm 0.2 \times 10^5$ cells/cm². B, time course: cortical cultures were treated with 1 μ M sPLA₂-IIA. MTT-reducing activity was determined at the indicated time points after sPLA₂-IIA treatment. Data are expressed as means $\pm$ S.E.M. ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with control by ANOVA followed by Dunnett's test.

TABLE 1

Effects of sPLA₂-IIA on the survival of various cells

Various cells were treated with 3 μ M sPLA₂-IIA. MTT-reducing activity was measured 48 h later. Data are expressed as means $\pm$ S.E.M. ($n = 4$). Comparisons with control were made by ANOVA followed by Dunnett's test.

Cell Type	Source	Species	MTT-Reducing Activity % of control
Neurons	Brain	Rat	5.97 $\pm$ 0.48**
Astrocytes	Brain	Human	103.22 $\pm$ 5.33
Astrocytes	Brain	Rat	98.73 $\pm$ 2.37
Myocytes	Heart	Rat	92.84 $\pm$ 8.17
Hepatocytes	Liver	Human	144.79 $\pm$ 8.08**
Mesangial cells	Kidney	Human	141.67 $\pm$ 2.30**
Mesangial cells	Kidney	Rat	150.23 $\pm$ 2.30**
Smooth muscle cells	Bronchia	Human	127.68 $\pm$ 5.14*
Smooth muscle cells	Arterial basilaris	Porcine	126.01 $\pm$ 3.82*
Endothelial cells	Brain	Human	94.44 $\pm$ 8.39
Endothelial cells	Aorta	Human	96.62 $\pm$ 5.26
Fibroblasts	Lung	Human	106.91 $\pm$ 3.24
Fibroblasts	Skin	Human	97.68 $\pm$ 4.06

* $P < 0.05$; ** $P < 0.01$.

TABLE 2

Effects of various cytokines on neuronal cell survival

Rat cortical neurons were treated with various cytokines. All cytokines except rat sPLA₂-IIA were human recombinants. MTT-reducing activity was determined 48 h later. Data are expressed as means $\pm$ S.E.M. ($n = 4$). Comparisons with control were made by ANOVA followed by Dunnett's test.

Cytokine	Concentration	MTT-Reducing Activity % of control
Control		100.0 $\pm$ 2.2
sPLA ₂ -IIA	1.0 μ M	55.4 $\pm$ 1.4**
sPLA ₂ -IIA (rat)	0.1 μ M	42.9 $\pm$ 5.3**
IL-1 β	100 ng/ml	102.0 $\pm$ 0.9
IL-2	100 ng/ml	102.0 $\pm$ 1.1
IL-6	10 ng/ml	100.8 $\pm$ 2.0
TNF- α	100 ng/ml	105.6 $\pm$ 2.1
IFN- γ	100 ng/ml	91.4 $\pm$ 11.4
M-CSF	100 ng/ml	89.6 $\pm$ 5.3
GM-CSF	100 ng/ml	105.9 $\pm$ 8.1

** $P < 0.01$.

post-treatment effects of indoxam on sPLA₂-IIA-induced neuronal cell death. As shown in Fig. 4B, indoxam suppressed neuronal cell death when applied within 10 h after PLA₂-IIA treatment. Treatment with the sPLA₂-IIA inhibitor after application of sPLA₂-IIA protected neurons from sPLA₂-IIA-induced cell death, similar to the cotreatment.

Light Microscopic Changes in Neurons by sPLA₂-IIA. The compositions of neurons and astrocytes in cortical cultures were determined by use of antibodies for MAP2,

GFAP, and microglial antigen, which are specific for neurons, astrocytes, and microglial cells, respectively. Approximately 95% of the cells were stained by anti-MAP2 antibody (Fig. 5A), whereas there were few anti-GFAP-positive cells (Fig. 5B) and anti-microglial antigen-positive cells (Fig. 5C). Examination of cultures treated with sPLA₂-IIA by light microscopy showed some disruption of neurites at 48 h. In control cultures, neurons had extended neurites and smooth, round cell bodies (Fig. 5D). On the other hand, some cell bodies shrank and lost their bright phase-contrast appearance at 36 h in sPLA₂-IIA-treated cultures. By 48 to 72 h, there were markedly fewer cells, and extensive debris was seen attached to the substratum (Fig. 5E). The morphologic disruption in sPLA₂-IIA-treated neurons was suppressed completely by indoxam (Fig. 5F).

Apoptotic Features of sPLA₂-IIA-Treated Neurons. We studied the condensation of chromatin, a characteristic feature of apoptosis, in neurons (Fig. 6). sPLA₂-IIA-treated neurons were stained with Hoechst 33258 fluorescent dye (Fig. 6A). In untreated cultures, cells showed little fluorescence in the nucleus. On the other hand, condensed and fragmented chromatin was clearly observed in sPLA₂-IIA-treated cultures. The amount of condensed chromatin in sPLA₂-IIA-treated neurons was decreased significantly by indoxam (Fig. 6A; Table 3).

