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Human Group IIA Secretory Phospholipase A_2 Induces Neuronal Cell Death via Apoptosis

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ABSTRACT

Expression of group IIA secretory phospholipase A_2 (sPLA$_2$-IIA) is documented in the cerebral cortex (CTX) after ischemia, suggesting that sPLA$_2$-IIA is associated with neurodegeneration. However, how sPLA$_2$-IIA is involved in the neurodegeneration remains obscure. To clarify the pathologic role of sPLA$_2$-IIA, we examined its neurotoxicity in rats that had the middle cerebral artery occluded and in primary cultures of cortical neurons. After occlusion, sPLA$_2$ activity was increased in the CTX. An sPLA$_2$ inhibitor, indoxam, significantly ameliorated not only the elevated activity of the sPLA$_2$ but also the neurodegeneration in the CTX. The neuroprotective effect of indoxam was observed even when it was administered after occlusion. In primary cultures, sPLA$_2$-IIA caused marked neuronal cell death. Morphologic and ultrastructural characteristics of neuronal cell death by sPLA$_2$-IIA were apoptotic, as evidenced by condensed chromatin and fragmented DNA. Before apoptosis, sPLA$_2$-IIA liberated arachidonic acid (AA) and generated prostaglandin D_2 (PGD_2), an AA metabolite, from neurons. Indoxam significantly suppressed not only AA release, but also PGD_2 generation. Indoxam prevented neurons from sPLA$_2$-IIA–induced neuronal cell death. The neuroprotective effect of indoxam was observed even when it was administered after sPLA$_2$-IIA treatment. Furthermore, a cyclooxygenase-2 inhibitor significantly prevented neurons from sPLA$_2$-IIA–induced PGD_2 generation and neuronal cell death. In conclusion, sPLA$_2$-IIA induces neuronal cell death via apoptosis, which might be associated with AA metabolites, especially PGD_2. Furthermore, sPLA$_2$ contributes to neurodegeneration in the ischemic brain, highlighting the therapeutic potential of sPLA$_2$-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 infarction 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 D_2 (PGD_2), are increased in the MCA-occluded rat brain (Gaudet et al., 1980). AA is liberated from cell membrane lipids by phospholipase A_2 (PLA_2), and PGs are metabolized from AA by cyclooxygenase (COX). PLA_2s are classified as secreted forms (sPLA$_2$), Ca^{2+}-dependent forms, and Ca^{2+}-independent forms. Human sPLA$_2$s are 14-kDa calcium-dependent enzymes and are classified into nine distinct types (Gelb et al., 2000). Among them, group IIA secretory PLA_2 (sPLA$_2$-IIA)

ABBREVIATIONS: MCA, middle cerebral artery; AA, arachidonic acid; PG, prostaglandin; PLA_2, phospholipase A_2; COX, cyclooxygenase; CTX, cerebral cortex; sPLA$_2$, secretory phospholipase A_2; sPLA$_2$-IIA, group IIA secretory phospholipase A_2; LPA, lysophosphatidic acid; OS_2, *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 PLA_2 inhibitor reduces the infarct size in MCA-occluded rats (Estevez and Phillis, 1997). Thus, sPLA$_2$-IIA seems to play an important role in neurodegeneration after ischemia.

sPLA$_2$-IIA is stimulated upon degradation of sphingomyelin and produces lysophosphatidic acid (LPA) (Fourcade et al., 1995). Moreover, the enzyme releases PGD_2 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 sPLA$_2$-IIA (Atsumi et al., 1997). sPLA$_2$-IIA causes cell death only in the presence of phosphatidylethanolamine (Vadas, 1997). Although sPLA$_2$ purified from *Oxyuranus scutellatus* (taipan) venom (OS_2) is responsible for neurotoxicity (Lambeau et al., 1989), it has not yet been clarified whether mammalian sPLA$_2$-IIA is involved in neuronal cell death. There are two approaches to examining the effects of sPLA$_2$-IIA. One is transfection of target cells with sPLA$_2$-IIA; another is exogenous addition of sPLA$_2$-IIA to target cells. In the present study, we evaluated the toxicity of sPLA$_2$-IIA on neurons by the latter approach, because neurons are differentiated cells and cannot be transfected. We provide the first evidence that mammalian sPLA$_2$-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 sPLA$_2$-IIA was provided by Lilly Research Laboratories (Indianapolis, IN) and rat sPLA$_2$-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-glial 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)-1β, 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). PGD_2, PGE_2, 9α-11β-PGF_2, $PGF_2\alpha$, PGI_2, Δ^{12}-PGJ_2, and leukotriene B_4 (LTB_4) were purchased from Cascade Biochem Ltd. (Berkshire, UK). U-46619, a stable agonist for thromboxane A_2 receptor, was synthesized in our laboratory (Arimura et al., 1998). An [^{3}H]AA and PGD_2 [^{3}H] 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 coronary 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 sPLA$_2$-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 μg/ml prostaglandin E_1. PLA_2 activity in the supernatant of the brain homogenates was measured in the presence or absence of 1 μM 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/cm^2 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% CO_2/9% O_2/86% N_2. On day 1 after plating, cultures were treated with 0.1 μM 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% CO_2. Human astrocytes were obtained from Clonetics (San Diego, CA) and were cultured at a density of 3.5×10^3 cells/cm^2 on 48-well plates in CCMD 190 medium (Clonetics, San Diego, CA) supplemented with 5% FBS, 50 μg/ml gentamicin, 50 ng/ml amphotericin, 20 ng/ml human epidermal growth factor, 25 μg/ml insulin, 25 ng/ml progesterone, and 50 μg/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/cm^2 on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cardiac myocytes were prepared from day-1 or -2 Sprague-Dawley rats as described previously (Hayasaki et al., 1996). Myocytes were cultured at a density of 2×10^5 cells/cm^2 on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/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/cm^2 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/cm^2 on 48-well plates in CCMD 180 medium (Clonetics) supplemented with 5% FBS, 50 μg/ml gentamicin, and 50 ng/ml amphotericin. Rat mesangial cells were prepared from 4-week-old Slc-Wistar rats (Jupan SLC Inc, Shizuoka, Japan) as described previously (Okuda et al., 1986). Mesangial cells were cultured at a density of 3.5×10^3 cells/cm^2 on 48-well plates in RPMI medium supplemented with 20% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Human bronchial smooth-muscle cells were obtained from Clonetics and were cultured at a density of 3.5×10^3 cells/cm^2 on 48-well plates in Molecular, Developmental, and Cellular Biology medium supplemented with 5% FBS, 50 μg/ml gentamicin, 50 ng/ml amphotericin, 0.5 ng/ml human epidermal growth factor, 5 μg/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/cm^2 on 48-well plates in DMEM supplemented with 10% FBS, 10 mM HEPES, 50 units/ml penicillin, and 50 μg/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/cm^2 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/cm^2 on 48-well plates in modified Czapek Dox broth medium supplemented with 2% FBS, 50 μg/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/cm^2 on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Human dermal fibroblasts were obtained from Clonetics and were cultured at a density of 5×10^4 cells/cm^2 on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Analysis of Neuronal Survival

Neurons (2.5×10^5 cells/cm^2) were treated with sPLA$_2$-IIA in the presence or absence of indoxam at 37°C. Two different methods were employed for assessment of neurotoxicity of sPLA$_2$-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/cm^2) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of 10 μM 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/cm^2) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of 10 μM 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/cm^2) were treated with 1 μM sPLA$_2$-IIA in the presence or absence of 10 μM 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 thiocarbohydrazide-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 [^{3}H]AA and Generated PGD$_2$

[^{3}H]AA. Neurons (2.5×10^5 cells/cm^2) and myocytes (2.5×10^5 cells/cm^2) were incubated for 24 h in culture medium containing 1 μCi/ml [^{3}H]AA, washed twice with culture medium, and treated with sPLA$_2$-IIA in the presence or absence of 10 μM indoxam at 37°C. The radioactivity of [^{3}H]AA into each supernatant was measured.

