

# Inhibition of Neuronal Apoptosis by Docosahexaenoic Acid (22:6n-3)

## ROLE OF PHOSPHATIDYLSERINE IN ANTIAPOPTOTIC EFFECT\*

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Hee-Yong Kim‡, Mohammed Akbar, Audrey Lau, and Lisa Edsall

From the Section of Mass Spectrometry, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, Maryland 20852

**Enrichment of Neuro 2A cells with docosahexaenoic acid (22:6n-3) decreased apoptotic cell death induced by serum starvation as evidenced by the reduced DNA fragmentation and caspase-3 activity. The protective effect of 22:6n-3 became evident only after at least 24 h of enrichment before serum starvation and was potentiated as a function of the enrichment period. During enrichment 22:6n-3 incorporated into phosphatidylserine (PS) steadily, resulting in a significant increase in the total PS content. Similar treatment with oleic acid (18:1n-9) neither altered PS content nor resulted in protective effect. Hindering PS accumulation by enriching cells in a serine-free medium diminished the protective effect of 22:6n-3. Membrane translocation of Raf-1 was significantly enhanced by 22:6n-3 enrichment in Neuro 2A cells. Consistently, *in vitro* biomolecular interaction between PS/phosphatidylethanolamine /phosphatidylcholine liposomes, and Raf-1 increased in a PS concentration-dependent manner. Collectively, enrichment of neuronal cells with 22:6n-3 increases the PS content and Raf-1 translocation, down-regulates caspase-3 activity, and prevents apoptotic cell death. Both the antiapoptotic effect of 22:6n-3 and Raf-1 translocation are sensitive to 22:6n-3 enrichment-induced PS accumulation, strongly suggesting that the protective effect of 22:6n-3 may be mediated at least in part through the promoted accumulation of PS in neuronal membranes.**

Mammalian brain is rich in long chain polyunsaturated fatty acids. Docosahexaenoic acid (22:6n-3), the major n-3 fatty acid found in brain, is highly enriched in neuronal cells (1). Growing evidences support the essential role of 22:6n-3 in neuronal function. In animal models n-3 fatty acid deficiency caused memory deficit (2), learning disability (3, 4), and visual acuity loss (5). In humans, various neurological disease states have been shown to be associated with a deficient 22:6n-3 status, implying the influence of this fatty acid in neuronal function (6, 7). In the case of preterm infants with underdeveloped brains, the inclusion of 22:6n-3 fatty acid in infant formula has been shown to improve visual attention (8). More recently, it has been shown that 22:6n-3 is required for the survival of rat retinal photoreceptors (9) and exerts a protective effect on apoptosis of retinal photoreceptors during development (10).

Neuronal apoptosis normally occurs during the development and maturation period (11–13). However, it has been shown

that various neurodegenerative conditions are also associated with apoptotic neuronal cell death (14–16). Neuronal cell survival is critically dependent on the supply of trophic factors, which influences downstream signaling pathways (17). For example, in many cells phosphatidylinositol 3-kinase-dependent Akt serine/threonine kinase transduces a survival signal through phosphorylating proapoptotic protein BAD, which in turn associates with 14-3-3, preventing the interaction of BAD with Bcl-2 and Bcl-X<sub>L</sub> (18–20). Deprivation of trophic factors inhibits phosphatidylinositol 3-kinase/Akt and subsequently BAD phosphorylation, which enables binding of BAD to Bcl-X<sub>L</sub>, resulting in mitochondrial damage. Subsequent release of cytochrome *c* activates caspases, ultimately leading to apoptotic cell death (21).

Growing evidence indicates that Raf-1 activation, which is known to be essential for transducing signals of many growth factors, can play an important role in the regulation of apoptotic processes (22–24). Activation of Raf-1 kinase has been shown to prevent apoptosis in hematopoietic cells (22). It has been also shown that inhibition of Raf-1 in cells expressing BCR/ABL, which protects these cells from apoptosis induced by growth factor deprivation, can induce apoptosis (23). In addition, expression of constitutively active mitochondrial Raf-1 has been shown to restore antiapoptotic potential of a transformation-deficient BCR/ABL mutant (24). Recently, it has been reported that activation of mitochondrial Raf-1 is involved in the antiapoptotic effect of Akt (25). Although mechanisms of Raf-1 activation is complex and still remains controversial, translocation of Raf-1 to the membrane and subsequent phosphorylation are considered to be important steps for its activation (26–29). It has been shown that Raf-1 kinase contains distinct binding domains for acidic phospholipids, phosphatidylserine, and phosphatidic acid (30), and therefore the membrane localization of Raf-1 may be dependent on the concentration of these phospholipids.

Phosphatidylserine is the major acidic phospholipid in mammalian cell membranes and is particularly enriched with 22:6n-3 fatty acid (1). We have previously demonstrated that 22:6n-3, which is abundantly present in neuronal cells, promotes the accumulation of phosphatidylserine in cell membranes (31, 32). In the present study, we explored the biological significance of 22:6n-3 by examining its effect on apoptotic behavior upon trophic factor removal in relation to its capacity to modulate phosphatidylserine accumulation. We found that enrichment of neuronal cells with 22:6n-3 increased the accumulation of PS and the membrane localization of Raf-1, down-regulated caspase-3 activity, and prevented apoptotic cell death under serum-free conditions. Its protective potential was sensitive to the extent of PS accumulation, suggesting that the observed antiapoptotic effect of 22:6n-3 may be mediated at

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‡ To whom correspondence should be addressed: Section of Mass Spectrometry, LMBB/NIAAA, NIH, 12420 Parklawn Dr., Rockville, MD 20852. Tel.: 301-402-8746; Fax: 301-594-0035; E-mail: hykim@nih.gov.

least in part through the enhanced PS accumulation in neuronal membranes.

#### EXPERIMENTAL PROCEDURES

Dulbecco's modified Eagle's medium (DMEM),<sup>1</sup> fetal bovine serum, and other tissue culture reagents were obtained from Life Technologies, Inc. Monoclonal antibodies for Raf-1 and caspase-3 were purchased from Transduction Laboratories (Lexington, KY), and horse radish peroxidase-conjugated secondary antibodies were from Amersham Pharmacia Biotech. Apoptotic DNA ladder kit was purchased from Roche Molecular Biochemicals. Hoechst dye #33258 (bisbenzimidazole trihydrochloride #33258) was purchased from Sigma. Silica gel 60 plates were obtained from Analtech (Newark, DE). Fatty acids were obtained from Nu-Check (Elysian, MN). [1-<sup>14</sup>C]Docosahexaenoic acid (50 mCi/mmol) and [<sup>3</sup>H]thymidine (15 Ci/mmol) were purchased from NEN Life Science Products and Amersham Pharmacia Biotech, respectively.

**Cell Culture Conditions and Fatty Acid Supplementation**—Rat pheochromocytoma PC12 cells were obtained from American Type Cell Culture (ATCC). Cells were maintained in RPMI medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum in a 37 °C incubator containing 5% CO<sub>2</sub> water saturated atmosphere. Mouse neuroblastoma Neuro 2A cells (ATCC) were maintained in DMEM (Life Technologies, Inc.) with 5% fetal bovine serum in 75-cm<sup>2</sup> Corning culture flasks under a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. The medium was changed twice weekly, and cells were subcultured when confluent. For DNA fragmentation assay by sedimentation or Hoechst staining, cells were plated on 6-well plates at a density of 5 × 10<sup>4</sup>/cm<sup>2</sup> and 2.5 × 10<sup>4</sup>/cm<sup>2</sup>, respectively. For DNA or mRNA isolation, cells were cultured in 10 ml of medium in 10-cm culture dishes. For direct exposure of cells to fatty acids, fatty acids were bound to fat-free bovine serum albumin and presented to cells in medium containing 40 μM vitamin E during serum starvation. To test the effect of fatty acid enrichment, Neuro 2A cells were supplemented with fatty acids for 24 or 48 h and then subjected to serum starvation. Fatty acid stock solutions in chloroform or methanol were dried, bound to fetal bovine serum in the presence of 40 μM vitamin E, and diluted in DMEM under the argon atmosphere so that final concentrations of fatty acids and fetal bovine serum became 25 μM and 0.5%, respectively. Non-enriched controls were treated similarly during the enrichment period, but fatty acids were omitted.