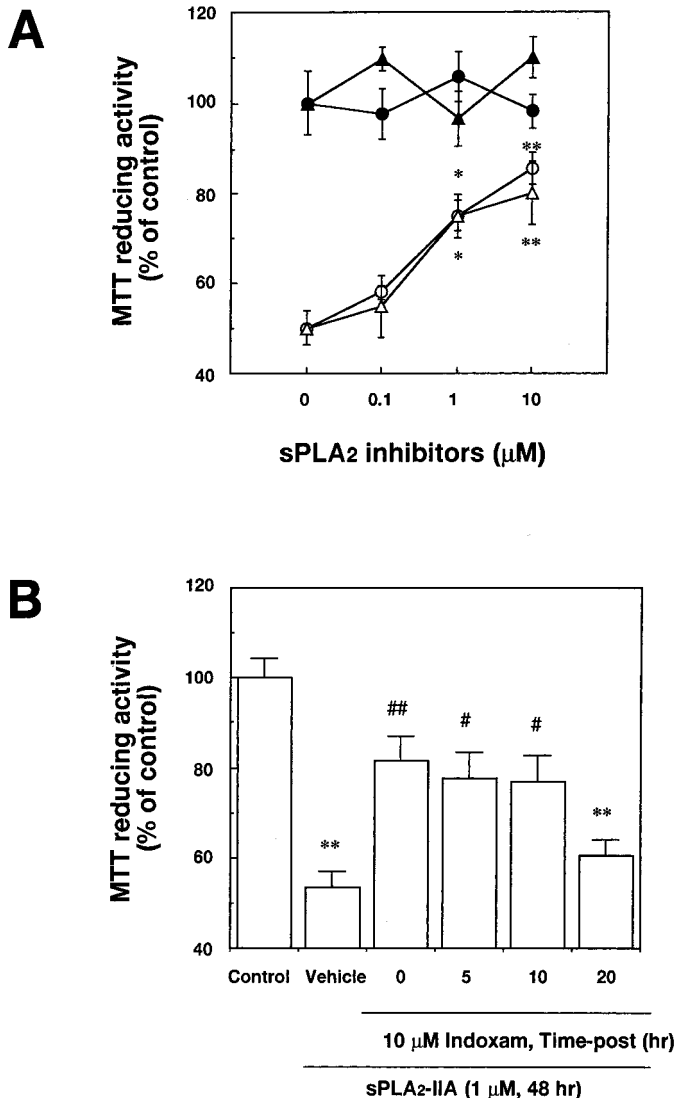


Fig. 4. Effects of PLA₂ inhibitors on sPLA₂-IIA-induced cell death of cortical cultures. A, dose response: *p*-BPB (triangles): vehicle (▲), or 100 μM sPLA₂-IIA (Δ) were preincubated with 0.01, 0.1, or 1 mM *p*-BPB for 20 min at 37°C. After preincubation, an aliquot was removed and diluted 100-fold in the culture medium (final concentration of sPLA₂-IIA = 1 μM). Indoxam (circles): cortical cultures were treated with vehicle (●) or 1 μM sPLA₂-IIA (○) in the presence of indoxam at the indicated concentrations. MTT-reducing activity was determined 48 h after sPLA₂-IIA treatment. Data are expressed as means ± S.E.M. (*n* = 4). *, *P* < 0.05; **, *P* < 0.01, compared with sPLA₂-IIA alone by ANOVA followed by Dunnett's test. B, treatment after application of sPLA₂-IIA: cortical cultures were treated with vehicle or 1 μM sPLA₂-IIA. Indoxam (10 μM) was applied at the indicated times after sPLA₂-IIA treatment. MTT-reducing activity was determined 48 h after sPLA₂-IIA treatment. Data are expressed as means ± S.E.M. (*n* = 4). **, *P* < 0.01, compared with control; #, *P* < 0.05; ##, *P* < 0.01, compared with vehicle by ANOVA followed by Dunnett's test.

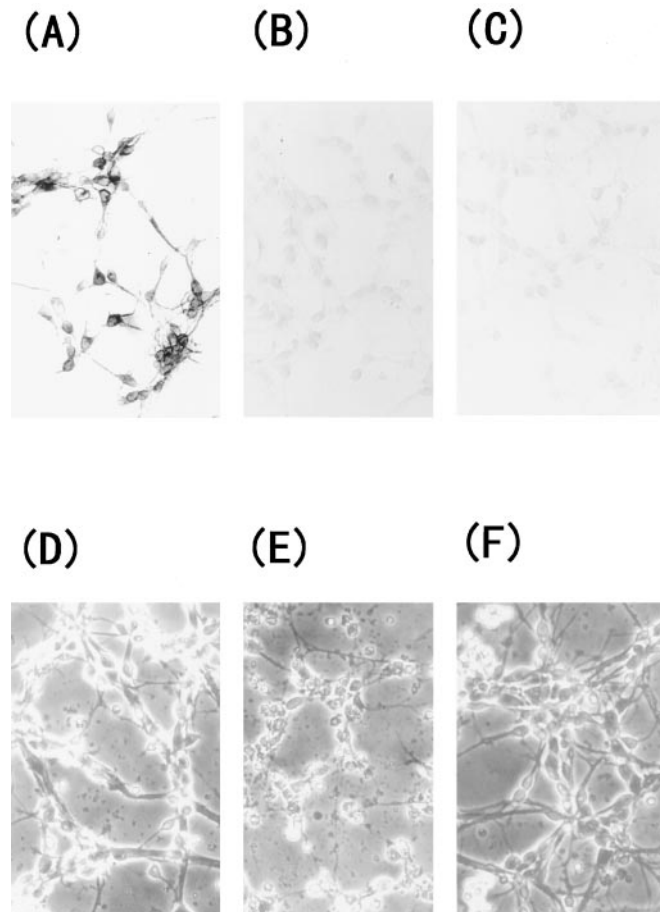


Fig. 5. Morphologic changes in cortical neurons by sPLA₂-IIA. Immunocytochemical analysis for anti-MAP2 (A), anti-GFAP (B), or anti-microglial antigen (C) was performed on cortical cultures. Cortical neurons were treated with control (D), 1 μM sPLA₂-IIA (E), or 1 μM sPLA₂-IIA + 10 μM indoxam (F). Neurons were examined by phase-contrast microscopy 48 h later. Magnification, 100×.

We also studied another apoptotic feature, fragmentation of DNA (Fig. 6B). With the TUNEL technique, it is possible to discriminate morphologically between the apoptotic nuclei by the presence of strand breaks in the DNA by labeling of the nicked ends of DNA. After neurons were incubated with or without sPLA₂-IIA for 24 h, the number of TUNEL-positive nuclei was increased in sPLA₂-IIA-treated neurons compared with untreated control rats. The number of TUNEL-

positive nuclei in sPLA₂-IIA-treated neurons was decreased significantly by indoxam (Fig. 6B; Table 3).

Ultrastructural Changes in Neuronal Cell Death. Investigation of sPLA₂-IIA-treated cultures by electron microscopy revealed neurons in different stages of cell death (Fig. 7). Although 98% of control neurons were healthy at 48 h (Fig. 7A), half of the sPLA₂-IIA-treated neurons showed characteristics of apoptotic death (Fig. 7, B and C). The neuronal size decreased progressively throughout the stages of cell death, as discussed below (Fig. 7, B and C). In the early stage of cell death, the plasma membrane became difficult to resolve, whereas features in the cytosol and the nucleus were unaltered. In the middle stage (Fig. 7B), microtubules, neurofilaments, and ribosomes appeared condensed as the neurons continued to shrink. Moreover, a decrease of the rough endoplasmic reticulum and progressive swelling of the Golgi cisternae were observed within the cytoplasm. The nucleus