PGD$_2$. Neurons (2.5×10^5 cells/cm^2) were treated with sPLA$_2$-IIA in the presence or absence of 10 μM indoxam at 37°C. At the times indicated in Figs. 9 to 11, PGD$_2$ was extracted according to the method described in a previous report (Powell, 1980). Supernatants of culture medium (1 ml) were mixed homogenously with cold ethanol (4 ml). The mixture was centrifuged at 1500*g* 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 PGD$_2$, PGE$_2$, and LTB$_4$. These eicosanoids were measured with their respective RIA kits (duplicate/sample). The cross-reactivity of PGJ$_2$ with the PGD$_2$ RIA kit is 7%, whereas that of other PGs, such as PGA$_2$, PGE$_1$, PGE$_2$, PGF$_{1\alpha}$, PGF$_{2\alpha}$, 6-keto PGE$_1$, 6-keto PGF$_{1\alpha}$, and TXB$_2$, is less than 1%.

Results

Alterations in sPLA$_2$-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 sPLA$_2$, acting on its catalytic site (Yokota et al., 1999). sPLA$_2$ 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), PLA$_2$ activity was increased rapidly and reached a maximum within 8 h after ischemia. In the contralateral cortex (right side), little change in PLA$_2$ activity was observed under the same conditions. On the other hand, PLA$_2$ 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 sPLA$_2$ activity within 24 h after the cessation of the photoirradiation. Indoxam intensely suppressed the occlusion-induced increase in sPLA$_2$ activity, indicating that sPLA$_2$ 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 infarction was also ameliorated by treatment with indoxam 2 h after occlusion (Fig. 1B). Treatment with the sPLA$_2$-IIA inhibitor, even after occlusion, significantly protected neurons from ischemia-induced cell death.

Effects of sPLA$_2$-IIA on Cell Survival. We examined the effect of sPLA$_2$-IIA on cell survival, including neurons. Primary cultures of dissociated cortical neurons were exposed to sPLA$_2$-IIA, and neuronal cell death was quantified 48 h later (Fig. 3A). There was a close correlation between MTT-reducing activity and morphologic criteria. sPLA$_2$-IIA at doses less than 0.1 μM did not reduce neuronal cell survival. In the absence of other cofactors or inflammatory factors, increasing concentrations of sPLA$_2$-IIA above 0.1 μM resulted in a significant increase in neuronal cell death in a dose-dependent manner, with a half-maximal concentration of 1.1 μM. On the other hand, sPLA$_2$-IIA had no effect on the survival of astrocytes (Table 1). sPLA$_2$-IIA triggered neuronal cell death after 24 h and killed most neurons within 72 h

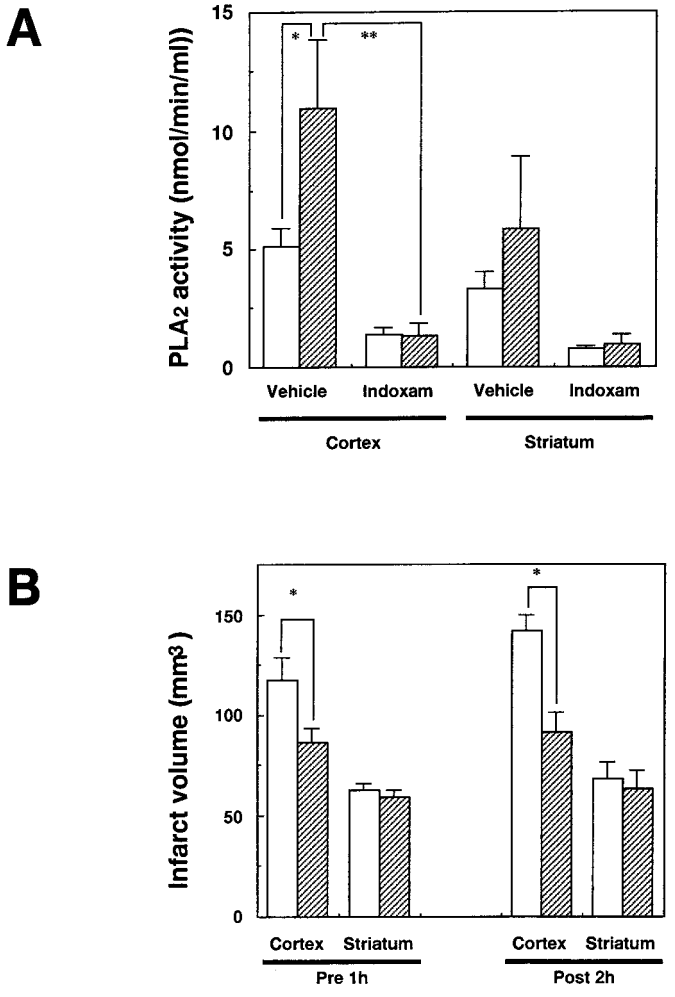

Fig. 1. Effect of sPLA$_2$-II inhibitor on sPLA$_2$ activity and infarct volume after PIT-MCA occlusion. Left MCA was occluded by PIT. A, sPLA$_2$ activity: homogenates of cerebral cortex and striatum were prepared for assay of sPLA$_2$ activity after MCA occlusion. sPLA$_2$ activity in the contralateral side (open bars) and the ipsilateral side (hatched bars) was measured in the absence or presence of 1 μM indoxam with phosphatidyl glycerol/sodium cholate mixed micelle used as substrate. Data are expressed as means ± 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 (□) 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 ± S.E.M. ($n = 6$). *, $P < 0.05$, compared with vehicle-treated conditions by ANOVA followed by Dunnett's test.

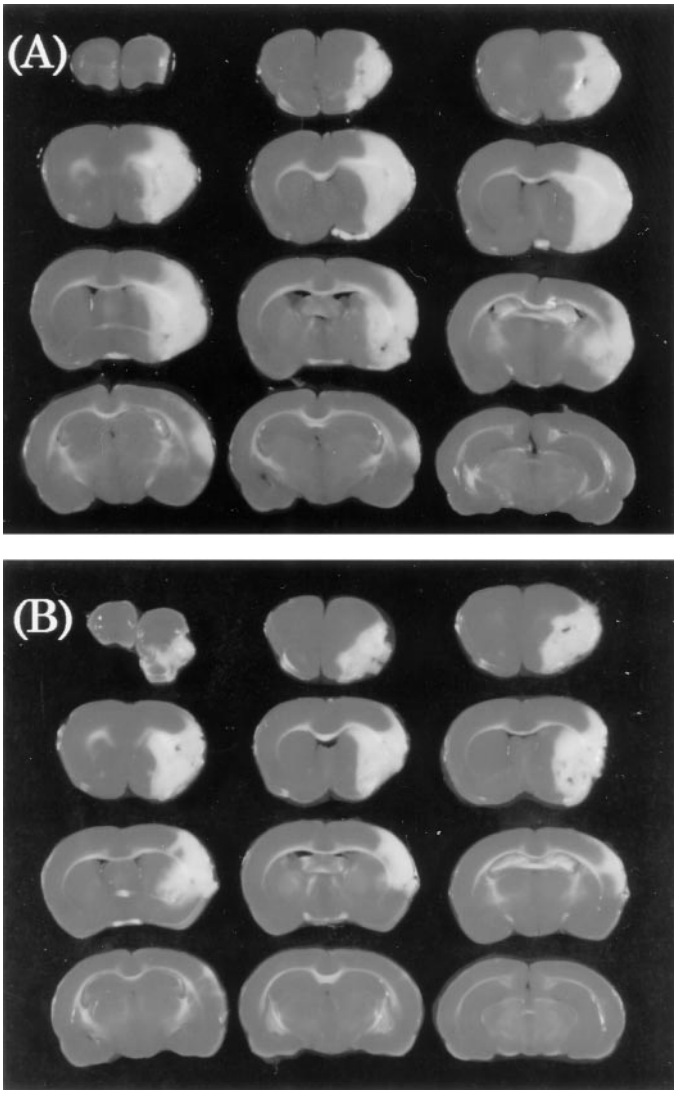

Fig. 2. Area of ischemic brain damage. Twenty-four hours after PIT-MCA, the cerebrum was coronary-sectioned in 1-mm-thick slices from 1.5 mm anterior to 4.5 mm posterior to the bregma; then nine consecutive slices were stained with triphenyltetrazolium chloride. A, the dorsolateral cortex and striatum 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, $sPLA_2$-IIA caused neuronal cell death, but only after a latent period.

Next, we examined the effects of $sPLA_2$-IIA on non-neuronal cell survival to ascertain whether $sPLA_2$-IIA induced the death of cells other than neurons (Table 1). $sPLA_2$-IIA caused complete neuronal cell death at 3 μM 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 $sPLA_2$-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-1β, 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 $sPLA_2$-IIA (ED_{50} = 68 nM) induced neuronal cell death more effectively than did human $sPLA_2$-IIA (ED_{50} = 1.1 μM).