**DNA Fragmentation Assay**—The DNA fragmentation assay by differential sedimentation was performed as reported earlier (33). Nuclei of PC12 or Neuro 2A cells were labeled with 1 μCi of [<sup>3</sup>H]thymidine for 24 h. To induce apoptosis, cells were washed gently twice with serum-free medium to remove unincorporated label and then incubated in the serum-free medium for 5–24 h. When cells were enriched with fatty acids before serum starvation, [<sup>3</sup>H]thymidine was added 24 h before the termination of enrichment. After serum starvation, cells were harvested and centrifuged at 200 × g for 10 min at 4 °C. An aliquot of the supernatant was then precipitated with 25% trichloroacetic acid. This fraction (S) reflects the amount of [<sup>3</sup>H]thymidine released during apoptosis induced by serum deprivation. The remaining cells were solubilized in a lysis buffer containing 0.2% Triton X-100 in 10 mM Tris/EDTA (TTE). The intact DNA (B) and the fragmented DNA (T) were then separated by centrifugation at 13,000 × g for 10 min at 4 °C. The fragmented DNA was precipitated from the supernatant with 25% trichloroacetic acid. The pellets were resuspended in 1% SDS and subjected to liquid scintillation. The percent DNA fragmentation is expressed as the sum of counts from (S + T)/(B + S + T) × 100.

**Analysis of DNA Ladder Formation**—Total genomic DNA was isolated from Neuro 2A cells by using an apoptotic DNA ladder assay kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. Briefly, after 48 h of serum withdrawal as mentioned above, the cells were harvested by trypsinization, suspended in 200 μl of PBS, and lysed with equal amount of lysis buffer and incubated at 70 °C for 10 min. After adding 100 μl of isopropanol, lysates were mixed, and the genomic DNA was sheared by passing a few times through a 25-gauge needle attached to a 1-ml disposable syringe. The whole lysate was

charged on glass filters and washed, and DNA was isolated. The isolated DNA was precipitated in ethanol and extracted with phenol/chloroform/isoamyl alcohol, air-dried, and suspended in Tris/EDTA buffer. Six to eight μg of total DNA was charged on 2% agarose gel (Bio-Rad) in loading buffer, electrophoresed in Tris-buffered EDTA buffer containing 1 μg/ml ethidium bromide at 75 volts, and photographed under UV illumination.

**Hoechst Staining**—After 48 h of serum deprivation, the medium was centrifuged gently at 100 × g to collect detached cells, which were subsequently fixed in 250 μl of 3.7% formaldehyde. The cells still attached to the plate were fixed directly on the plate with 750 μl of 3.7% formaldehyde for 15 min. Cells were combined and centrifuged, and then 100 μl of Hoechst dye (24 μg/ml) dissolved in 50% glycerol/PBS was added. After incubating for at least 10 min, cells were observed by fluorescence microscopy with a 365-nm filter.

**Immunoblotting of Caspase-3**—Neuro 2A cells were washed twice with cold PBS, and the pellet was suspended in 100 μl of lysis buffer that contained 20% Triton X-100, 50 mM NaCl, 25 mM Tris/HCl, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was determined by BCA assay using bicinchoninic acid reagent (34). Ten micrograms of protein were loaded onto a 15% SDS-polyacrylamide gel and electrophoresed at a constant current of 30 mA, then transferred from the gel to a polyvinylidene difluoride membrane at 45 volts for 1 h. Procaspase-3 (32 kDa) and the 17-kDa fragment were immunoblotted with anti-caspase-3 (Transduction Laboratories) and visualized by enhanced chemoluminescence detection.

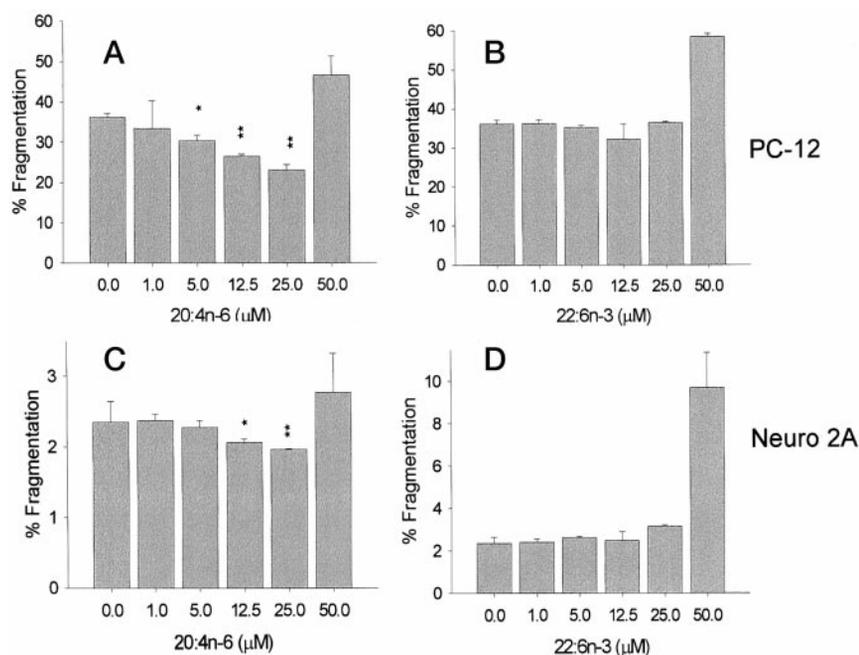
**Caspase-3 Activity Measurement**—Caspase-3 activity was measured using a colorimetric assay kit (Biomol, Plymouth Meeting, PA) according to the manufacturer's protocol. Briefly, cell lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and protein concentrations in the resulting supernatants were determined by BCA assay. Aliquots were incubated with acetyl-DEVD-p-nitroanilide for 2 h at 37 °C, and the absorbance at 405 nm was measured spectrophotometrically.

**Reverse Transcription-PCR Analysis of Raf-1 mRNA**—Total RNA was isolated from Neuro 2A cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol and quantified spectrophotometrically. One microgram of isolated RNA was treated with DNase 1 and used for first-strand cDNA synthesis. The treated RNA was incubated with 1 μl (0.5 μg) of oligo(dT)<sub>12–18</sub> primer for 10 min at 70 °C and reverse-transcribed by using 1 μl (200 units of Moloney murine leukemia virus reverse transcriptase) of Superscript II RT (Life Technologies, Inc.) in 20 μl of reaction buffer containing 2 μl of 2 × PCR buffer, 25 mM MgCl<sub>2</sub>, 1 μl of 10 mM dNTP mix, and 2 μl of 0.1 M of dithiothreitol. The mixture was placed in a Perkin-Elmer 2400 Gene Amp PCR system set at 42 °C for a 50-min cycle followed by a 15-min incubation at 70 °C and a 4 °C soak. After reaction, the prepared cDNA was recovered from the mixture after RNA was digested by incubating with 1 μl of RNase H (2 units) for 20 min at 37 °C. To 1–2 μl of template cDNA, 25 μl of PCR reaction buffer (PCR master mix, Roche Molecular Biochemicals) was added along with 1 μl (40 pmol) each of an upstream primer GTC CAG TAG CCC CAA CAA TC-3' (a 20-mer positioned at 202–221) and a downstream primer 5'-GCG CAG AAC AGC CAC CTC AT-3' (a 20-mer positioned at 517–498) obtained from Lofstrand Lab, Ltd (Gaithersburg, MD). PCR was then performed using 35 cycles programmed as follows: initial denaturation for 2 min at 94 °C and 15 s at 94 °C, annealing for 30 s at 55 °C, and primer extension for 1 min at 72 °C. One microliter (20 pmol) of each G3PDH primers (CLONTECH, California, CA) was used as a control providing a 450-base pair band. A band of 316 base pairs was visualized by illuminating under UV light after 4 μl of the PCR product was charged on 2% agarose gel containing 1 μg/ml ethidium bromide (Sigma) and electrophoresed for 1 h at 80 V.