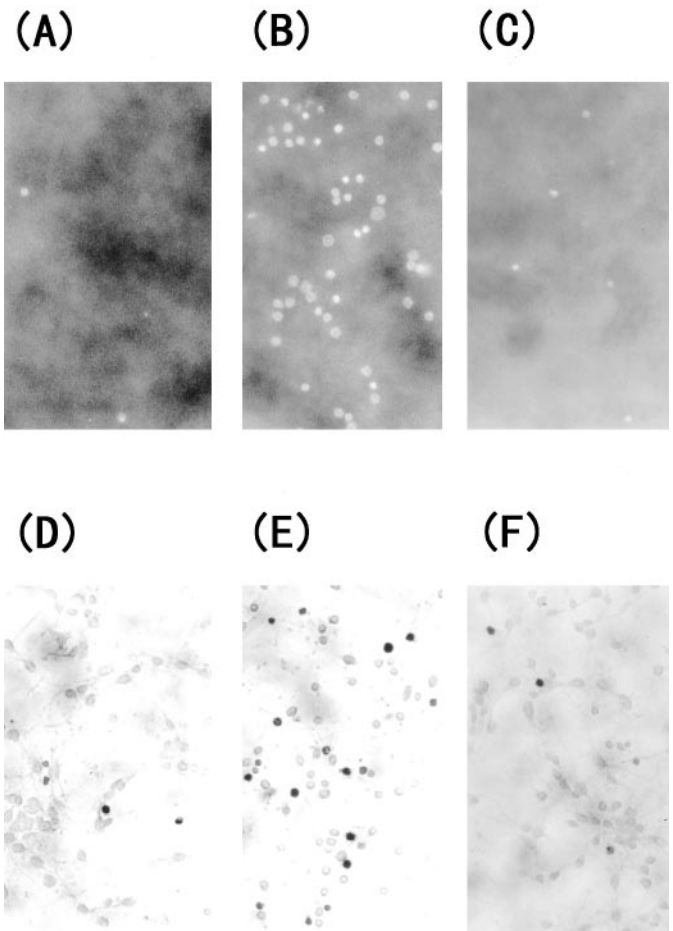


Fig. 6. Apoptotic features of cortical neurons induced by sPLA₂-IIA. Cortical neurons were treated with vehicle (A and D), 1 μ M sPLA₂-IIA (B and E), or 1 μ M sPLA₂-IIA + 10 μ M indoxam (C and F). Neurons were stained with 10 μ M Hoechst 33258 for 10 min 48 h later (A–C). Neurons were fixed with 4% paraformaldehyde, washed twice with PBS, and stained by the TUNEL technique 48 h later (D–F). Magnification, 100 $\times$.

TABLE 3
Effects of indoxam on sPLA₂-IIA-induced apoptotic features
Cortical neurons were treated with 1 μ M sPLA₂-IIA in the absence or presence of 10 μ M indoxam or 10 μ M NS-398. Hoechst 33258- and TUNEL-positive neurons were detected 48 h later. Data are expressed as means $\pm$ S.E.M. ($n = 4$). Comparisons were made by ANOVA followed by Dunnett's test.

Treatment	Hoechst 33258	TUNEL
	cells/cm ²	
Control	634.9 $\pm$ 84.0	253.8 $\pm$ 6.7
sPLA ₂ -IIA	9,079.6 $\pm$ 1,008.1**	523.8 $\pm$ 69.2**
sPLA ₂ -IIA + indoxam	730.0 $\pm$ 69.0##	285.7 $\pm$ 27.7##
sPLA ₂ -IIA + NS-398	802.4 $\pm$ 71.6##	306.3 $\pm$ 28.4##

** $P < 0.01$, compared with control.
$P < 0.01$, compared with sPLA₂-IIA.

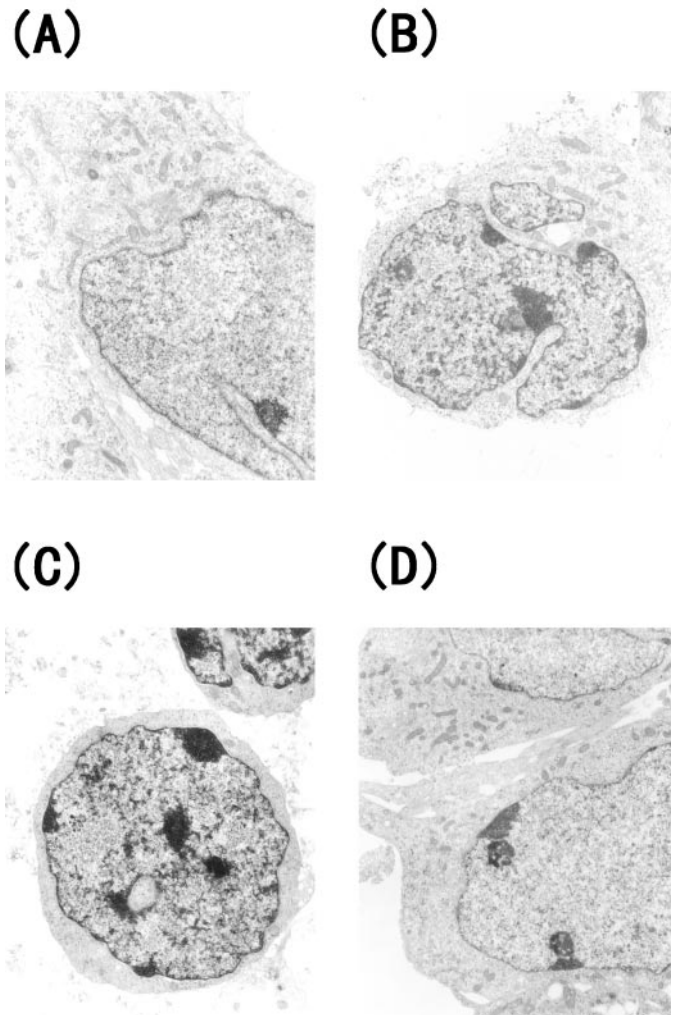


Fig. 7. Ultrastructural changes in cortical neurons by sPLA₂-IIA. Cortical neurons were treated with vehicle (A), 1 μ M sPLA₂-IIA (B and C), or 1 μ M sPLA₂-IIA + 10 μ M indoxam (D). The cultures were examined 36 h (B) or 48 h (A, C, and D) later by electron microscopy. Neurons showed ultrastructural details of cytoplasmic organelles such as endoplasmic reticulum, Golgi apparatus, and mitochondria (A and D). Middle-stage neurons showed blebbing of plasma membranes (B). Late-stage neurons had shrunk markedly. Nuclear chromatin was now condensed and fragmented (C). These cells no longer contained cytoplasmic organelles other than mitochondria. Plasma membranes became less distinct just before cell lysis. Magnification, 3000 $\times$.

shrank progressively, and chromatin clumps became increasingly electron-dense. In the late stage (Fig. 7C), intracellular organelles such as the endoplasmic reticulum and the Golgi apparatus were lost, but the mitochondria remained intact. Condensation and fragmentation of chromatin were noted in the nucleus. The dying cells finally fragmented into small pieces. The ultrastructural disruption in sPLA₂-IIA-treated neurons was completely suppressed by indoxam (Fig. 7D).

sPLA₂-IIA-Induced Liberation of AA. In cooperation with other inflammatory stimuli, sPLA₂-IIA hydrolyzes phospholipids in membranes to liberate free AA (Murakami et al., 1991). We examined free [³H]AA release during sPLA₂-IIA-induced neuronal cell death (Fig. 8). Neurons were incubated with [³H]AA for 24 h for equilibration among phospholipids. Free [³H]AA was spontaneously released from the neurons into the medium (Fig. 8A). In the absence of other cofactors or inflammatory stimuli, sPLA₂-IIA significantly increased the liberation of [³H]AA from neurons before neuronal cell death (Fig. 8A). The level of free [³H]AA was

dependent on the concentration of sPLA₂-IIA from 0.1 to 3 μ M at 25 h (Fig. 8B). On the contrary, regardless of the presence or absence of sPLA₂-IIAs, little liberation of free AA was observed from myocytes (Fig. 8A), which were insensitive to sPLA₂-IIA (Table 1). These results indicated that there was a close correlation between sPLA₂-IIA-induced AA release and neuronal cell death.