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-1β, IL-2, IL-6, TNF-α, IFN-γ, M-CSF, and M-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 $sPLA_2$ Inhibitors on $sPLA_2$-IIA–Induced Neuronal Cell Death. To determine how the enzymatic activity of $sPLA_2$-IIA is involved in neuronal cell death, we examined the effects of $sPLA_2$ inhibitors on $sPLA_2$-IIA–induced neuronal cell death (Fig. 4). Indoxam prevented $sPLA_2$-IIA–induced neuronal cell death in a concentration-dependent manner (Fig. 4A). Similarly, *p*-BPB a putative irreversible $sPLA_2$ inhibitor, showed a concentration-dependent neuroprotective effect (Fig. 4A).

The delayed neuronal cell death induced by $sPLA_2$-IIA suggested that $sPLA_2$-IIA inhibitors might prevent the death of neurons after $sPLA_2$-IIA treatment. We examined the

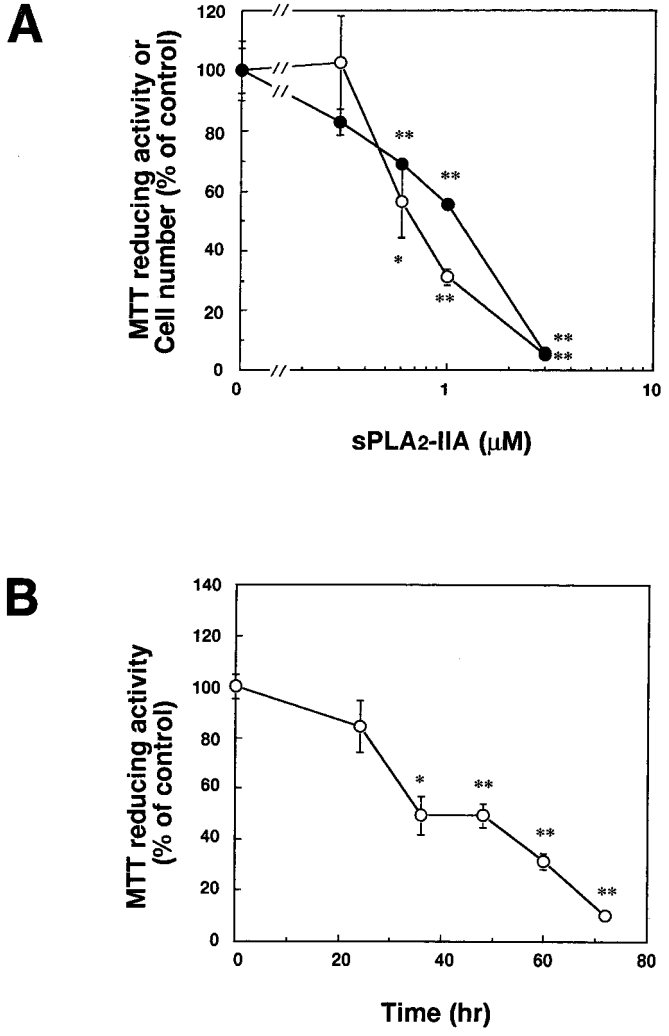

Fig. 3. Effect of $sPLA_2$-IIA on cell survival of cortical cultures. A, dose response: cortical cultures were treated with the indicated concentrations of $sPLA_2$-IIA. MTT reducing activity (○) and cell densities (●) were determined 48 h later. The cell density of control cultures was $2.7 \pm 0.2 \times 10^5$ cells/cm^2. B, time course: cortical cultures were treated with 1 μM $sPLA_2$-IIA. MTT-reducing activity was determined at the indicated time points after $sPLA_2$-IIA treatment. Data are expressed as means ± 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 $sPLA_2$-IIA on the survival of various cells

Various cells were treated with 3 μM $sPLA_2$-IIA. MTT-reducing activity was measured 48 h later. Data are expressed as means ± 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 ± 0.48**
Astrocytes	Brain	Human	103.22 ± 5.33
Astrocytes	Brain	Rat	98.73 ± 2.37
Myocytes	Heart	Rat	92.84 ± 8.17
Hepatocytes	Liver	Human	144.79 ± 8.08**
Mesangial cells	Kidney	Human	141.67 ± 2.30**
Mesangial cells	Kidney	Rat	150.23 ± 2.30**
Smooth muscle cells	Bronchia	Human	127.68 ± 5.14*
Smooth muscle cells	Arterial basilaris	Porcine	126.01 ± 3.82*
Endothelial cells	Brain	Human	94.44 ± 8.39
Endothelial cells	Aorta	Human	96.62 ± 5.26
Fibroblasts	Lung	Human	106.91 ± 3.24
Fibroblasts	Skin	Human	97.68 ± 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 $sPLA_2$-IIA were human recombinants. MTT-reducing activity was determined 48 h later. Data are expressed as means ± 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 ± 2.2
$sPLA_2$-IIA	1.0 μM	55.4 ± 1.4**
$sPLA_2$-IIA (rat)	0.1 μM	42.9 ± 5.3**
IL-1β	100 ng/ml	102.0 ± 0.9
IL-2	100 ng/ml	102.0 ± 1.1
IL-6	10 ng/ml	100.8 ± 2.0
TNF-α	100 ng/ml	105.6 ± 2.1
IFN-γ	100 ng/ml	91.4 ± 11.4
M-CSF	100 ng/ml	89.6 ± 5.3
GM-CSF	100 ng/ml	105.9 ± 8.1

** $P < 0.01$.

post-treatment effects of indoxam on sPLA$_2$-IIA–induced neuronal cell death. As shown in Fig. 4B, indoxam suppressed neuronal cell death when applied within 10 h after PLA$_2$-IIA treatment. Treatment with the sPLA$_2$-IIA inhibitor after application of sPLA$_2$-IIA protected neurons from sPLA$_2$-IIA–induced cell death, similar to the cotreatment.

Light Microscopic Changes in Neurons by sPLA$_2$-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 sPLA$_2$-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 sPLA$_2$-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 sPLA$_2$-IIA–treated neurons was suppressed completely by indoxam (Fig. 5F).

Apoptotic Features of sPLA$_2$-IIA–Treated Neurons. We studied the condensation of chromatin, a characteristic feature of apoptosis, in neurons (Fig. 6). sPLA$_2$-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 sPLA$_2$-IIA–treated cultures. The amount of condensed chromatin in sPLA$_2$-IIA–treated neurons was decreased significantly by indoxam (Fig. 6A; Table 3).

