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Induction of Apoptosis by Phosphatidylserine

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Treatment of Chinese hamster ovary (CHO) cells with phosphatidylserine (PS) caused cell death in a dose-dependent manner. Other phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid, had no effect on cell viability. The cells incubated with PS became round and underwent a dramatic reduction of cellular volume while maintaining the membrane containment of cellular contents. The PS-treatment induced chromatin condensation and extensive DNA fragmentation, with a pattern characteristic of internucleosomal fragmentation on agarose gel electrophoresis. These results indicate that PS-treatment induces apoptosis of CHO cells. This apoptosis-inducing activity was highly specific for PS, and neither of the synthetic PS analogs 1,2-diacyl-*sn*-glycero-3-phospho-D-serine (D-PS) and 2,3-diacyl-*sn*-glycero-1-phospho-L-serine induced apoptosis. Analysis using fluorescence-labeled phospholipids showed that both PS and D-PS were taken up equally and then transported to intracellular membranes, suggesting that the PS-specific induction of apoptosis was not the result of its specific internalization. These observations suggest that certain molecules which may recognize the stereo-specific configuration of PS are involved in the apoptotic process triggered by PS.

Key words: apoptosis, Chinese hamster ovary cell, lipid transport, phosphatidylserine.

Phosphatidylserine (PS) is a major anionic lipid component of mammalian cell membranes. Recent studies have shown that PS contributes to many regulatory processes of biological responses. It is well known that PS is an essential cofactor for the activation of protein kinase C (*1-3*) and blood coagulation (*4, 5*). PS has also been shown to regulate the activities of various enzymes such as Na^+/K^+-ATPase (*6*), diacylglycerol kinase (*7*), B-Raf protein kinase (*8*), and dynamin GTPase (*9*), and the channel functions of receptors for acetylcholine (*10*) and glutamate (*11*). Using a CHO-K1 cell mutant defective in PS biosynthesis, Kuge *et al.* (*12*) showed that PS plays an essential role in cell viability.

In eukaryotic plasma membranes, aminophospholipids such as PS and phosphatidylethanolamine (PE) reside in the inner leaflet of plasma membranes (*13-16*) and are translocated to the outer leaflet in the early stages of apoptosis, which allows phagocytes to recognize and engulf the apoptotic cells (*17-19*). The transbilayer movement of membrane phospholipids is regulated partly by an enzyme, aminophospholipid translocase, which catalyzes the transport of PS and PE from the outer to the inner leaflet of plasma membrane (*20, 21*). In CHO cells, exogeneously supplied PS was shown to be incorporated, translocated to the inner leaflet of plasma membrane, then transported to intracellular organellae such as the Golgi apparatus and mitochondria (*22-24*). This internalization of exogenous PS was thought to be catalyzed by translocase, since the endocytotic pathway for exogenous phospholipids is not well developed in CHO cells (*25, 26*).

Recently, Endo *et al.* showed that excess PS exogenously added to CHO-K1 cells was cytotoxic to the cells, while the same amount of phosphatidylcholine (PC) had no effect (*27*). They showed that cell surface aminophospholipid translocase was responsible for the uptake of exogenous PS and isolated mutant cells defective in the cell surface translocation of PS. However, the process by which exogenous PS induces cell death and whether the cytotoxic effect is specific for PS remain to be determined. In this study, we showed that PS induces apoptosis in CHO-K1 cells and that PS-induced apoptosis is highly specific for 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (natural PS). Other closely related PS analogs, such as 1,2-diacyl-*sn*-glycero-3-phospho-D-serine (D-PS) and 2,3-diacyl-*sn*-glycero-1-phospho-L-serine, showed no effects on cell growth, suggesting the existance of cellular machinery specifically recognizing the structure of PS in the apoptotic pathway.

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Abbreviations: CHO, Chinese hamster ovary; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; lyso-PS, lysophosphatidylserine; PKC, protein kinase C; D-PS, 1,2-diacyl-*sn*-glycero-3-phospho-D-serine; C6-NBD, 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-capyroyl; C6-NBD-PS, 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphoserine.