sPLA₂-IIA-Induced Generation of PGD₂. sPLA₂-IIA hydrolyzes phospholipids in membranes to generate PGD₂ from mast cells (Murakami et al., 1991). We examined the sPLA₂-IIA-induced generation of PGD₂ from neurons in the absence of other inflammatory stimuli (Fig. 9). The basal level of PGD₂ was not altered throughout the period of culture. When neurons were exposed to sPLA₂-IIA, generation of PGD₂ was not observed within 10 h, but increased transiently at 15 h and decreased thereafter (Fig. 9A). Moreover, sPLA₂-IIA generated PGD₂ from neurons in a concentration-dependent manner at concentrations ranging from 0.1 to 3

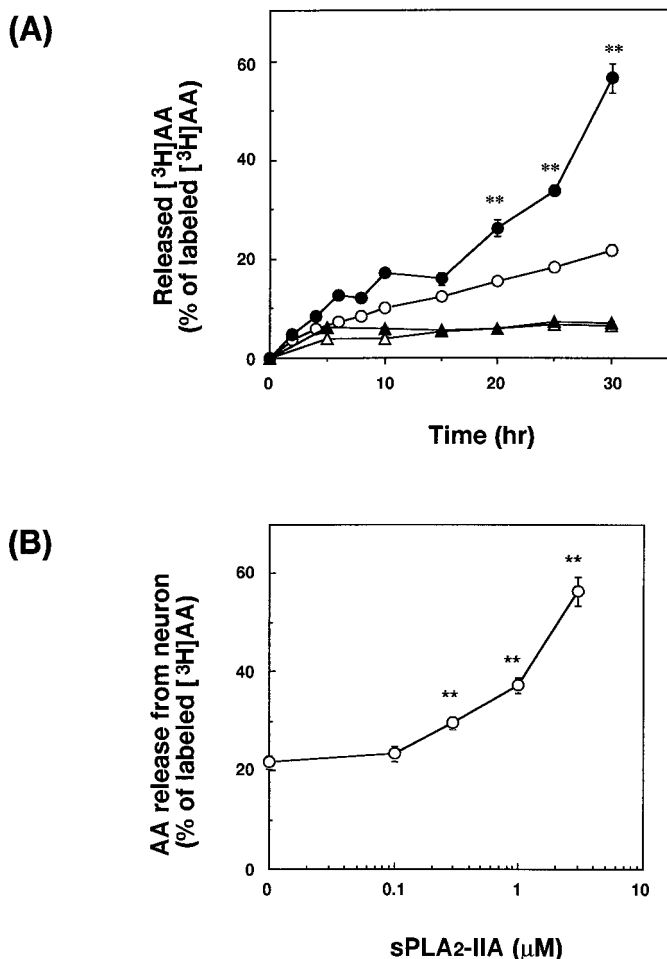


Fig. 8. sPLA₂-IIA-induced liberation of [³H]AA from neurons. Cortical neurons were labeled with 1 μ Ci/ml [³H]AA for 24 h. A, time course: labeled neurons were treated without (○) or with 1 μ M sPLA₂-IIA (●). Labeled myocytes were treated without (△) or with 1 μ M sPLA₂-IIA (▲). The radioactivity of [³H]AA in the supernatant was measured at the indicated times after treatment. B, dose response: labeled neurons were treated with the indicated concentrations of sPLA₂-IIA. The radioactivity of [³H]AA in the supernatant was measured 25 h after treatment. Data are expressed as means $\pm$ S.E.M. ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with untreated conditions by ANOVA followed by Dunnett's test.

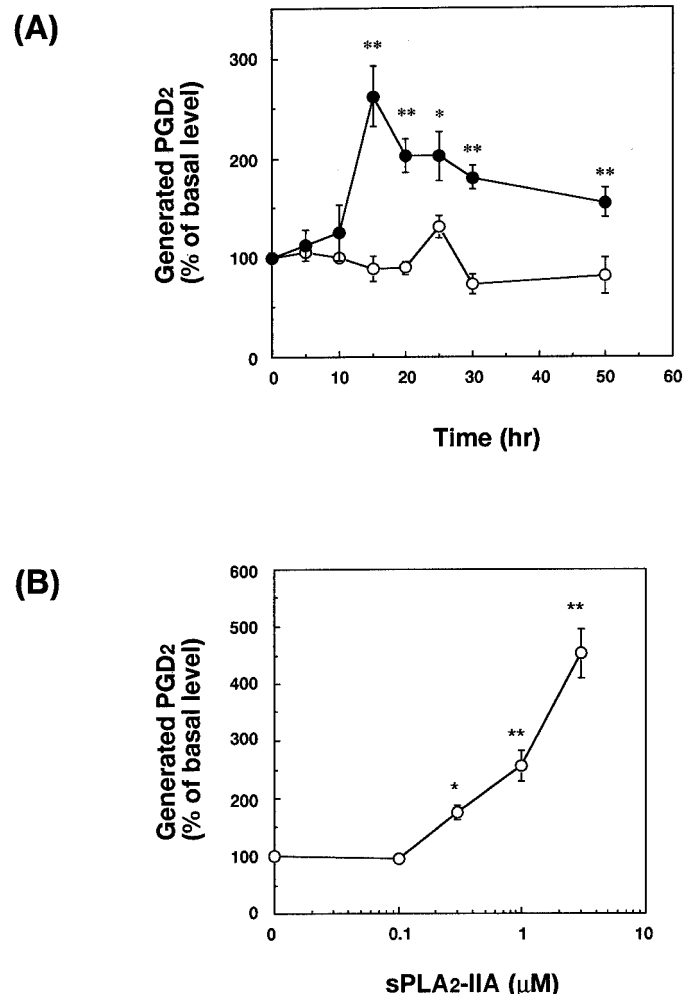


Fig. 9. sPLA₂-IIA-induced generation of PGD₂ from neurons. A, time course: cortical neurons were treated with vehicle (○) or 1 μ M sPLA₂-IIA (●). PGD₂ released into the supernatant was measured at the indicated times after treatment. B, dose response: cortical neurons were treated with the indicated concentrations of sPLA₂-IIA. PGD₂ released into the supernatant was measured 15 h after treatment. Each level of PGD₂ generated from untreated neurons was measured as a control level. The control level of PGD₂ was 72 ± 3 pg/ml. Data are expressed as means $\pm$ S.E.M. ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with vehicle-treated conditions by ANOVA followed by Dunnett's test.

μM (Fig. 9B). These results indicated that sPLA₂-IIA induced PGD₂ generation from neurons without cofactors or inflammatory costimuli before apoptosis.