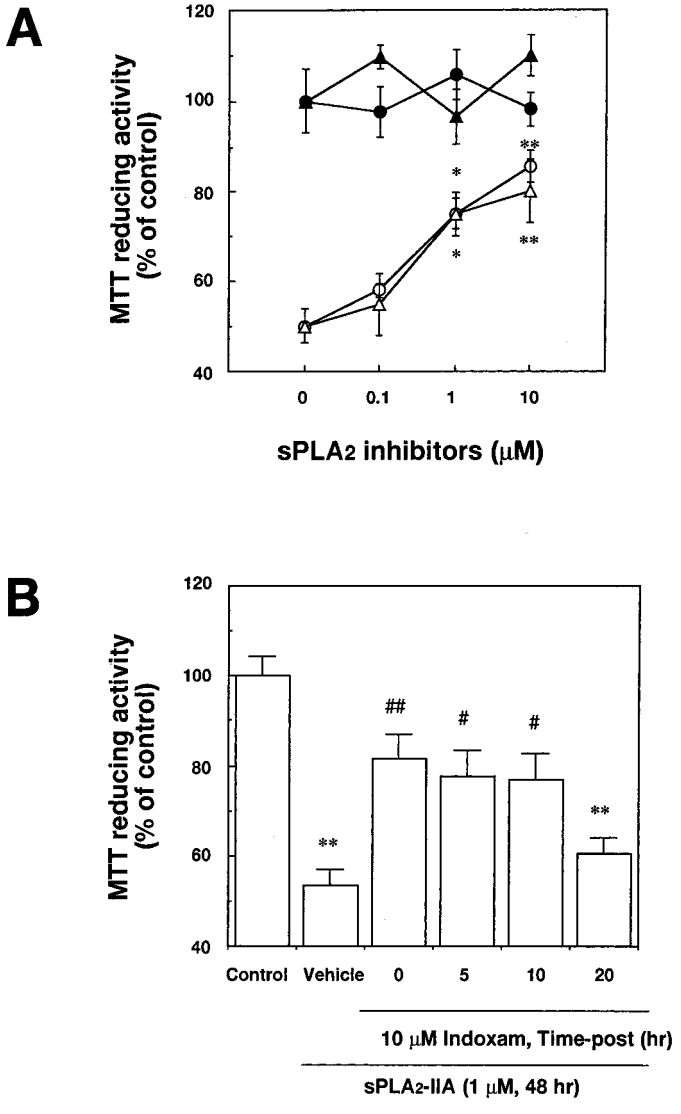

Fig. 4. Effects of PLA$_2$ inhibitors on sPLA$_2$-IIA–induced cell death of cortical cultures. A, dose response: *p*-BPB (triangles): vehicle (▲), or 100 μM sPLA$_2$-IIA (△) were preincubated with 0.01, 0.1, or 1 mM *p*-BPB for 20 min at 37°C. After preincubation, an aliquot was removed and diluted 100-fold in the culture medium (final concentration of sPLA$_2$-IIA = 1 μM). Indoxam (circles): cortical cultures were treated with vehicle (●) or 1 μM sPLA$_2$-IIA (○) in the presence of indoxam at the indicated concentrations. MTT-reducing activity was determined 48 h after sPLA$_2$-IIA treatment. Data are expressed as means ± S.E.M. (n = 4). *, $P < 0.05$; **, $P < 0.01$, compared with sPLA$_2$-IIA alone by ANOVA followed by Dunnett's test. B, treatment after application of sPLA$_2$-IIA: cortical cultures were treated with vehicle or 1 μM sPLA$_2$-IIA. Indoxam (10 μM) was applied at the indicated times after sPLA$_2$-IIA treatment. MTT-reducing activity was determined 48 h after sPLA$_2$-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.

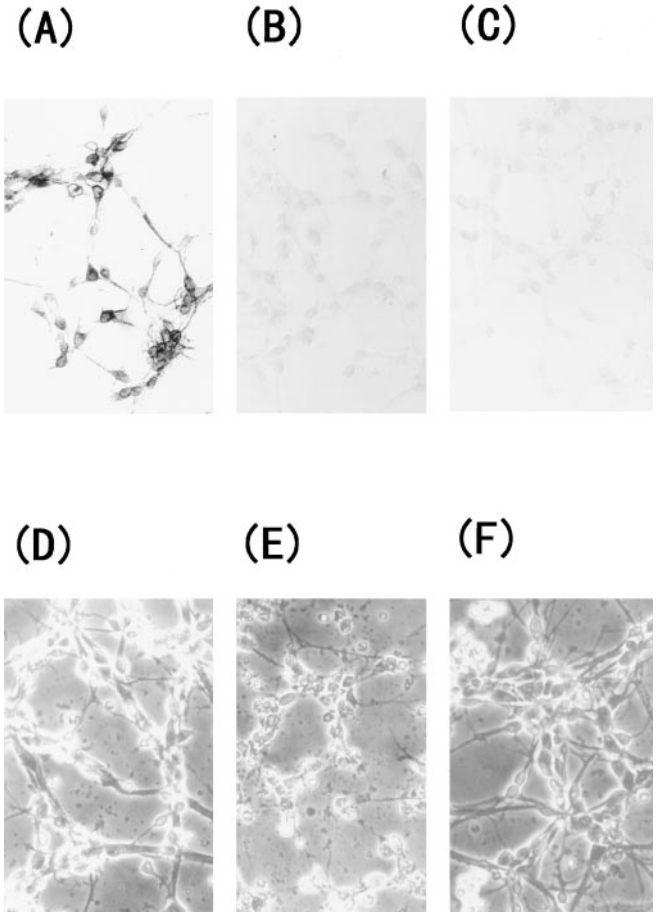

Fig. 5. Morphologic changes in cortical neurons by sPLA$_2$-IIA. Immunocytochemical analysis for anti-MAP2 (A), anti-GFAP (B), or anti-microglial antigen (C) was performed on cortical cultures. Cortical neurons were treated with control (D), 1 μM sPLA$_2$-IIA (E), or 1 μM sPLA$_2$-IIA + 10 μM indoxam (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 $sPLA_2$-IIA for 24 h, the number of TUNEL-positive nuclei was increased in $sPLA_2$-IIA–treated neurons compared with untreated control rats. The number of TUNEL-positive nuclei in $sPLA_2$-IIA–treated neurons was decreased significantly by indoxam (Fig. 6B; Table 3).

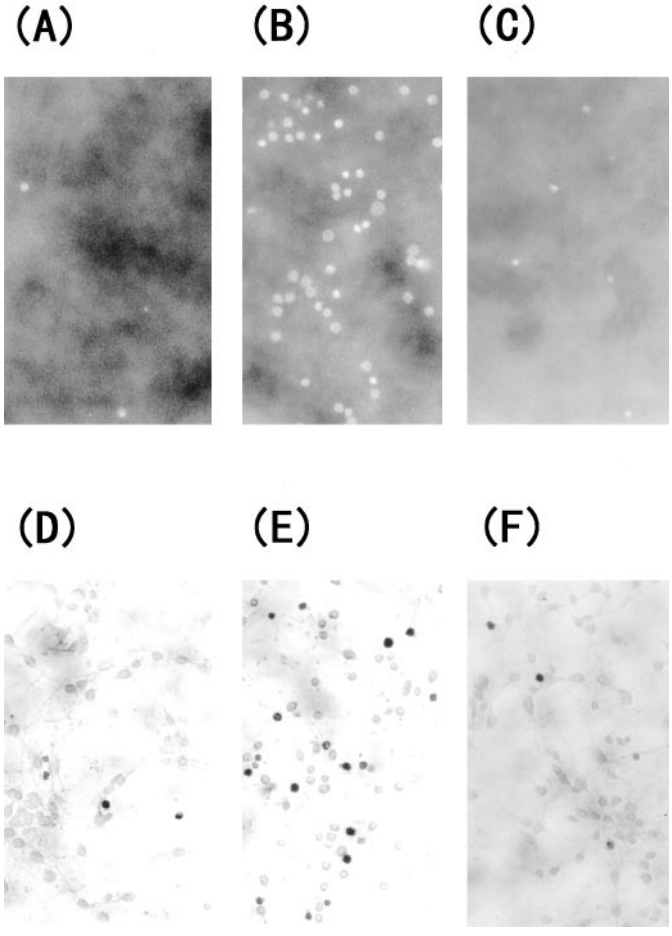

Fig. 6. Apoptotic features of cortical neurons induced by $sPLA_2$-IIA. Cortical neurons were treated with vehicle (A and D), 1 μM $sPLA_2$-IIA (B and E), or 1 μM $sPLA_2$-IIA + 10 μM indoxam (C and F). Neurons were stained with 10 μM Hoechst 33258 for 10 min 48 h later (A–C). Neurons were fixed with 4% paraformaldehyde, washed twice with PBS, and stained by the TUNEL technique 48 h later (D–F). Magnification, 100×.

TABLE 3

Effects of indoxam on $sPLA_2$-IIA-induced apoptotic features

Cortical neurons were treated with 1 μM $sPLA_2$-IIA in the absence or presence of 10 μM indoxam or 10 μM NS-398. Hoechst 33258- and TUNEL-positive neurons were detected 48 h later. Data are expressed as means ± S.E.M. (n = 4). Comparisons were made by ANOVA followed by Dunnett's test.