**Fatty acid Incorporation Time Course**—Neuro 2A cells were seeded on 6-well plates at a density of 4 × 10<sup>5</sup>/cm<sup>2</sup> in 2 ml of DMEM containing 5% fetal bovine serum. On the next day, 0.5 μCi of [<sup>3</sup>H]22:6n-3 was added in 2 ml of DMEM containing 0.5% fetal bovine serum and 40 μM vitamin E. The final concentration of the fatty acid was adjusted to 20 μM with unlabeled fatty acids. After 5, 11, 24, and 48 h of incubation, the medium was removed, and the cells were washed with medium containing 0.2% bovine serum albumin twice. Cells were collected in methanol containing 0.5% (w/v) 2,6-di-*tert*-butyl-*p*-cresol (BHT), and lipids were extracted according to the method of Bligh and Dyer (35). The lipid extracts were dried and reconstituted in chloroform, and aliquots were taken for radioactivity counting. The rest of the extracts were mixed with 25 μmol each of standard phospholipids and loaded on the TLC plates. Lipids were separated, and each lipid band was scraped and subjected to liquid scintillation counting as described earlier (36). Separately, cells were enriched with 20 μM nonlabeled 18:1n-9 or 22:6n-3, and lipids were extracted as described above. Phosphatidylserine

<sup>1</sup> The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; 22:6n-3, docosahexaenoic acid; 20:4n-6, arachidonic acid; 18:1n-9, oleic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; LC/MS, liquid chromatography/mass spectrometry; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BDNF, brain derived neurotrophic factor; iPLA<sub>2</sub>, Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub>.

**FIG. 1. Effect of fatty acids on DNA fragmentation induced by serum starvation.** PC-12 or Neuro 2A cells were prelabeled with [<sup>3</sup>H]thymidine for 24 h and then incubated in serum-free media for 5 h in the presence of fatty acids at 0–50  $\mu$ M. DNA fragmentation was evaluated by sedimentation assay. The data presented are representative of three experiments, each of which was performed using triplicate cultures. Data are expressed as means  $\pm$  S.D. Unpaired Student's *t* test was performed in comparison to the control value. \*, *p* < 0.05; \*\*, *p* < 0.01.

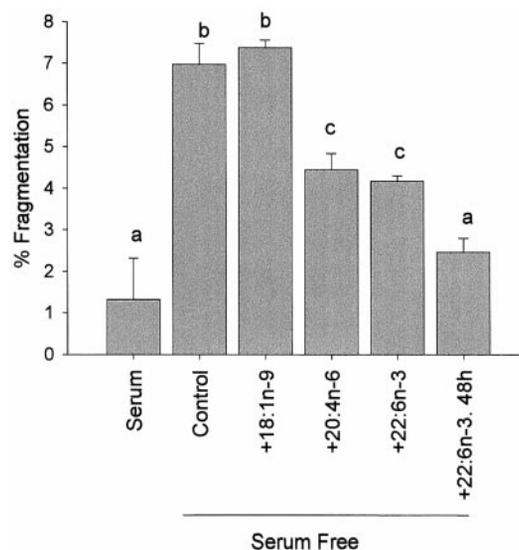


molecular species were determined by electrospray liquid chromatography/mass spectrometry as described previously (31, 32, 37).

**Preparation of Unilamellar Vesicles**—Liposomes of varying concentrations of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) were prepared by the following methods. Desired amounts of 18:0–22:6 PC, PE, and PS (Avanti Polar Lipids, Alabaster, AL) in chloroform were mixed, then dried under argon. Lipids were reconstituted in 2 ml of 75  $\mu$ M 2,6-di-*tert*-butyl-*p*-cresol (BHT) in cyclohexane. Samples were frozen on dry ice and then lyophilized under vacuum until only a lipid film remained. The samples were purged under argon before removing to an argon box, whereupon the lipids were reconstituted with an appropriate volume of 50  $\mu$ M diethylenetriamine pentaacetic acid in PBS. Solutions were mixed with a Vortex until a colloidal suspension formed and then passed through a 0.1- $\mu$ m polycarbonate filter on a mini-extruder (Avanti Polar Lipids) 11 times to make unilamellar vesicles.

**Analysis of Raf-1 and Membrane Interaction**—Anti-Raf-1 antibody (Transduction Laboratories) was immobilized on a CM5 sensor chip (Biacore, Uppsala, Sweden) using Biacore X as directed by manufacturer's instructions. Briefly, upon activating the chip with *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysuccinimide, bovine serum albumin-free anti-Raf was coupled to the sensor chip for a total of approximately 5000 RUs bound. After the coupling was finished, the chip surface was deactivated with ethanolamine/HCl, pH 8.5. The wash buffer used throughout was PBS. Raf-1 was captured on the chip using cell lysate from Neuro-2A cells (ATCC). The cell lysate was collected in radioimmune precipitation buffer (1 $\times$  PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) containing 100  $\mu$ g/ml phenylmethylsulfonyl fluoride. One flow cell was kept as the control cell, and no lysate was passed over this cell. The experimental cell had 15–20 RUs of Raf-1 captured on the surface, allowing liposome and Raf-1 interaction at approximately a 1:1.5 ratio. The liposomes were injected into the flow cells, and the association with and dissociation from Raf-1 was measured. Regeneration of the anti-Raf surface was completed in a two-step process. First, 10 mM acetate, pH 4.0, was injected into the cells, followed by an injection of radioimmune precipitation buffer. After allowing the chip surface to equilibrate with PBS, the chip was again ready for use. The recovered protein was analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blotting using Raf-1 antibody to confirm the identity of the captured protein.

**Translocation of Raf-1 in Neuro 2 Cells**—After enrichment of Neuro 2A cells with fatty acids for 48 h, Neuro-2A cells were washed and grown overnight in serum-free DMEM at 37  $^{\circ}$ C. The next day, experimental cells were stimulated with 250 ng of recombinant human BDNF (Promega, Madison, WI) in 5 ml of serum-free DMEM for 5–30 min at 37  $^{\circ}$ C. Membrane and cytosolic fractions were separated as described earlier (30), with slight modifications. Briefly, after the incubation, cells were washed with ice-cold PBS buffer, collected in 10 ml of Buffer A (10 mM Hepes, pH 7.4, 2 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM phenylmeth-