Effects of the sPLA₂-IIA Inhibitor on the sPLA₂-IIA-Induced Liberation of AA and Generation of PGD₂. To ascertain whether the liberation of AA and the generation of PGD₂ were associated with neuronal cell death, we evaluated the effect of indoxam on the sPLA₂-IIA-induced liberation of AA and the generation of PGD₂ from neurons (Fig. 10). Indoxam attenuated the sPLA₂-IIA-induced AA liberation in a concentration-dependent manner. At 10 μM , the inhibitor suppressed the AA release completely by cotreatment with sPLA₂-IIA (Fig. 10A). Furthermore, indoxam significantly

inhibited AA release from neurons within 10 h after the treatment with sPLA₂-IIA (Fig. 10A).

Next, we estimated the effect of indoxam on sPLA₂-IIA-induced PGD₂ generation from neurons (Fig. 10B). The inhibitor attenuated PGD₂ generation in a concentration-dependent manner. At 10 μM , the inhibitor suppressed the PGD₂ generation completely by cotreatment with sPLA₂-IIA (Fig. 10B). Treatment with indoxam, even after occlusion, significantly blocked the sPLA₂-IIA-induced generation of PGD₂ within 10 h after sPLA₂-IIA-treatment (Fig. 10B). Thus, the sPLA₂-IIA inhibitor prevented neurons from the sPLA₂-IIA-induced liberation of AA and generation of PGD₂ not only by cotreatment, but also by treatment after occlusion.

Effect of a COX-2 Inhibitor on sPLA₂-IIA-Induced Neuronal Cell Death. To determine how PGD₂ is involved in neuronal cell death, we examined the effect of COX-2 inhibitor on sPLA₂-IIA-induced PGD₂ generation (Fig. 11A) and neuronal cell death (Fig. 11B). NS-398 inhibited PGD₂ generation in a concentration-dependent manner ($\text{IC}_{50} = 13 \text{ nM}$). At 10 μM , NS-398 lowered the elevated level of PGD₂ significantly. There was no statistical difference in PGD₂ generation between control and 10 μM NS-398-added, sPLA₂-IIA-treated neurons. Thus, NS-398 markedly suppressed the generation of PGD₂ from sPLA₂-IIA-treated neurons.

Next, we investigated the effects of NS-398 on sPLA₂-IIA-induced neuronal cell death (Fig. 11B). NS-398 markedly prevented neurons from sPLA₂-IIA-induced cell death in a concentration-dependent manner ($\text{IC}_{50} = 15 \text{ nM}$). At 10 μM , NS-398 significantly inhibited sPLA₂-IIA-induced neuronal cell death. Moreover, NS-398 ameliorated apoptotic features of sPLA₂-IIA-treated neurons (Table 3). There was no statistical difference in the number of chromatin-condensed neurons and TUNEL-positive nuclei between control and 10 μM NS-398-added, sPLA₂-IIA-treated neurons. Thus COX-2 metabolites were suggested to be critically involved in sPLA₂-IIA-induced apoptosis of neurons.

Effects of Eicosanoids and Inflammatory Factors on Neuronal Cell Survival. To determine how eicosanoids are involved in sPLA₂-IIA-induced cell death, we examined the effects of various eicosanoids on neuronal survival (Table 4). As shown in Fig. 12A, PGD₂ caused neuronal cell death in a concentration-dependent manner ($\text{ED}_{50} = 8 \mu\text{M}$). PGD₂ exhibited minimal toxicity during the first 6 h of treatment and displayed significant neurotoxicity only after prolonged exposure (Fig. 12B). Moreover, Δ^{12} -PGJ₂, one of the PGD₂ metabolites, killed neurons more potently than did PGD₂ as shown in Fig. 12A ($\text{ED}_{50} = 1.1 \mu\text{M}$). Δ^{12} -PGJ₂ caused neuronal cell death more rapidly than did PGD₂, without time lag (Fig. 12B). On the other hand, other eicosanoids such as PGE₂, 9 α -11 β -PGF₂, PGF₂ α , PGI₂, U-46619, and LTB₄ had no effect on neuronal cell survival (Table 4). Among the eicosanoids tested, only PGD₂ and Δ^{12} -PGJ₂ showed neurotoxicity.

sPLA₂-IIA has been reported to be stimulated upon degradation of sphingomyelin to produce LPA and ceramide (Fourcade et al., 1995), and these products are known to induce apoptosis (Obeid et al., 1993). The effects of sphingomyelinase, LPA, and ceramide, a sphingomyelin metabolite, on neuronal cell survival were estimated (Table 4). These

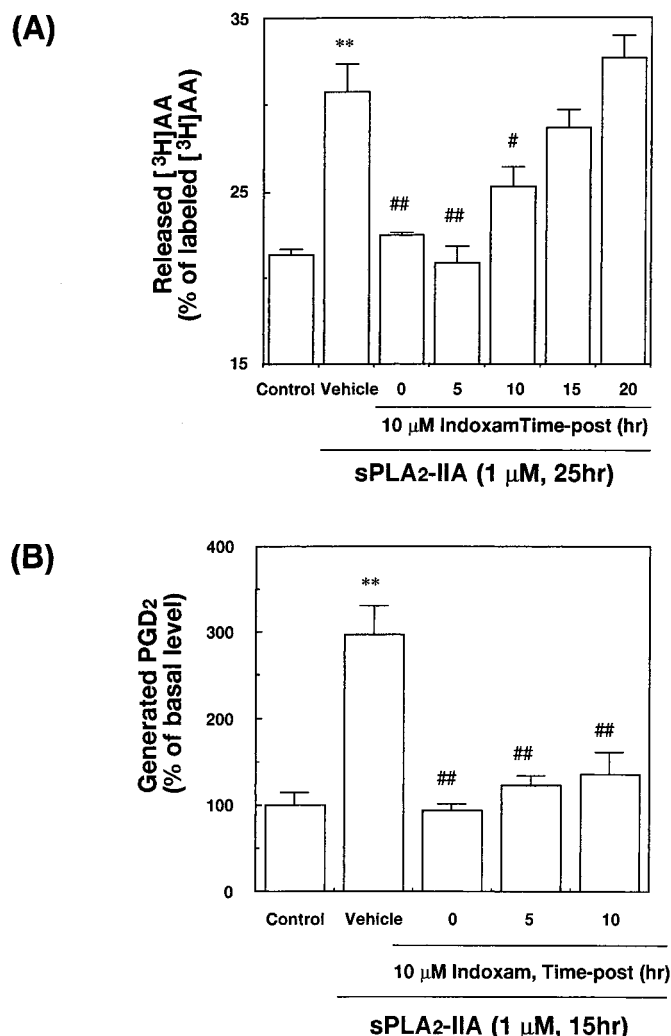


Fig. 10. Effects of indoxam on sPLA₂-IIA-induced liberation of [³H]AA and generation of PGD₂ from neurons. A, AA: cortical neurons were labeled with 1 $\mu\text{Ci}/\text{ml}$ [³H]AA at 37°C for 24 h. Labeled neurons were treated with 1 μM sPLA₂-IIA or not treated. Vehicle or 10 μM indoxam was applied at the indicated times after sPLA₂-IIA treatment. The radioactivity of [³H]AA in the supernatant was measured 25 h later. B, PGD₂: cortical neurons were treated with 1 μM sPLA₂-IIA or not treated. Indoxam (10 μM) was applied at the indicated time points after sPLA₂-IIA treatment. PGD₂ released into the supernatant was measured 15 h later. The level of PGD₂ generated from untreated neurons was measured as the control level. The control level was $64 \pm 9 \text{ pg}/\text{ml}$. Data are expressed as means $\pm$ S.E.M. ($n = 4$). **, $P < 0.01$, compared with control; #, $P < 0.05$; ##, $P < 0.01$, compared with vehicle by ANOVA followed by Dunnett's test.

agents neither caused cell death nor modulated sPLA₂-IIA-induced neuronal cell death.