Treatment	Hoechst 33258	TUNEL
	cells/cm²	
Control	634.9 ± 84.0	253.8 ± 6.7
$sPLA_2$-IIA	9,079.6 ± 1,008.1**	523.8 ± 69.2**
$sPLA_2$-IIA + indoxam	730.0 ± 69.0##	285.7 ± 27.7##
$sPLA_2$-IIA + NS-398	802.4 ± 71.6##	306.3 ± 28.4##

** $P < 0.01$, compared with control.
$P < 0.01$, compared with $sPLA_2$-IIA.

Ultrastructural Changes in Neuronal Cell Death. Investigation of $sPLA_2$-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 $sPLA_2$-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), microtubles, 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

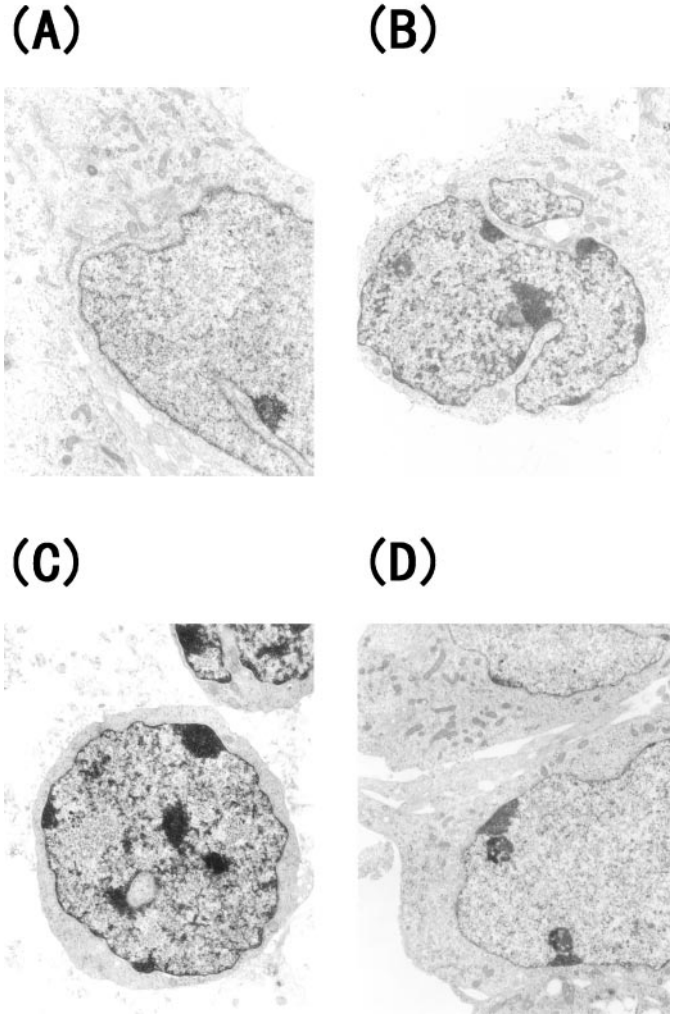

Fig. 7. Ultrastructural changes in cortical neurons by $sPLA_2$-IIA. Cortical neurons were treated with vehicle (A), 1 μM $sPLA_2$-IIA (B and C), or 1 μM $sPLA_2$-IIA + 10 μM indoxam (D). The cultures were examined 36 h (B) or 48 h (A, C, and D) later by electron microscopy. Neurons showed ultrastructural 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×.

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 sPLA$_2$-IIA–treated neurons was completely suppressed by indoxam (Fig. 7D).

sPLA$_2$-IIA–Induced Liberation of AA. In cooperation with other inflammatory stimuli, sPLA$_2$-IIA hydrolyzes phospholipids in membranes to liberate free AA (Murakami et al., 1991). We examined free [^{3}H]AA release during sPLA$_2$-IIA–induced neuronal cell death (Fig. 8). Neurons were incubated with [^{3}H]AA for 24 h for equilibration among phospholipids. Free [^{3}H]AA was spontaneously released from the neurons into the medium (Fig. 8A). In the absence of other cofactors or inflammatory stimuli, sPLA$_2$-IIA significantly increased the liberation of [^{3}H]AA from neurons before neuronal cell death (Fig. 8A). The level of free [^{3}H]AA was dependent on the concentration of sPLA$_2$-IIA from 0.1 to 3 μM at 25 h (Fig. 8B). On the contrary, regardless of the presence or absence of sPLA$_2$-IIAs, little liberation of free AA was observed from myocytes (Fig. 8A), which were insensitive to sPLA$_2$-IIA (Table 1). These results indicated that there was a close correlation between sPLA$_2$-IIA–induced AA release and neuronal cell death.

sPLA$_2$-IIA–Induced Generation of PGD$_2$. sPLA$_2$-IIA hydrolyzes phospholipids in membranes to generate PGD$_2$ from mast cells (Murakami et al., 1991). We examined the sPLA$_2$-IIA–induced generation of PGD$_2$ from neurons in the absence of other inflammatory stimuli (Fig. 9). The basal level of PGD$_2$ was not altered throughout the period of culture. When neurons were exposed to sPLA$_2$-IIA, generation of PGD$_2$ was not observed within 10 h, but increased transiently at 15 h and decreased thereafter (Fig. 9A). Moreover, sPLA$_2$-IIA generated PGD$_2$ from neurons in a concentration-dependent manner at concentrations ranging from 0.1 to 3

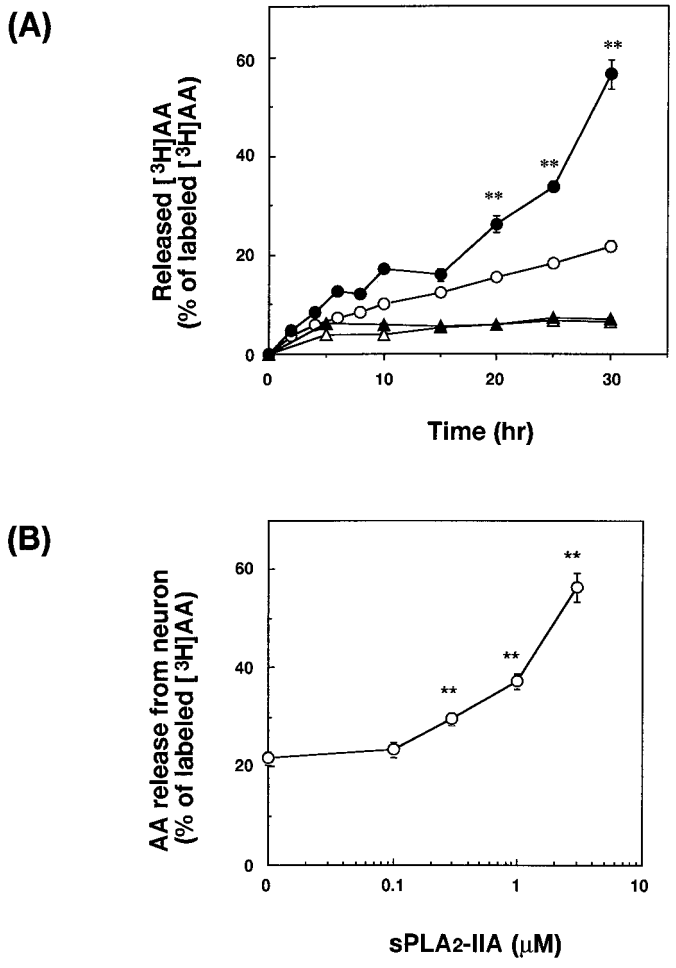

Fig. 8. sPLA$_2$-IIA–induced liberation of [^{3}H]AA from neurons. Cortical neurons were labeled with 1 μCi/ml [^{3}H]AA for 24 h. A, time course: labeled neurons were treated without (○) or with 1 μM sPLA$_2$-IIA (●). Labeled myocytes were treated without (△) or with 1 μM sPLA$_2$-IIA (▲). The radioactivity of [^{3}H]AA in the supernatant was measured at the indicated times after treatment. B, dose response: labeled neurons were treated with the indicated concentrations of sPLA$_2$-IIA. The radioactivity of [^{3}H]AA in the supernatant was measured 25 h after treatment. Data are expressed as means ± S.E.M. (n = 4). *, $P < 0.05$; **, $P < 0.01$, compared with untreated conditions by ANOVA followed by Dunnett's test.