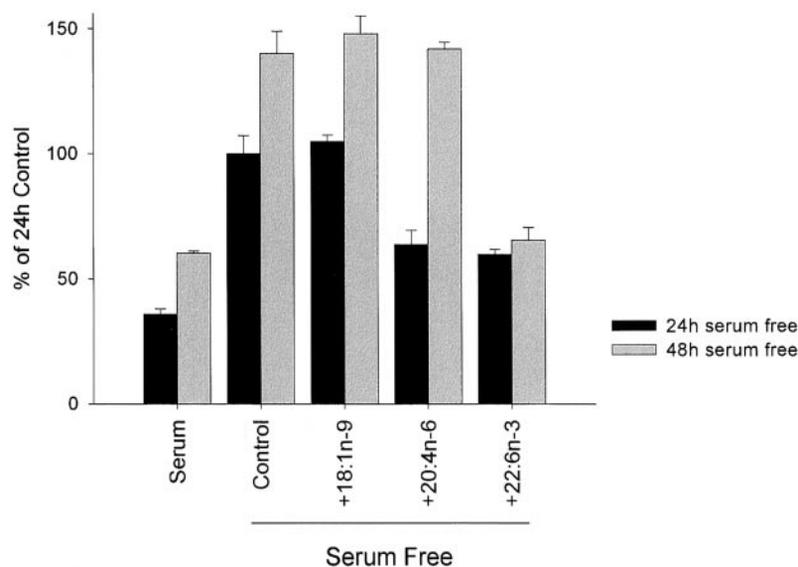


**FIG. 2. Effect of the fatty acid enrichment on DNA fragmentation induced by serum starvation in Neuro 2A cells.** Cells were enriched with fatty acids at 25  $\mu$ M for 24–48 h and subsequently exposed to serum-free conditions. DNA fragmentation was then evaluated by sedimentation assay. For labeling, [<sup>3</sup>H]thymidine was added 24 h before the termination of enrichment. The data presented are representative of three experiments, each of which was performed using triplicate cultures. Data are expressed as means  $\pm$  S.D. Statistical significance was tested using Bonferroni/Dunn *post hoc* analysis. The values designated with different letters are significantly different from each other (*p* < 0.05).

ylsulfonyl fluoride), pelleted, and lysed by sonication in 40  $\mu$ l of Buffer B (Buffer A plus 50 mM NaF, 10  $\mu$ g/ml aprotinin, and 10 mg/ml leupeptin). Unbroken cells and nuclei were removed by centrifuging at 1000  $\times$  *g* for 5 min at 4  $^{\circ}$ C. The supernatant was further centrifuged at 100,000  $\times$  *g* for 80 min at 4  $^{\circ}$ C. The supernatant (cytosol fraction) was collected, and the membrane pellet was solubilized by sonication in 80  $\mu$ l of Buffer B containing 100 mM NaCl and 1% Triton X-100. The protein content was measured by the BCA protein assay. The Raf-1 protein from cytosol and membrane fractions was detected by SDS-polyacrylamide gel electrophoresis and Western blotting.

**Statistical Analysis**—Statistical analysis was performed using the Student's *t* test or Bonferroni/Dunn *post hoc* analysis.

**FIG. 3. Effect of the serum starvation period on the DNA fragmentation in Neuro 2A cells.** Cells were first enriched with various fatty acids for 24 h in the presence of [ $^3$ H]thymidine and subsequently exposed to the serum-free condition for 24 or 48 h. The data presented are representative of two experiments, each of which was performed using triplicate cultures. Data are expressed as % of control value at 24-h serum starvation (means  $\pm$  S.D.).



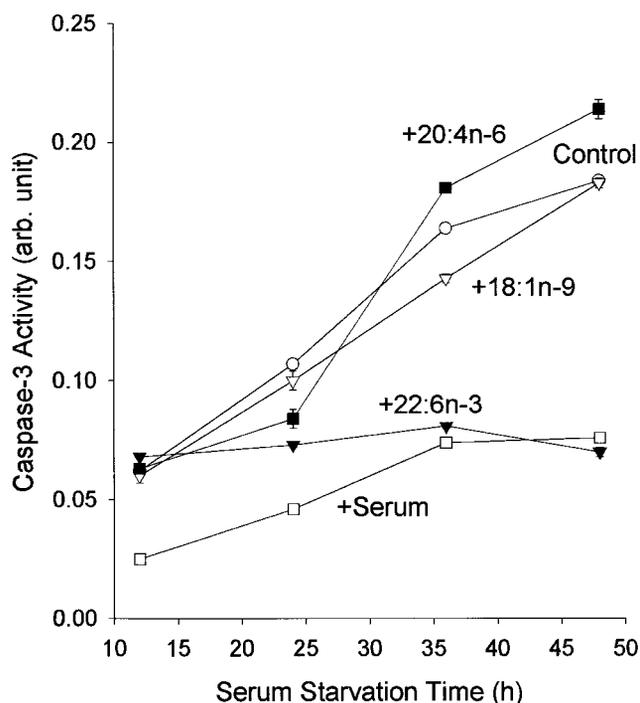
## RESULTS

*Direct Exposure of Neuronal Cells to 22:6n-3 Does Not Prevent DNA Fragmentation Induced by Serum Starvation*—Incubation of PC-12 or Neuro 2A cells under the serum-free conditions for 5 h induced apoptotic cell death, as determined by genomic DNA fragmentation, although Neuro 2A cells yielded much less fragmentation. Although coincubation of cells with 20:4n-6 during the serum deprivation period dose-dependently decreased the genomic DNA fragmentation induced by serum starvation, 22:6n-3 (1–25  $\mu$ M) was not effective at all (Fig. 1). At a higher concentration (50  $\mu$ M), both fatty acids were toxic, and DNA fragmentation increased significantly. The protective effect appeared to be 20:4n-6-specific, since 12.5–25  $\mu$ M 18:1n-9 did not have any significant effect as was the case with 22:6n-3.

*Prolonged Enrichment with 22:6n-3 Reduces DNA Fragmentation by Serum Starvation*—Since 22:6n-3 fatty acid exists mainly as membrane phospholipids in neuronal cells, accumulation of this fatty acid in membrane phospholipids may play an important role rather than the free fatty acid itself. Therefore, Neuro 2A cells were first enriched with 25  $\mu$ M 22:6n-3, and the DNA fragmentation induced by subsequent serum starvation was examined (Fig. 2). Unlike the case with direct exposure of 22:6n-3 during 5-h serum starvation periods, Neuro 2A cells enriched with 22:6n-3 for 24 h before serum starvation showed considerably less DNA fragmentation in comparison with the cells enriched with 18:1n-9 or non-enriched controls. Enrichment of cells with 22:6n-3 for 48 h further protected cells, as was indicated by even less DNA fragmentation. It was observed that the extent of protection or the degree of DNA fragmentation induced by serum starvation differed depending on the cell conditions or lot to lot variations in serum or medium constituents. However, it was consistently observed that the protective effect of 22:6n-3 was improved as the cells were enriched for a longer period up to 48 h.

*The Antiapoptotic Effect of 22:6n-3 Enrichment Is Maintained during Prolonged Serum Starvation Period*—The cells enriched with 20:4n-6 also showed similarly less DNA fragmentation (Fig. 2); however, the protective effect was sensitive to the duration of serum starvation as shown in Fig. 3. The protective effect of 20:4n-6 observed during the 24-h serum-free conditions was abolished when the cells were deprived of serum for 48 h. Only 22:6n-3 remained protective, with up to 48 h of serum starvation.