Discussion

In the present study, we demonstrated that mammalian sPLA₂-IIA induces cell death of cortical neurons via apoptosis. We propose a mechanism showing how sPLA₂-IIA is involved in stroke, as follows. First, cerebral ischemia triggers an increment in inflammatory factors such as TNF- α and IL-1 β (Qin, 1998). Second, these cytokines increase secretion of sPLA₂-IIA from astrocytes (Oka and Arita, 1991), leading to the expression of sPLA₂-IIA in the ischemic brain (Lauritzen et al., 1994). Third, sPLA₂-IIA activity is elevated in the ischemic area such as the CTX. Fourth, sPLA₂-IIA liberates AA and generates excessively PGD₂, leading to

apoptosis of neurons in the penumbra. Finally, apoptosis in neurons is followed by neurologic malfunctions in patients with stroke.

MCA occlusion causes irreversible necrosis and infarction, which is a consequence of the loss of ATP and energy failure in the ischemic core of a stroke. On the other hand, according to Li et al. (1995), in the penumbra around the core, cell death was induced via apoptosis and necrosis. Focal cerebral ischemia was caused in the rat by PIT-MCA occlusion (Umemura et al., 1995). After occlusion, sPLA₂ activity was significantly increased in the cortex, in which the ischemic core and the penumbra coexist. Both the elevated activity of sPLA₂ and the protein expression of sPLA₂-IIA were followed by neurologic damage in both areas. The sPLA₂ inhibitor reduced the elevated PLA₂ activity completely and the infarct volume significantly in the cortex. The inhibitor ameliorated occlusion-induced inflammation and neurodegeneration in the penumbra, suggesting that sPLA₂ might play an important role in apoptosis in the penumbra.

Among various sPLA₂s, sPLA₂-IIE, sPLA₂-V, and sPLA₂-X, but not sPLA₂-IIA, are expressed in the normal brain (Suzuki et al., 2000). On the other hand, the level of sPLA₂-IIA is elevated during the inflammatory state after ischemia (Lauritzen et al., 1994). Therefore, we ascertained whether sPLA₂-IIA causes cell death via apoptosis or necrosis in primary cultures of rat cortical neurons. The process of neuronal cell death induced by sPLA₂-IIA showed apoptotic features such as progressive cell shrinkage, blebbing of the plasma membrane, loss of cytosolic organelles, clumping of chromatin, and fragmentation of DNA. During apoptosis, no DNA ladder formation (180- to 200-bp oligonucleosome inter-ger fragments) was detected by conventional agarose gel electrophoresis (data not shown). Thus, human sPLA₂-IIA-induced apoptosis in neurons was accompanied by large DNA fragmentation. Despite overwhelming evidence in favor of internucleosomal DNA cleavage activity as a characteristic of apoptosis, there have been several accounts of apoptosis in the absence of this DNA cleavage pattern (Oberhammer et al., 1992). In our previous study, amyloid- β protein induced neuronal cell death, accompanied by small DNA fragmenta-

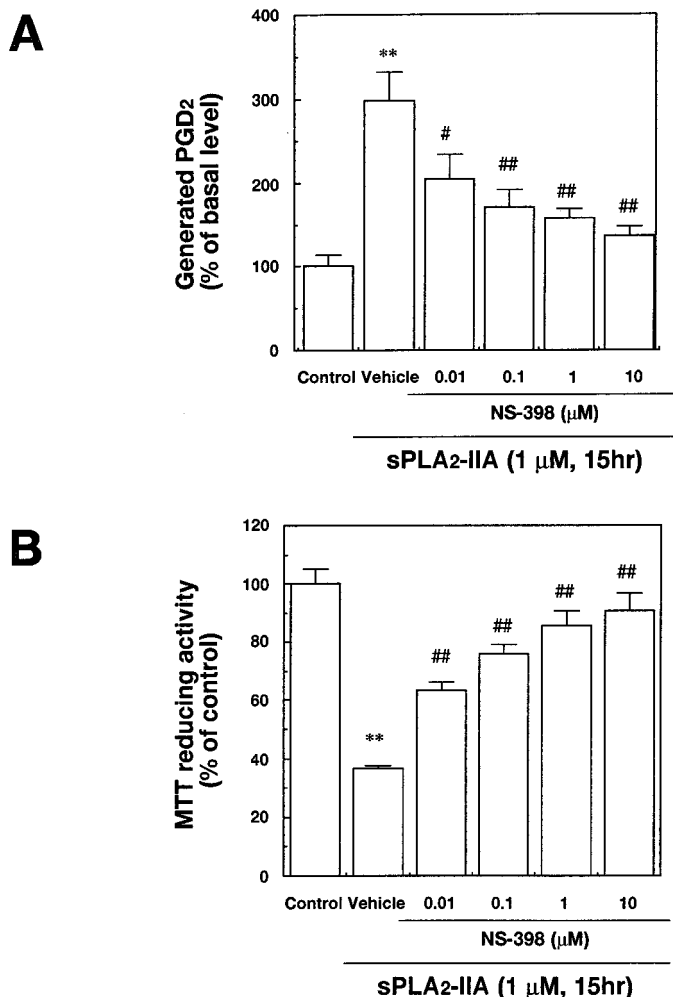


Fig. 11. Effects of NS-398 on sPLA₂-IIA-induced generation of PGD₂ and neuronal cell death. A, generation of PGD₂: cortical neurons were treated with 1 μ M sPLA₂-IIA in the presence of NS-398 at the indicated concentrations. PGD₂ released into the supernatant was measured at 15 h after sPLA₂-IIA-treatment. The level of PGD₂ generated from untreated neurons was measured as the control level. The control level was 64 ± 9 pg/ml. Data are expressed as means $\pm$ S.E.M. ($n = 4$). **, $P < 0.01$, compared with untreated neurons; #, $P < 0.05$; ##, $P < 0.01$, compared with sPLA₂-IIA alone-treated neurons by ANOVA followed by Dunnett's test. B, neuronal cell death: neurons were treated with vehicle 1 μ M sPLA₂-IIA in the presence of NS-398 at the indicated concentrations. MTT-reducing activity was determined 48 h after sPLA₂-IIA treatment. Data are expressed as means $\pm$ S.E.M. ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with sPLA₂-IIA alone by ANOVA followed by Dunnett's test.