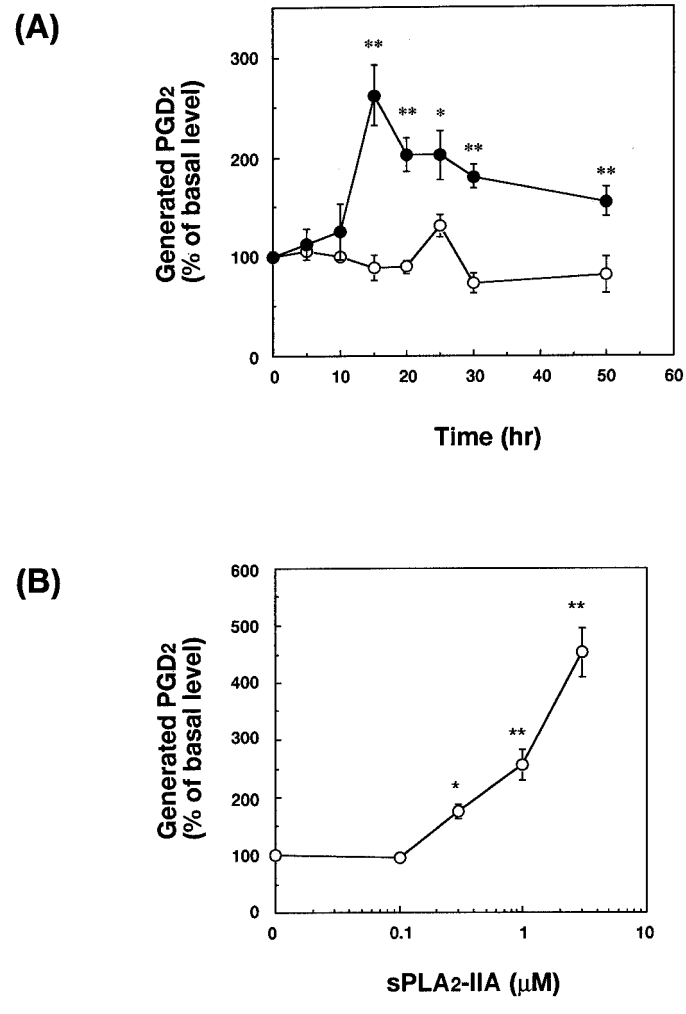

Fig. 9. sPLA$_2$-IIA–induced generation of PGD$_2$ from neurons. A, time course: cortical neurons were treated with vehicle (○) or 1 μM sPLA$_2$-IIA (●). PGD$_2$ released into the supernatant was measured at the indicated times after treatment. B, dose response: cortical neurons were treated with the indicated concentrations of sPLA$_2$-IIA. PGD$_2$ released into the supernatant was measured 15 h after treatment. Each level of PGD$_2$ generated from untreated neurons was measured as a control level. The control level of PGD$_2$ was 72 ± 3 pg/ml. Data are expressed as means ± 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 $sPLA_2$-IIA induced PGD_2 generation from neurons without cofactors or inflammatory costimuli before apoptosis.

Effects of the $sPLA_2$-IIA Inhibitor on the $sPLA_2$-IIA–Induced Liberation of AA and Generation of PGD_2. To ascertain whether the liberation of AA and the generation of PGD_2 were associated with neuronal cell death, we evaluated the effect of indoxam on the $sPLA_2$-IIA–induced liberation of AA and the generation of PGD_2 from neurons (Fig. 10). Indoxam attenuated the $sPLA_2$-IIA–induced AA liberation in a concentration-dependent manner. At 10 μM, the inhibitor suppressed the AA release completely by cotreatment with $sPLA_2$-IIA (Fig. 10A). Furthermore, indoxam significantly inhibited AA release from neurons within 10 h after the treatment with $sPLA_2$-IIA (Fig. 10A).

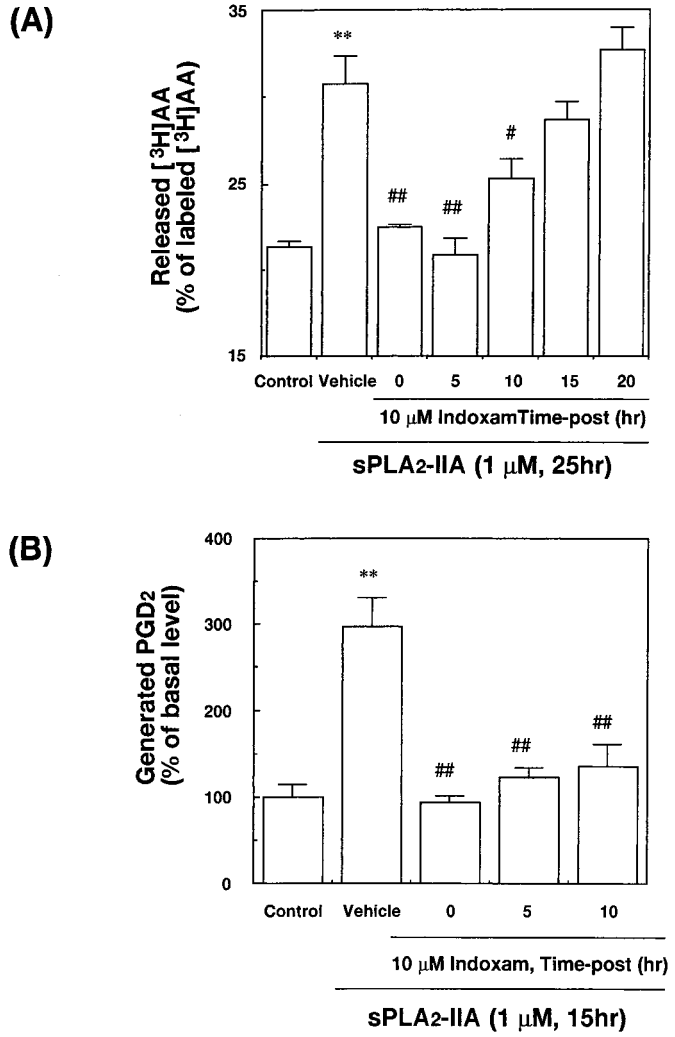

Fig. 10. Effects of indoxam on $sPLA_2$-IIA–induced liberation of [^{3}H]AA and generation of PGD_2 from neurons. A, AA: cortical neurons were labeled with 1 μCi/ml [^{3}H]AA at 37°C for 24 h. Labeled neurons were treated with 1 μM $sPLA_2$-IIA or not treated. Vehicle or 10 μM indoxam was applied at the indicated times after $sPLA_2$-IIA treatment. The radioactivity of [^{3}H]AA in the supernatant was measured 25 h later. B, PGD_2: cortical neurons were treated with 1 μM $sPLA_2$-IIA or not treated. Indoxam (10 μM) was applied at the indicated time points after $sPLA_2$-IIA treatment. PGD_2 released into the supernatant was measured 15 h later. The level of PGD_2 generated from untreated neurons was measured as the control level. The control level was 64 ± 9 pg/ml. 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.

Next, we estimated the effect of indoxam on $sPLA_2$-IIA–induced PGD_2 generation from neurons (Fig. 10B). The inhibitor attenuated PGD_2 generation in a concentration-dependent manner. At 10 μM, the inhibitor suppressed the PGD_2 generation completely by cotreatment with $sPLA_2$-IIA (Fig. 10B). Treatment with indoxam, even after occlusion, significantly blocked the $sPLA_2$-IIA–induced generation of PGD_2 within 10 h after $sPLA_2$-IIA–treatment (Fig. 10B). Thus, the $sPLA_2$-IIA inhibitor prevented neurons from the $sPLA_2$-IIA–induced liberation of AA and generation of PGD_2 not only by cotreatment, but also by treatment after occlusion.

Effect of a COX-2 Inhibitor on $sPLA_2$-IIA–Induced Neuronal Cell Death. To determine how PGD_2 is involved in neuronal cell death, we examined the effect of COX-2 inhibitor on $sPLA_2$-IIA–induced PGD_2 generation (Fig. 11A) and neuronal cell death (Fig. 11B). NS-398 inhibited PGD_2 generation in a concentration-dependent manner (IC_{50} = 13 nM). At 10 μM, NS-398 lowered the elevated level of PGD_2 significantly. There was no statistical difference in PGD_2 generation between control and 10 μM NS-398-added, $sPLA_2$-IIA–treated neurons. Thus, NS-398 markedly suppressed the generation of PGD_2 from $sPLA_2$-IIA–treated neurons.

Next, we investigated the effects of NS-398 on $sPLA_2$-IIA–induced neuronal cell death (Fig. 11B). NS-398 markedly prevented neurons from $sPLA_2$-IIA–induced cell death in a concentration-dependent manner (IC_{50} = 15 nM). At 10 μM, NS-398 significantly inhibited $sPLA_2$-IIA–induced neuronal cell death. Moreover, NS-398 ameliorated apoptotic features of $sPLA_2$-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, $sPLA_2$-IIA–treated neurons. Thus COX-2 metabolites were suggested to be critically involved in $sPLA_2$-IIA–induced apoptosis of neurons.