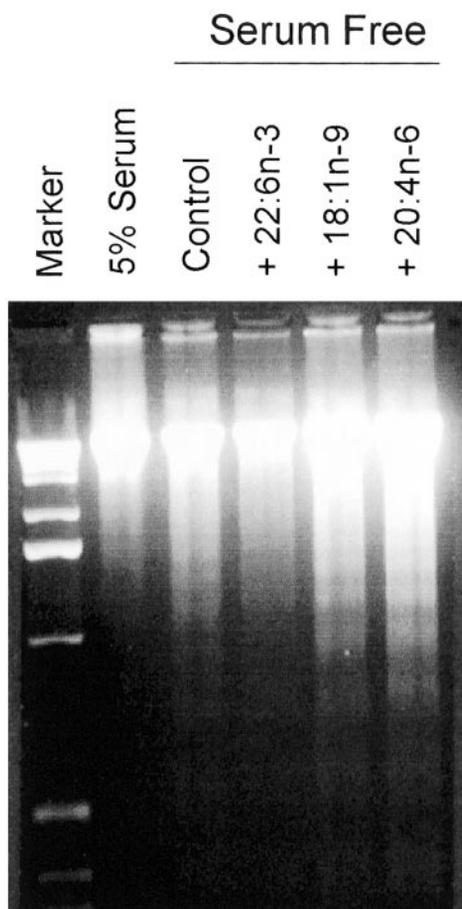
Similar results were obtained for the activity of caspase-3, a member of cysteine protease family that has been shown to



**FIG. 4. Effect of the serum starvation period on caspase-3 activity in Neuro 2A cells.** Cells were first enriched with various fatty acids (25  $\mu$ M) for 48 h and subsequently exposed to the serum-free condition for up to 48 h. Data are expressed as means  $\pm$  S.D. obtained from triplicate cultures.

mediate apoptosis in mammalian cells. Neuro 2A cells were first enriched with various fatty acids for 48 h and then exposed to the serum-free medium for up to 48 h, during which period caspase-3 activity was followed. Fig. 4 shows the increase of caspase-3 activity as a function of the starvation period, with the exception of cells enriched with 22:6n-3. After 24 h of serum starvation, both 20:4n-6- and 22:6n-3-treated cells showed less caspase-3 activity in comparison to non-enriched control or 18:1n-9-enriched cells. Upon prolonged serum starvation, however, the protective effect of 20:4n-6 was no longer observed, and only 22:6n-3-treated cells maintained caspase-3 activity at a level similar to 5% serum control.

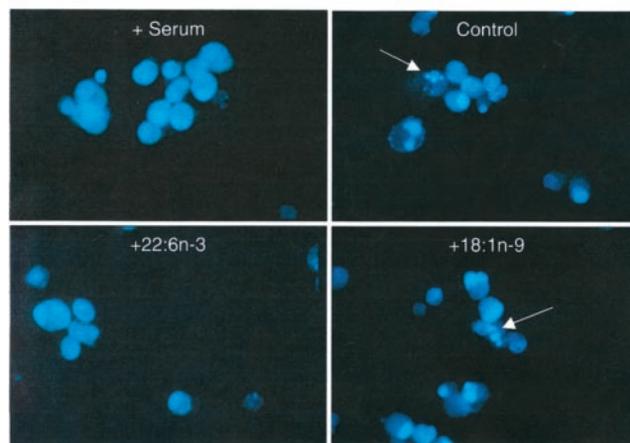
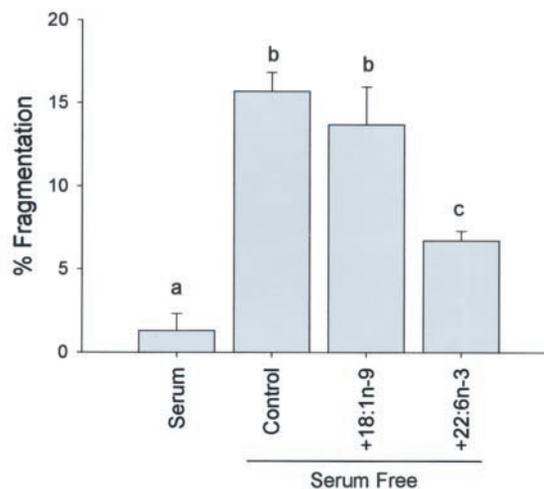
Neuro 2A cells exhibited a characteristic DNA ladder pattern on an agarose gel after serum starvation, confirming the occurrence of apoptotic cell death. Neuro 2A cells required at least



**FIG. 5. Effect of fatty acid enrichment on DNA fragmentation.** Neuro 2A cells were enriched with various fatty acids ( $25 \mu\text{M}$ ) for 48 h and then subjected to serum deprivation for 48 h. Isolated DNA was analyzed by agarose gel electrophoresis. The representative data from at least three experiments is presented.

48 h of serum starvation for DNA ladder formation. Shown in Fig. 5 is the DNA ladder observed after the cells were enriched with fatty acids for 48 h and subsequently deprived of serum for 48 h. In agreement with the data shown in Figs. 3 and 4, DNA ladder formation was significantly reduced after enrichment with 22:6n-3, whereas other fatty acids such as 18:1n-9 or 20:4n-6 did not decrease the level of fragmentation. Consistent results were also obtained using Hoechst staining as illustrated in Fig. 6. Serum-starved, non-enriched cells contained condensed, bright, and fragmented nuclei, representing cells undergoing apoptotic cell death. After enrichment with 22:6n-3 for 48 h before serum deprivation, the cells with fragmented nuclei were much less visible, whereas the same treatment with 18:1n-9 showed the results similar to the serum-starved, non-enriched control cells. All these data strongly suggest that the anti-apoptotic effect of fatty acid enrichment may be specific for 22:6n-3.

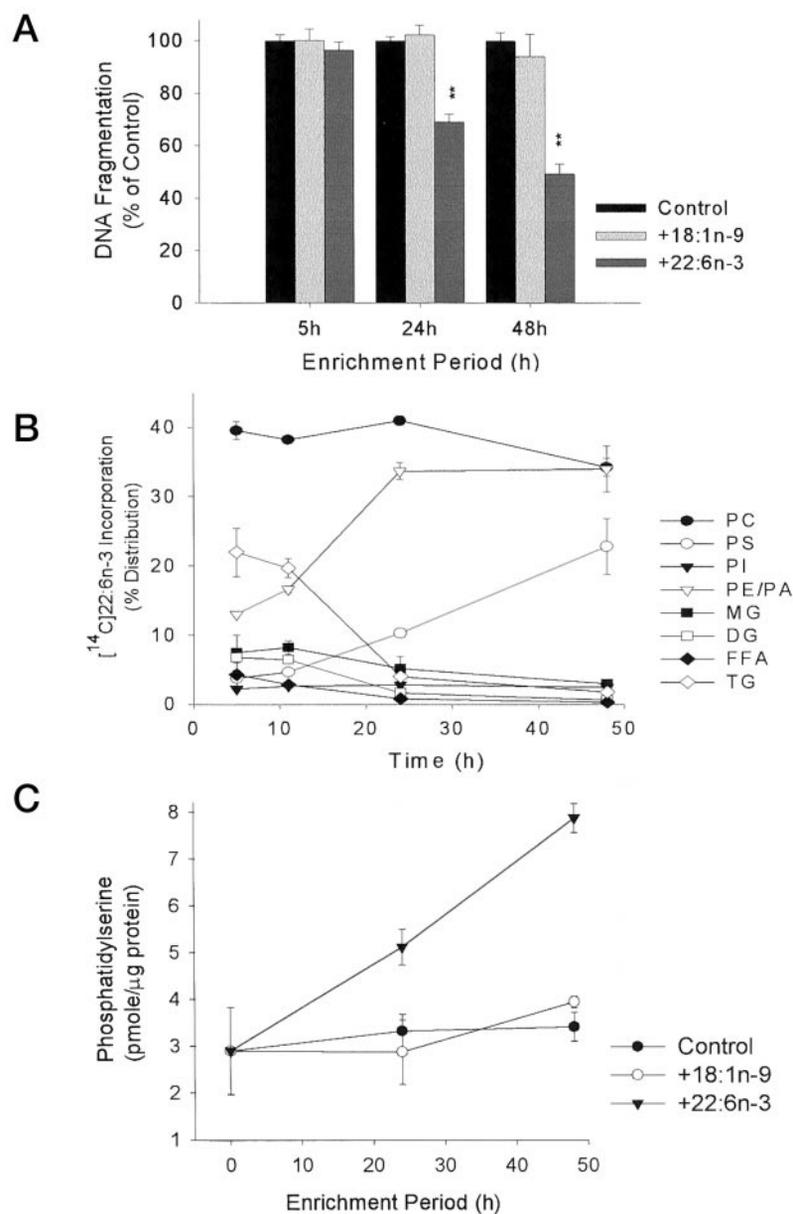
*The Protective Effect of 22:6n-3 Correlated with the Extent of PS Accumulation*—To relate biochemical mechanisms to the protective effect of 22:6n-3 after a prolonged enrichment period, the incorporation profile of this fatty acid was monitored in relation to its protective effect. As shown in Fig. 7B, [ $^{14}\text{C}$ ]22:6n-3, which was initially incorporated into neutral lipids, was progressively remodeled to phospholipids, especially PE and PS. When [ $^{14}\text{C}$ ]22:6n-3 was exposed to cells for 5 h, radioactivity was distributed into neutral lipids and phospholipids in the ratio of 41:59. Among these, triacylglycerol and PC were the major lipid classes labeled, and their distribution was  $22.0 \pm 3.5\%$  and  $39.6 \pm 1.3\%$ , respectively. The free fatty acid fraction