TABLE 4

Effects of eicosanoids and inflammatory factors on neuronal cell survival

Rat cortical neurons were treated with various prostaglandins and inflammatory factors. MTT-reducing activity was determined 48 h later. Data are expressed as means $\pm$ S.E.M. ($n = 4$). Comparisons with control were made by ANOVA followed by Dunnett's test.

Eicosanoids	Concentration	MTT-Reducing Activity
		% of control
Control		100.0 $\pm$ 4.0
AA	30 μ M	88.3 $\pm$ 11.9
PGD ₂	10 μ M	36.9 $\pm$ 3.1**
PGE ₂	10 μ M	102.6 $\pm$ 6.4
9 α , 11 β -PGF ₂	10 μ M	105.1 $\pm$ 2.7
PGF ₂ α	10 μ M	107.3 $\pm$ 1.3
PGI ₂	10 μ M	104.1 $\pm$ 2.5
U-46619	10 μ M	96.5 $\pm$ 4.7
LTB ₄	10 μ M	90.3 $\pm$ 2.4
LPA	100 μ M	98.1 $\pm$ 1.0
C ₂ -ceramide	10 μ M	121.7 $\pm$ 7.0
SPHase	0.1 U/ml	99.3 $\pm$ 0.9
sPLA ₂ -IIA	0.1 μ M	104.4 $\pm$ 0.1
SPHase + sPLA ₂ -IIA	0.1 U/ml, 0.1 μ M	100.2 $\pm$ 1.0

** $P < 0.01$, compared with controls by ANOVA followed by Dunnett's test. SPHase, sphingomyelinase.

tion (Ueda et al., 1996). Because our cortical cultures were prepared according to a method reported previously (Ueda et al., 1996), the possibility that the internucleosomal DNA cleavage activity in neurons was absent or simply undetected could be excluded. How the internucleosomal DNA cleavage activity was attenuated remains unclear. To our knowledge, this is the first report that mammalian sPLA₂-IIA elicits neuronal cell death via apoptosis.

Apoptosis is stimulated through receptors for death-inducing cytokines such as Fas ligand and TNF- α . These death factors stimulate sphingomyelin hydrolysis, and sphingomyelin degradation products including ceramide have been shown to induce apoptosis (Obeid et al., 1993). A number of phospholipases, including sPLA₂-IIA, have been shown to degrade phospholipids in intact cells much more efficiently in the presence of sphingomyelinase (Fourcade et al., 1995). However, sphingomyelinase did not affect sPLA₂-IIA-induced neuronal cell death, and neither LPA nor ceramide mimicked the action of sPLA₂-IIA on neurons in the present study. Thus, neither sphingomyelinase nor LPA seemed to be involved in the sPLA₂-IIA-induced apoptosis.

sPLA₂-IIA exhibited neurotoxicity at concentrations around 1 μ M in vitro. Does the concentration of endogenous sPLA₂-IIA reach to such a high level in vivo? Concentrations of in vivo sPLA₂-IIA were calculated under the assumption that sPLA₂-IIA contributed to most of the sPLA₂ activity shown in Fig. 2B. Its concentration was about 3.5 nM in the area of neurodegeneration induced by MCA occlusion, if spread uniformly in that area. This gave rise to the question why the concentration of sPLA₂-IIA required in vitro was approximately 300 times higher than that expressed in vivo. This discrepancy could be explained by the following reports. First, sPLA₂-IIA generated from cytokine-stimulated astrocytes might reach to such a high concentration microenvironmentally at surfaces of neurons via attachment to heparan sulfate proteoglycan (Koduri et al., 1998). Second, sPLA₂-IIA might cause neuronal cell death at lower concentrations in the presence of cofactors (Murakami et al., 1991; Fourcade et al., 1995). This was not found in the present study. Third, the sensitivity of the cells to endogenously produced sPLA₂-IIA is higher than exogenously added sPLA₂-IIA (Murakami et al., 1999). The striking difference between the amounts of sPLA₂-IIA required by different systems [e.g., exogenously added (micromolar) versus endogenously produced (nanomolar)] implies that the continued supply of sPLA₂-IIA, which occurs in the latter situation, may be an important factor for its adequate action during cellular (particularly prolonged) responses.

Intact cells are resistant to injury induced by mammalian sPLA₂-II (Morgan et al., 1993). Indeed, sPLA₂-IIA was not toxic to non-neuronal cells such as mesangial cells, smooth-muscle cells, endothelial cells, fibroblasts, hepatocytes, or myocytes in the present study. On the other hand, apoptotic cell membranes are potent targets for the enzyme (Atsumi et al., 1997). Neuronally differentiated PC12 cells deprived of nerve growth factor and serum, mast cells deprived of hematopoietic cytokines, and anti-Fas antibody-treated U937 monocytic leukemia cells, all of which display the classical changes of apoptosis, become sensitive to sPLA₂-II-mediated liberation of AA. Under our culture conditions, sPLA₂-IIA liberated AA significantly from neurons before apoptosis but not from myocytes, which are resistant to the enzyme. A specific sPLA₂ inhibitor, indoxam, significantly suppressed the sPLA₂-IIA-induced liberation of AA from neurons. Furthermore, indoxam and *p*-BPB prevented neurons from sPLA₂-IIA-induced apoptosis, suggesting that the neurotoxicity of sPLA₂-IIA depends on the susceptibility of cells to sPLA₂-IIA-mediated liberation of AA.

AA is metabolized to PGs and LTs by COX and lipoxygenase, respectively. sPLA₂-IIA significantly generated PGD₂ from neurons before cell death. AA increased gradually from 10 h and significantly from 20 h, whereas PGD₂ peaked sharply at 15 h and remained significantly elevated for 50 h. Why then did PGD₂ generation precede any significant release of AA? Besides PGD₂ (Murakami et al., 1991), sPLA₂-IIA generates PGE₂ from kidney 293 cells (Murakami et al., 1999) and LTB₄ from neutrophils (Mayer and Marshall, 1993). In the present study, PGE₂ was increased by sPLA₂-IIA, whereas LTB₄ was not (data not shown). However, the control level of PGE₂ and LTB₄ was two and four times as high as that of PGD₂ (data not shown). Moreover, AA released from neurons included not only the AA itself, but also its metabolites, such as PGD₂, LTB₄, and PGE₂. The content