Effects of Eicosanoids and Inflammatory Factors on Neuronal Cell Survival. To determine how eicosanoids are involved in $sPLA_2$-IIA–induced cell death, we examined the effects of various eicosanoids on neuronal survival (Table 4). As shown in Fig. 12A, PGD_2 caused neuronal cell death in a concentration-dependent manner (ED_{50} = 8 μM). PGD_2 exhibited minimal toxicity during the first 6 h of treatment and displayed significant neurotoxicity only after prolonged exposure (Fig. 12B). Moreover, Δ^{12}-PGJ_2, one of the PGD_2 metabolites, killed neurons more potently than did PGD_2 as shown in Fig. 12A (ED_{50} = 1.1 μM). Δ^{12}-PGJ_2 caused neuronal cell death more rapidly than did PGD_2, without time lag (Fig. 12B). On the other hand, other eicosanoids such as PGE_2, 9α-11β-PGF_2, $PGF_2\alpha$, PGI_2, U-46619, and LTB_4 had no effect on neuronal cell survival (Table 4). Among the eicosanoids tested, only PGD_2 and Δ^{12}-PGJ_2 showed neurotoxicity.

$sPLA_2$-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

agents neither caused cell death nor modulated sPLA$_2$-IIA–induced neuronal cell death.

Discussion

In the present study, we demonstrated that mammalian sPLA$_2$-IIA induces cell death of cortical neurons via apoptosis. We propose a mechanism showing how sPLA$_2$-IIA is involved in stroke, as follows. First, cerebral ischemia triggers an increment in inflammatory factors such as TNF-α and IL-1β (Qin, 1998). Second, these cytokines increase secretion of sPLA$_2$-IIA from astrocytes (Oka and Arita, 1991), leading to the expression of sPLA$_2$-IIA in the ischemic brain (Lauritzen et al., 1994). Third, sPLA$_2$-IIA activity is elevated in the ischemic area such as the CTX. Fourth, sPLA$_2$-IIA liberates AA and generates excessively PGD$_2$, leading to apoptosis of neurons in the penumbra. Finally, apoptosis in neurons is followed by neurologic malfunctions in patients with stroke.

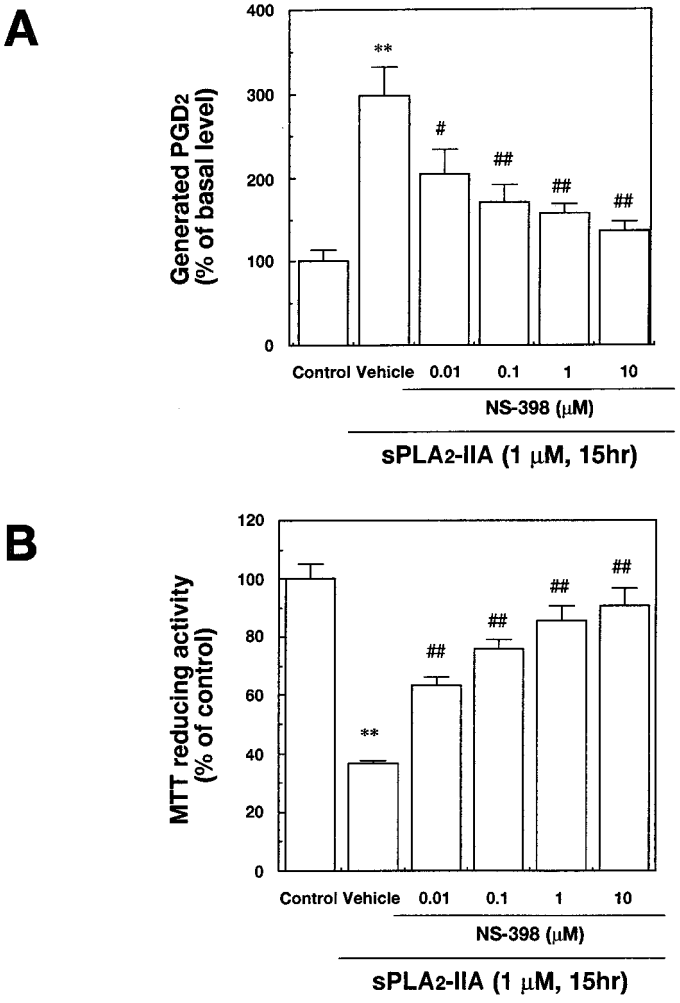

Fig. 11. Effects of NS-398 on sPLA$_2$-IIA–induced generation of PGD$_2$ and neuronal cell death. A, generation of PGD$_2$: cortical neurons were treated with 1 μM sPLA$_2$-IIA in the presence of NS-398 at the indicated concentrations. PGD$_2$ released into the supernatant was measured at 15 h after sPLA$_2$-IIA–treatment. The level of PGD$_2$ generated from untreated neurons was measured as the control level. The control level was 64 ± 9 pg/ml. Data are expressed as means ± S.E.M. (n = 4). **, $P < 0.01$, compared with untreated neurons; #, $P < 0.05$; ##, $P < 0.01$, compared with sPLA$_2$-IIA alone-treated neurons by ANOVA followed by Dunnett's test. B, neuronal cell death: neurons were treated with vehicle 1 μM sPLA$_2$-IIA in the presence of NS-398 at the indicated concentrations. MTT-reducing activity was determined 48 h after sPLA$_2$-IIA treatment. Data are expressed as means ± S.E.M. (n = 4). *, $P < 0.05$; **, $P < 0.01$, compared with sPLA$_2$-IIA alone by ANOVA followed by Dunnett's test.

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, sPLA$_2$ activity was significantly increased in the cortex, in which the ischemic core and the penumbra coexist. Both the elevated activity of sPLA$_2$ and the protein expression of sPLA$_2$-IIA were followed by neurologic damage in both areas. The sPLA$_2$ inhibitor reduced the elevated PLA$_2$ activity completely and the infarct volume significantly in the cortex. The inhibitor ameliorated occlusion-induced inflammation and neurodegeneration in the penumbra, suggesting that sPLA$_2$ might play an important role in apoptosis in the penumbra.

Among various sPLA$_2$s, sPLA$_2$-IIE, sPLA$_2$-V, and sPLA$_2$-X, but not sPLA$_2$-IIA, are expressed in the normal brain (Suzuki et al., 2000). On the other hand, the level of sPLA$_2$-IIA is elevated during the inflammatory state after ischemia (Lauritzen et al., 1994). Therefore, we ascertained whether sPLA$_2$-IIA causes cell death via apoptosis or necrosis in primary cultures of rat cortical neurons. The process of neuronal cell death induced by sPLA$_2$-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 integer fragments) was detected by conventional agarose gel electrophoresis (data not shown). Thus, human sPLA$_2$-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-

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 ± 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 ± 4.0
AA	30 μM	88.3 ± 11.9
PGD$_2$	10 μM	36.9 ± 3.1**
PGE$_2$	10 μM	102.6 ± 6.4
9α, 11β-PGF$_2$	10 μM	105.1 ± 2.7
PGF$_2\alpha$	10 μM	107.3 ± 1.3
PGI$_2$	10 μM	104.1 ± 2.5
U-46619	10 μM	96.5 ± 4.7
LTB$_4$	10 μM	90.3 ± 2.4
LPA	100 μM	98.1 ± 1.0
C$_2$-ceramide	10 μM	121.7 ± 7.0
SPHase	0.1 U/ml	99.3 ± 0.9
sPLA$_2$-IIA	0.1 μM	104.4 ± 0.1
SPHase + sPLA$_2$-IIA	0.1 U/ml, 0.1 μM	100.2 ± 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 $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-IIA–induced neuronal cell death, and neither LPA nor ceramide mimicked the action of $sPLA_2$-IIA on neurons in the present study. Thus, neither sphingomyelinase nor LPA seemed to be involved in the $sPLA_2$-IIA–induced apoptosis.