**FIG. 6. Effect of fatty acid enrichment on DNA fragmentation detected by Hoechst staining.** Neuro 2A cells were enriched with various fatty acids ( $25 \mu\text{M}$ ) for 48 h and then subjected to serum starvation for 48 h, followed by staining with bisbenzimidazole trihydrochloride (Hoechst 33258). Fragmented nuclei are indicated by arrows in the representative field of each sample. DNA fragmentation was evaluated by counting condensed or fragmented nuclei in each field where 500–800 cells were typically scored. Statistical significance was tested using Bonferroni/Dunn *post hoc* analysis. The values designated with different letters are significantly different from each other ( $p < 0.05$ ).

contained  $4.3 \pm 0.4\%$  of [ $^{14}\text{C}$ ]22:6n-3. At 24 h, [ $^{14}\text{C}$ ]22:6n-3 was significantly enriched in PE ( $33.7 \pm 1.2\%$ ) and PS ( $10.3 \pm 0.3\%$ ) in comparison to the 5-h point, where PE and PS contained only  $13.0 \pm 0.1$  and  $3.8 \pm 1.1\%$  of the [ $^{14}\text{C}$ ] label, respectively. Further enrichment for an additional 24 h significantly increased the incorporation of [ $^{14}\text{C}$ ]22:6n-3 in PS ( $22.8 \pm 4.0\%$ ), whereas [ $^{14}\text{C}$ ] label in PE appeared to be maintained ( $34.0 \pm 3.3\%$ ) at the level of the 24-h time point. After 48 h, more than 90% of the [ $^{14}\text{C}$ ] label was found in the phospholipid fraction. Phospholipid molecular species analysis by electrospray LC/MS revealed that the actual PS content of 22:6n-3-treated cells also increased steadily during 48 h of the enrichment period (Fig. 7C), corroborating the results obtained with radiolabeled 22:6n-3. The increase was mainly due to the 18:0,22:6-PS species, which changed from  $1.0 \pm 0.2$  to  $5.7 \pm 0.2 \text{ pmol}/\mu\text{g}$  of protein in 48 h. In contrast, non-enriched control or 18:1n-9-treated cells did not show any significant change in the PS content during the same enrichment period. Consequently, 22:6n-3-enriched cells showed a significantly higher total PS level ( $7.9 \pm 0.3 \text{ pmol}/\mu\text{g}$  of protein) in comparison with that of non-enriched control or 18:1n-9-treated cells ( $3.4 \pm 0.3$  and  $3.9 \pm 0.1 \text{ pmol}/\mu\text{g}$  of protein, respectively) at 48 h of enrichment. The protective effect of 22:6n-3 on DNA fragmentation induced by serum starvation

**FIG. 7. Fatty acid enrichment time course in relation to the protective effect of 22:6n-3.** Neuro 2A cells were enriched with 18:1n-9 or 22:6n-3 fatty acid (25  $\mu\text{M}$ ) for 5, 24, or 48 h, and the DNA fragmentation induced by subsequent 24-h serum starvation was evaluated (A). In parallel, incorporation of [ $^{14}\text{C}$ ]22:6n-3 (final concentration, 25  $\mu\text{M}$ ) into various lipid classes was followed (B), and PS accumulation was determined by LC/MS during the time course of enrichment with 25  $\mu\text{M}$  fatty acids (C). All data points were obtained from triplicate samples. Two independent experiments generated the similar results. In A, unpaired Student's *t* test was performed in comparison to the control value. \*\*,  $p < 0.01$ . TG, triacylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; MG, monoacylglycerol; DG, diacylglycerol; FFA, free fatty acid.



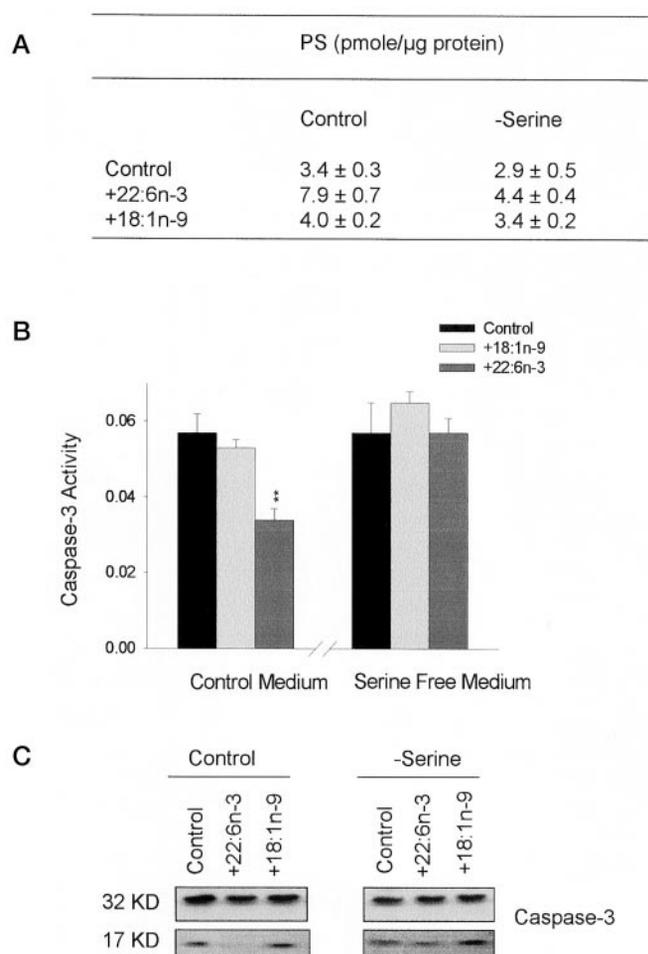
was potentiated as the cells were enriched for a longer period of time (Fig. 7A), and this increase paralleled the PS accumulation during the time course of enrichment.

In mammalian cells, PS is biosynthesized by the serine base exchange reaction (38). Therefore, to modulate the PS accumulation, Neuro 2A cells were enriched with fatty acids in a serine-free medium before serum starvation, and caspase-3 activation was examined in relation to PS accumulation (Fig. 8). Although depleting serine from the media did not completely offset the PS accumulation enhanced by 22:6n-3, increase in the PS content was significantly lessened in comparison with the cells enriched in the control medium (Fig. 8A). Concurrently, the protective effect of 22:6n-3 on caspase-3 activation, as shown by enzymatic activity (Fig. 8B) or Western blotting (Fig. 8C), was either not observed or trivial in the cells kept in the serine-free medium, supporting the notion that PS accumulation has a role in the protective effect of 22:6n-3. Neither the caspase-3 activity nor the PS content in 18:1n-9-treated or non-enriched control cells was significantly affected by the serine-free conditions.