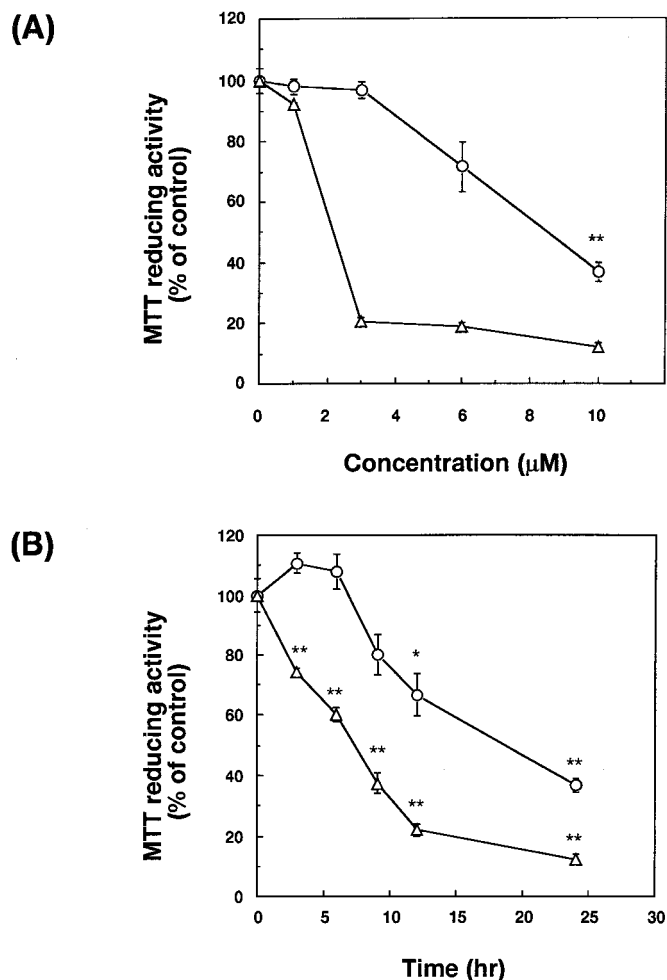


Fig. 12. Effect of PGD₂ and Δ^{12} -PGJ₂ on the survival of neurons. A, dose response: cortical neurons were treated with PGD₂ (○) and Δ^{12} -PGJ₂ (Δ) at the indicated concentrations. MTT-reducing activity was determined 24 h later. B, time course: cortical neurons were treated with 10 μ M PGD₂ (○) and Δ^{12} -PGJ₂ (Δ). MTT-reducing activity was determined at the indicated times after the PG treatment. Data are expressed as mean $\pm$ S.E.M. values ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with untreated conditions by ANOVA followed by Dunnett's test.

ratio of PGD₂ was so low among AA metabolites that the increment in PGD₂ could not sufficiently reflect the elevation of released AA, which could explain the generation of PGD₂ from neurons before any significant release of AA.

PGD₂ is the major PG in the brain of mammalian species, and PGD₂ levels were markedly increased in the brain during reperfusion after ischemia, suggesting the involvement of PGD₂ in neurodegeneration (Gaudet et al., 1980). Indeed, PGD₂ caused marked neuronal cell death in our cortical cultures, whereas other eicosanoids such as PGE₂ and LTB₄ did not. The sPLA₂-IIA inhibitor prevented sPLA₂-IIA-treated neurons from PGD₂ generation and neuronal cell death. Furthermore, the COX-2 inhibitor also suppressed sPLA₂-IIA-treated neurons from PGD₂ generation and apoptosis. Taken together, these results indicate that PGD₂ was involved in sPLA₂-IIA-induced neuronal apoptosis. However, interpretation of PGD₂ as an apoptosis inducer requires circumspection for several reasons. First, the LD₅₀ value of PGD₂ was high compared with the affinity for its receptors. Second, there was a latent period for PGD₂ to induce neuronal cell death. Third, little PGD₂ receptor was detected in the rat brain (data not shown). Finally, a PGD₂ receptor blocker did not prevent neurons from PGD₂ induced cell death (data not shown). How did PGD₂ induce cell death? In the present study, neurotoxicity was observed in one of the PGD₂ metabolites, Δ^{12} -PGJ₂. At low concentration, this metabolite caused neuronal cell death more potently than did PGD₂. In comparison with PGD₂, Δ^{12} -PGJ₂ killed neurons rapidly without a latent period. Δ^{12} -PGJ₂ induces apoptosis in other cells (Kim et al., 1993). Taken together, the sPLA₂-IIA-induced neuronal apoptosis could be ascribed to PGD₂ metabolites, possibly Δ^{12} -PGJ₂, but not to PGD₂ itself. Further studies are necessary for determining the pathologic role of PGD₂ and its metabolites in sPLA₂-IIA-induced neuronal cell death and stroke.

Optimal action of sPLA₂ generally requires membrane perturbation with induction of microdomains of membrane asymmetry. This microheterogeneity may be induced by co-factors such as venom-derived peptides and highly positively charged molecules. In contrast, human sPLA₂-IIA could hydrolyze membrane glycerophospholipids without costimuli or prior modifications of the cell membrane. sPLA₂-IIA required a latent period to induce the liberation of AA, the generation of PGD₂, and neuronal cell death. Furthermore, treatment with sPLA₂ inhibitor reduced the liberation of AA and the generation of PGD₂ after sPLA₂-IIA treatment. This raises the question of what happens in sPLA₂-IIA-treated neurons during the latent period. Pancreatic group I sPLA₂ (sPLA₂-IB) binds to its specific receptor (sPLA₂ receptor) and elicits a variety of biologic responses including DNA synthesis, eicosanoid formation, hormone release, and chemokinetic cell migration (Hanasaki and Arita, 1999). On the other hand, high affinity-receptors for mammalian sPLA₂-IIA have not yet been identified. Neurotoxic sPLA₂ are present in the venoms from several snake species. In particular, the venom from the Tipan snake *O. scutellatus* contains multiple phospholipases, including a single-chain sPLA₂ called OS₂ that, when injected into mouse brain, is lethal at very low doses (Lambeau et al., 1989). Studies using I-labeled OS₂ have revealed very high-affinity binding sites, N-type binding sites, in rat brain, which is interfered by bee venom sPLA₂ but not by mammalian sPLA₂ (Lambeau et al., 1989). Al-

though we cannot rule out the possibility that membrane rearrangement of neurons might occur and provide substrates preferentially for sPLA₂-IIA via its own receptor during the latent period, we have not yet obtained any evidence proving the possibility. However, it should be noted that sPLA₂-IIA-treated neurons in the latent period might be the proapoptotic state, which could revert to the viable state. Indeed, neuronal cell death was significantly terminated by the treatment with the sPLA₂ inhibitor after the sPLA₂-IIA treatment in cultured neurons and the MCA occlusion in rats.

In conclusion, sPLA₂-IIA induces apoptosis of neurons, possibly via metabolites of PGD₂, suggesting that sPLA₂-IIA plays a crucial role in apoptosis of neurons in the penumbra of the patients with stroke. Furthermore, the sPLA₂-IIA inhibitors can rescue neurons from apoptosis during the latent period, when they seem to terminate the reversible proapoptotic state in neurons or revert neurons from a proapoptotic state to a viable state. Thus, the present study sheds light on the sPLA₂-IIA inhibitor as a prelude to a new generation of drug treatments for stroke.

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