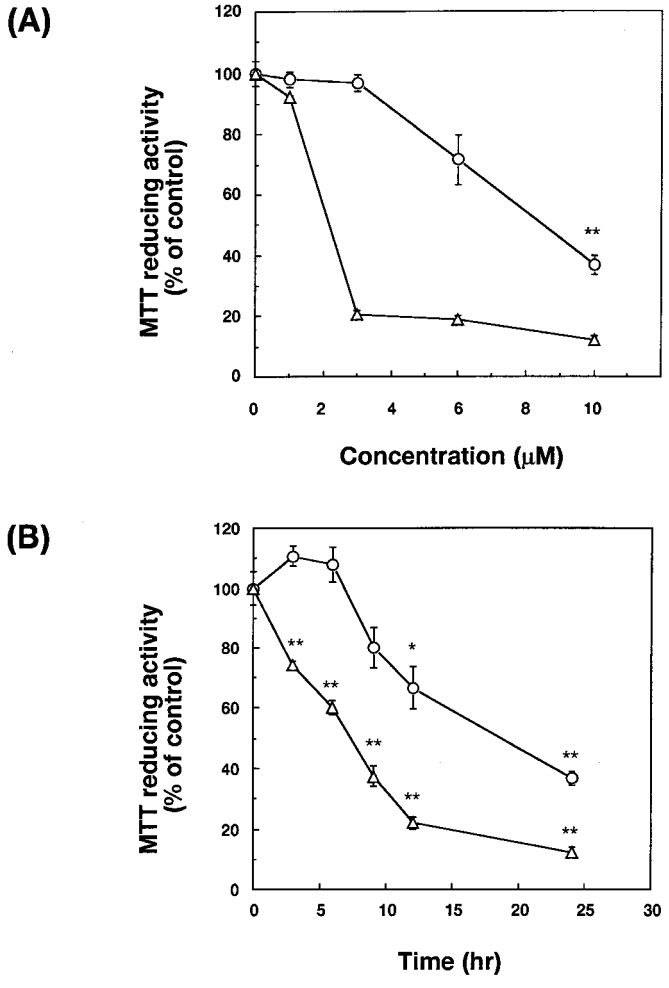

Fig. 12. Effect of PGD_2 and Δ^{12}-PGJ_2 on the survival of neurons. A, dose response: cortical neurons were treated with PGD_2 (○) and Δ^{12}-PGJ_2 (△) at the indicated concentrations. MTT-reducing activity was determined 24 h later. B, time course: cortical neurons were treated with 10 μM PGD_2 (○) and Δ^{12}-PGJ_2 (△). MTT-reducing activity was determined at the indicated times after the PG treatment. Data are expressed as mean ± S.E.M. values ($n = 4$). *, $P < 0.05$; **, $P < 0.01$, compared with untreated conditions by ANOVA followed by Dunnett's test.

$sPLA_2$-IIA exhibited neurotoxicity at concentrations around 1 μM in vitro. Does the concentration of endogenous $sPLA_2$-IIA reach to such a high level in vivo? Concentrations of in vivo $sPLA_2$-IIA were calculated under the assumption that $sPLA_2$-IIA contributed to most of the $sPLA_2$ 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 $sPLA_2$-IIA required in vitro was approximately 300 times higher than that expressed in vivo. This discrepancy could be explained by the following reports. First, $sPLA_2$-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, $sPLA_2$-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 $sPLA_2$-IIA is higher than exogenously added $sPLA_2$-IIA (Murakami et al., 1999). The striking difference between the amounts of $sPLA_2$-IIA required by different systems [e.g., exogenously added (micromolar) versus endogenously produced (nanomolar)] implies that the continued supply of $sPLA_2$-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 $sPLA_2$-II (Morgan et al., 1993). Indeed, $sPLA_2$-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 $sPLA_2$-II-mediated liberation of AA. Under our culture conditions, $sPLA_2$-IIA liberated AA significantly from neurons before apoptosis but not from myocytes, which are resistant to the enzyme. A specific $sPLA_2$ inhibitor, indoxam, significantly suppressed the $sPLA_2$-IIA–induced liberation of AA from neurons. Furthermore, indoxam and *p*-BPB prevented neurons from $sPLA_2$-IIA–induced apoptosis, suggesting that the neurotoxicity of $sPLA_2$-IIA depends on the susceptibility of cells to $sPLA_2$-IIA–mediated liberation of AA.

AA is metabolized to PGs and LTs by COX and lipoxygenase, respectively. $sPLA_2$-IIA significantly generated PGD_2 from neurons before cell death. AA increased gradually from 10 h and significantly from 20 h, whereas PGD_2 peaked sharply at 15 h and remained significantly elevated for 50 h. Why then did PGD_2 generation precede any significant release of AA? Besides PGD_2 (Murakami et al., 1991), $sPLA_2$-IIA generates PGE_2 from kidney 293 cells (Murakami et al., 1999) and LTB_4 from neutrophils (Mayer and Marshall, 1993). In the present study, PGE_2 was increased by $sPLA_2$-IIA, whereas LTB_4 was not (data not shown). However, the control level of PGE_2 and LTB_4 was two and four times as high as that of PGD_2 (data not shown). Moreover, AA released from neurons included not only the AA itself, but also its metabolites, such as PGD_2, LTB_4, and PGE_2. The content

ratio of PGD_2 was so low among AA metabolites that the increment in PGD_2 could not sufficiently reflect the elevation of released AA, which could explain the generation of PGD_2 from neurons before any significant release of AA.

PGD_2 is the major PG in the brain of mammalian species, and PGD_2 levels were markedly increased in the brain during reperfusion after ischemia, suggesting the involvement of PGD_2 in neurodegeneration (Gaudet et al., 1980). Indeed, PGD_2 caused marked neuronal cell death in our cortical cultures, whereas other eicosanoids such as PGE_2 and LTB_4 did not. The sPLA$_2$-IIA inhibitor prevented sPLA$_2$-IIA–treated neurons from PGD_2 generation and neuronal cell death. Furthermore, the COX-2 inhibitor also suppressed sPLA$_2$-IIA–treated neurons from PGD_2 generation and apoptosis. Taken together, these results indicate that PGD_2 was involved in sPLA$_2$-IIA–induced neuronal apoptosis. However, interpretation of PGD_2 as an apoptosis inducer requires circumspection for several reasons. First, the LD_{50} value of PGD_2 was high compared with the affinity for its receptors. Second, there was a latent period for PGD_2 to induce neuronal cell death. Third, little PGD_2 receptor was detected in the rat brain (data not shown). Finally, a PGD_2 receptor blocker did not prevent neurons from PGD_2 induced cell death (data not shown). How did PGD_2 induce cell death? In the present study, neurotoxicity was observed in one of the PGD_2 metabolites, Δ^{12}-PGJ_2. At low concentration, this metabolite caused neuronal cell death more potently than did PGD_2. In comparison with PGD_2, Δ^{12}-PGJ_2 killed neurons rapidly without a latent period. Δ^{12}-PGJ_2 induces apoptosis in other cells (Kim et al., 1993). Taken together, the sPLA$_2$-IIA–induced neuronal apoptosis could be ascribed to PGD_2 metabolites, possibly Δ^{12}-PGJ_2, but not to PGD_2 itself. Further studies are necessary for determining the pathologic role of PGD_2 and its metabolites in sPLA$_2$-IIA–induced neuronal cell death and stroke.

Optimal action of sPLA$_2$ generally requires membrane perturbation with induction of microdomains of membrane asymmetry. This microheterogeneity may be induced by cofactors such as venom-derived peptides and highly positively charged molecules. In contrast, human sPLA$_2$-IIA could hydrolyze membrane glycerophospholipids without costimuli or prior modifications of the cell membrane. sPLA$_2$-IIA required a latent period to induce the liberation of AA, the generation of PGD_2, and neuronal cell death. Furthermore, treatment with sPLA$_2$ inhibitor reduced the liberation of AA and the generation of PGD_2 after sPLA$_2$-IIA treatment. This raises the question of what happens in sPLA$_2$-IIA–treated neurons during the latent period. Pancreatic group I sPLA$_2$ (sPLA$_2$-IB) binds to its specific receptor (sPLA$_2$ 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 sPLA$_2$-IIA have not yet been identified. Neurotoxic sPLA$_2$ 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 sPLA$_2$ called OS_2 that, when injected into mouse brain, is lethal at very low doses (Lambeau et al., 1989). Studies using I-labeled OS_2 have revealed very high-affinity binding sites, *N*-type binding sites, in rat brain, which is interfered by bee venom sPLA$_2$ but not by mammalian sPLA$_2$ (Lambeau et al., 1989). Although we cannot rule out the possibility that membrane rearrangement of neurons might occur and provide substrates preferentially for sPLA$_2$-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 sPLA$_2$-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 sPLA$_2$ inhibitor after the sPLA$_2$-IIA treatment in cultured neurons and the MCA occlusion in rats.

In conclusion, sPLA$_2$-IIA induces apoptosis of neurons, possibly via metabolites of PGD_2, suggesting that sPLA$_2$-IIA plays a crucial role in apoptosis of neurons in the penumbra of the patients with stroke. Furthermore, the sPLA$_2$-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 sPLA$_2$-IIA inhibitor as a prelude to a new generation of drug treatments for stroke.

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