Fatty acid enrichment did not alter sphingolipid contents significantly, although serine depletion decreased sphingolmy-

elin (SM) levels. The SM contents were estimated to be  $8.2 \pm 1.7$ ,  $9.8 \pm 0.8$ , or  $8.6 \pm 0.6$  pmol/ $\mu\text{g}$  of protein under serine adequate conditions and  $4.8 \pm 0.5$ ,  $3.8 \pm 0.6$  or  $5.6 \pm 0.5$  pmol/ $\mu\text{g}$  of protein under serine-deprived conditions for control or 22:6n-3- or 18:1n-9-enriched cells, respectively. The ceramide content could not be accurately quantified due to lack of a proper internal standard. Nevertheless, it was possible to compare ceramide contents in various cell culture samples based on the area ratios of ceramide peaks calculated against the deuterium-labeled PC internal standard peak in mass chromatograms. The area ratios thus obtained did not alter significantly after various fatty acid treatments or serine deprivation, indicating that ceramide levels were maintained at a relatively constant level.

*Enrichment of Neuro 2A Cells with 22:6n-3 Promotes the BDNF-induced Raf-1 Translocation to Membranes*—Raf-1 kinase has been suggested to be involved in cell survival (23–25). It has been shown that membrane translocation is required for the activation of Raf-1 (26, 27) and the regulatory domain interacting with PS plays an important role in this process (30). Therefore, modulation of PS accumulation by 22:6n-3 may influence the translocation of Raf-1. Neuro 2A cells, first enriched



**FIG. 8. Effect of serine deprivation on the accumulation of PS (A), caspase-3 activity (B), and cleavage of caspase-3 to the 17KD active fragment (C).** Neuro-2A cells were enriched with 18:1n-9 or 22:6n-3 fatty acid (25  $\mu$ M) for 48 h in the serine-free or control media. The PS content was determined by LC/MS at the end of the enrichment period in comparison to non-enriched controls. Enriched and non-enriched control cells were subsequently deprived of serum for 24 h, and caspase-3 activity and caspase-3 cleavage were evaluated after using acetyl-DEVD as a substrate and by Western blotting, respectively. The data in A and B were obtained from triplicate cultures, and the Western blot data shown in C is representative of triplicate samples. Two independent experiments generated similar results. In B, unpaired Student's *t* test was performed in comparison to the control value. \*\*,  $p < 0.01$

with 22:6n-3 for 48 h, were stimulated with BDNF for 30 min, and the membrane translocation of Raf-1 was examined in comparison with unstimulated cells. As shown in Fig. 9A, Raf-1 translocated from cytosol to membrane fraction in response to BDNF. Cells enriched with 22:6n-3 showed significantly higher levels of membrane-associated Raf-1 in comparison to non-enriched control or 18:1n-9-enriched cells under both basal and stimulated conditions. It was consistently observed that the expression of total Raf-1 kinase was highest in 22:6n-3-treated cells, as shown for protein and mRNA levels in Fig. 9B. These results indicated that 22:6n-3 enrichment not only facilitated the membrane translocation of Raf-1 but also up-regulated its expression in Neuro 2A cells.

**Interaction of Raf-1 with Lipid Membranes Is Dependent on the PS Concentration**—As indicated above, the enrichment of Neuro 2A cells significantly enhances PS accumulation as well as translocation of Raf-1. To determine whether the enhanced PS concentration in cell membranes played a role in the promoted translocation of Raf-1, the interaction of this protein with liposomes with varying proportions of PS was examined *in*

*vitro* using biomolecular interaction analysis. On a biosensor chip, Raf-1 protein was captured via immobilized anti-Raf-1 antibody, and the binding of liposomes to the captured Raf-1 was monitored by the changes in the surface plasmon resonance signal resulting from the changes of the mass concentration on the sensor chip surface due to the binding (39). The PS/PE/PC liposomes contained 50% PE and 0–50% PS and PC. As indicated in Fig. 10, the liposomes devoid of PS did not interact with Raf-1, whereas the interaction was greater as the PS proportion increased. All the phospholipids used contained 18:0, 22:6n-3 species, and the use of other fatty acid containing species also produced similar results (data not shown).

## DISCUSSION

In this study, we evaluated the role docosahexaenoic acid (22:6n-3) in neuronal apoptosis induced by serum starvation. We demonstrated that 22:6n-3 enhances PS accumulation and protects neuronal cells from apoptotic cell death as a membrane component. The observed positive correlation between PS accumulation and the protective effect by 22:6n-3 strongly suggested that the antiapoptotic effect of 22:6n-3 enrichment may be mediated at least in part through the promoted accumulation of PS in neuronal membranes. We also demonstrated that positive modulation of PS accumulation by 22:6n-3 facilitates the translocation of Raf-1 to membranes.

When 22:6n-3 was simply added to medium during the serum starvation period, the protective effect was not observed. In addition, the absence of serine during the enrichment period diminished the protective effect of 22:6n-3, suggesting that 22:6n-3, not as the free fatty acid form but as a membrane component, particularly PS, may exert its protective effect. The inhibition of caspase-3 activity, DNA fragmentation by sedimentation method, DNA ladder formation, Hoechst staining as well as caspase-3 cleavage to the 17-kDa active fragment consistently indicated the anti-apoptotic effect of 22:6n-3 after enrichment. In HL-60 cells, inhibition of sphingosine-induced apoptosis by 22:6n-3 only after 24 h of enrichment has been reported previously (40), similarly suggesting that 22:6n-3 as a membrane component may be responsible for the observed effect. Along with the modification of membrane phospholipid profile, the possibility of altered gene transcription by 22:6n-3 during the enrichment period cannot be excluded, since we observed the overexpression of Raf-1 in 22:6n-3-treated cells. In fact, a preliminary study identified several genes whose expression is affected by 22:6n-3 enrichment (data not shown). Nevertheless, the PS accumulation appears to be an important factor in these processes.

It has been documented that the final steps of 22:6n-3 biosynthesis in brain occurs in astroglia, where this fatty acid is readily released (36, 41), suggesting that one of the supporting roles of astroglia may include supplying 22:6n-3 fatty acid to neuronal cells for its enrichment. The 22:6n-3 thus provided appears to accumulate in neuronal membranes, since this fatty acid has been shown to be resistant to the PLA<sub>2</sub> action in neuronal cells (42, 43), in contrast to well documented 20:4n-6 release in response to various neurotransmitters (44). Among phospholipid classes in neuronal membranes, PS is particularly enriched with 22:6n-3, which composes 35–40% of the total fatty acid in PS (45). Concomitantly, the PS content has been shown to be higher in neuronal cells in comparison to non-neuronal cells (46, 47). All these data suggest that neuronal cells may incorporate and retain 22:6n-3 in phospholipids to sustain particularly high levels of PS in neuronal membranes. The present study also indicated that 22:6n-3 is a positive modulator of PS accumulation in Neuro 2A cells. Deprivation of serine from the media diminished the effect of 22:

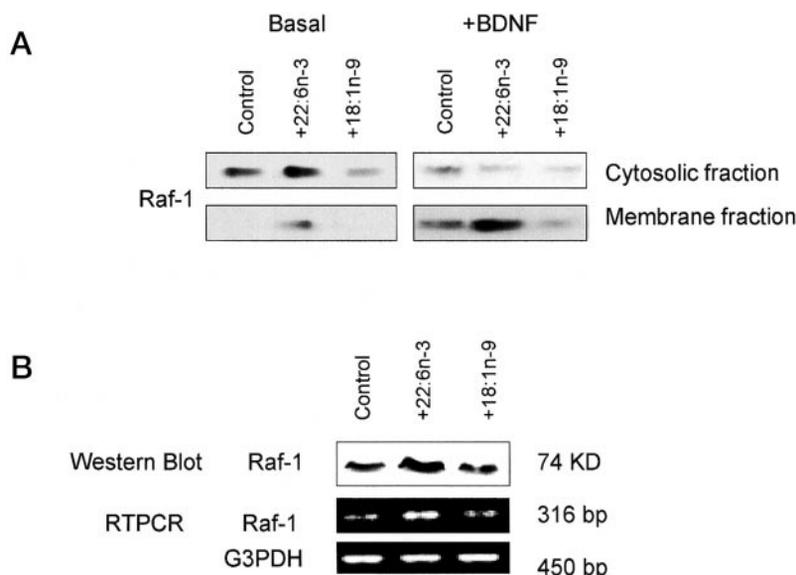


FIG. 9. Effect of fatty acid enrichment on the membrane translocation of Raf-1 (A) and Raf-1 expression (B). Neuro 2A cells were enriched with 25  $\mu$ M 22:6n-3 or 18:1n-9 fatty acid for 48 h and subjected to overnight serum starvation along with the non-enriched control cells. After cells were stimulated with BDNF (50 ng/ml) for 30 min, membrane and cytosolic fractions were separated. Stimulated Raf-1 translocation was evaluated in comparison to basal translocation by Western blotting (A). Separately, the levels of Raf-1 mRNA or protein in the whole cells were evaluated without fractionation of membrane and cytosol by Western blotting and reverse transcription-PCR (RT-PCR) (B). Three independent experiments generated the similar data. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; *bp*, base pairs.

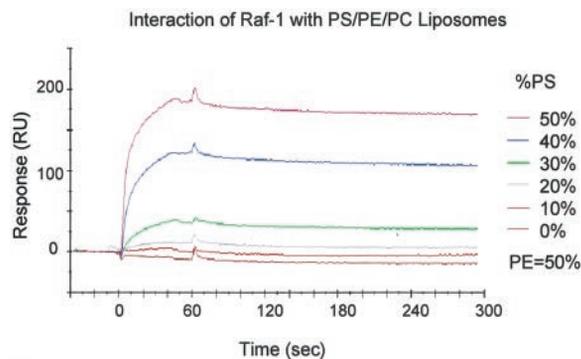


FIG. 10. Interaction of Raf-1 with PS evaluated *in vitro* using biomolecular interaction analysis. Unilamellar vesicles containing 18:0,22:6 species of PS/PE/PC in the ratio ranging from 0/50/50 to 50/50/0 were interacted with Raf-1 captured on a gold surface via immobilized anti-Raf-1 antibody as described under "Experimental Procedures." *RU*, relative units.

6n-3 on PS accumulation, indicating that PS biosynthesis is the major target for the enhanced PS accumulation.

The positive correlation between the antiapoptotic effect of 22:6n-3 enrichment and PS accumulation was evident since the protective effect consistently responded to the PS content modulated by the enrichment with various fatty acids, by varied enrichment period, and by depleting the serine supply needed for the PS biosynthesis. In all cases, the PS content paralleled the extent of the protective effect, strongly suggesting that 22:6n-3 as a positive modulator of PS accumulation may play a role in preventing neuronal apoptosis induced by serum starvation. The electrospray LC/MS analysis of phospholipid molecular species as described earlier (31, 37) indicated that Neuro 2A cells enriched with 22:6n-3 in serine-free media did not produce phosphatidylthreonine that has been previously observed in hippocampal neurons (48).

Involvement of sphingolipids, particularly ceramides, in neuronal apoptosis has been documented (49). The observed reduction of SM levels in serine-free media (Fig. 8) corroborated the expected inhibition of sphingolipid biosynthesis that requires serine (50). However, neither fatty acid enrichment nor serine

depletion affected ceramide levels significantly, suggesting that there may be tight regulation in maintaining basal levels of this signaling molecule involved in apoptosis. There was little influence of 22:6n-3 enrichment on sphingolipid levels regardless of the serine status, but serine was required for the antiapoptotic effect of 22:6n-3 enrichment, strongly suggesting sphingolipid involvement to be highly unlikely.

It has been indicated that 20:4n-6 released by the activation of PLA<sub>2</sub> modulates the activities of various signaling molecules such as protein kinase C, G-proteins, and adenylate and guanylate cyclases in response to stimulus (51). Initial protection of apoptosis, which was observed uniquely with 20:4n-6 fatty acid in this study, may be due to the fatty acid itself since cyclooxygenase or lipoxygenase inhibitors did not affect the protective effect of 20:4n-6 (data not shown). 20:4n-6 also increased the PS accumulation after 48 h of enrichment ( $5.9 \pm 0.6$  pmol/ $\mu$ g of protein) but was unable to effectively prevent apoptosis during long term serum starvation. The loss of the protective effect in 20:4n-6-enriched cells after prolonged serum starvation suggests that other signaling pathways may also be involved. Recently, it has been reported that caspase-mediated cleavage of iPLA<sub>2</sub> during apoptosis augments spontaneous fatty acid release (52). It has been further implied that cytosolic PLA<sub>2</sub> and iPLA<sub>2</sub> functionally cooperate so that Ca<sup>2+</sup>-dependent cytosolic PLA<sub>2</sub>-mediated 20:4n-6 release can be promoted by iPLA<sub>2</sub> (52). Therefore, it is possible that the neuronal membranes enriched with 20:4n-6 may be more susceptible to the iPLA<sub>2</sub> activity increased during serum starvation, so that local free fatty acid levels may reach the toxic concentration range. Alternatively, other death-signaling pathways sensitive to 20:4n-6 may develop during the apoptotic process and may compromise the initial protective effect.

Events underlying the process of neuronal cell proliferation, differentiation, and apoptosis are becoming better established. Key events in this process focus on signaling pathways derived from the Ras/Raf-1/MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase)/mitogen-activated protein kinase cascade, as well as other pathways such as the activation of phosphatidylinositol 3-kinase/Akt (53). It has been well documented that PS is involved in various signaling

pathways including protein kinase C and Raf-1 kinase activation (30, 54, 55). Translocation of Raf-1 kinase to cell membranes, an important step in growth factor signaling, has been shown to be aided by Ras and membrane PS (30, 56). It has been also shown that Raf-1 interacts with PS through the PS binding domain (30); however, the effect of PS concentration on their interaction has not been elucidated. The configuration of Raf-1 employed in the present *in vitro* study may not be exactly compared with the free form due to its binding to anti-Raf-1 antibody in the biomolecular interaction analysis. Nevertheless, the observed PS dependence in the interaction of Raf-1 and liposomes suggests that antibody binding to Raf-1 may not significantly obscure its interaction with PS. The *in vitro* demonstration of PS dependence (Fig. 10) corroborates the enhanced translocation of Raf-1 observed in 22:6n-3-enriched cells (Fig. 9), where PS concentration increased significantly (Fig. 8). Besides the role of Ras/Raf-1 activation in proliferation, growing evidence indicates the importance of its interaction with the phosphatidylinositol 3-kinase/Akt pathway for ensuring cell survival in an either cooperative (26–28) or antagonistic manner (56, 57). To define where exactly in signaling pathways the enhanced translocation of Raf-1 by 22:6n-3 enrichment will participate to promote the survival signal under the serum-starved conditions, further studies will be necessary.

In summary, enrichment of neuronal cells with 22:6n-3 increases the PS content and Raf-1 translocation, down-regulates caspase-3 activity, and prevents apoptotic cell death. The correlation observed between the degree of protective effect and the extent of PS accumulation as well as the Raf-1 translocation enhanced by 22:6n-3 enrichment strongly suggests that the protective effect of 22:6n-3 may be mediated at least in part through the promoted accumulation of PS in neuronal membranes. Therefore, we propose that 22:6n-3 as a positive modulator of the membrane PS concentration may be an important biochemical mechanism underlying the well recognized need for 22:6n-3 fatty acid in the neuronal system.

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