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MATERIALS AND METHODS

Materials—Egg phosphatidylcholine was prepared by chromatography on aluminium oxide neutral and Iatrobeads. Phosphatidylserine (from bovine brain), phosphatidylethanolamine (from *Escherichia coli*), phosphatidylinositol (from bovine liver), phosphatidic acid (from egg yolk), lysophosphatidylserine, and 1-palmitoyl-2-C_6-NBD-phosphatidylserine (C_6-NBD-PS) were purchased from Avanti Polar Lipids (Birmingham, AL). 1,2-Acyl-*sn*-glycero-3-phospho-D-serine and 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phospho-D-serine were synthesized as described previously (*28*). Other synthetic PS analogs were synthesized as described previously (*29*).

Cell Culture—CHO-K1 fibroblasts were grown in Ham's F12 medium supplemented with 10% newborn calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco, Grand Island, NY) at 37°C in a 5% CO_2/95% air incubator.

Preparation of Phospholipid Suspensions—Phospholipids in chloroform solution were thoroughly dried in a glass tube under a stream of nitrogen, resuspended in phosphate-buffered saline (PBS), then sonicated three times on ice for 5 min. The suspensions were then sterilized by passage through a 0.22-μm pore filter. In the case of phosphatidylethanolamine, the lipid was resuspended in growth medium and then sonicated for 10–20 min.

Measurement of Phospholipid Cytotoxicities—Cells were plated in 96-well plates at 1×10^4 cells/well in 0.1 ml of medium. After 2 days, phospholipid vesicles were added. Cells were incubated for additional periods at 37°C. Media were then changed to a medium containing 300 μg/ml XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (Boehringer Mannheim) and the cells were incubated for an additional 4 h. Viable cell numbers were estimated by measuring A_{492}.

DNA Isolation and Gel Electrophoresis—DNA was prepared using a standard method (*30*). In brief, 5×10^5 cells were washed with cold PBS, suspended in 200 μl of lysis buffer (1% SDS, 0.5 mg/ml RNase, 0.5 mg/ml Proteinase K, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and incubated for 30 min at 37°C. Then 300 μl of NaI solution (6 M NaI, 13 mM EDTA, 0.5% sodium-*N*-lauroylsarcosinate, 10 mg/ml glycogen, 26 mM Tris-HCl, pH 8.0) was added to the lysate, and the mixture was incubated for 15 min at 60°C. The lysates were precipitated overnight with 500 μl of isopropanol at 4°C, then centrifuged at 20,000× *g* for 15 min. Pellets were washed with isopropanol twice, air-dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Electrophoresis was performed in 1.8% agarose for 1 h. DNA was visualized by staining with ethidium bromide under UV light and photographed.

Analysis of Fluorescence-Labeled Phosphatidylserine Uptake by CHO-K1 Cells—Monolayer cultures were rinsed twice with Hanks' buffered saline (HBS) and further incubated in cold HBS for 5 min. The cells were then incubated with HBS containing 20 μM C_6-NBD-phosphatidyl-L-serine or C_6-NBD-phosphatidyl-D-serine vesicles for 30 min on ice. The cells were washed with cold HBS three times, then incubated at 37°C for various periods. The monolayers were washed with cold HBS containing 2% fetal calf serum (FCS) and incubated with the same solution for 10 min, three times, to remove C_6-NBD-lipids from the cell surface (back-exchange procedure). The cells were harvested by scraping, and the lipid fractions were extracted by the method of Bligh and Dyer (*31*). The relative fluorescence of the lipid extracts was measured using a Fluorescence Spectrophotometer F-2000 (HITACHI), and the absolute amounts of fluorescent lipids were determined from the calibration curve.

Fluorescence Microscopy—Cells were incubated with 3 μM C_6-NBD-phosphatidyl-L-serine or C_6-NBD-phosphatidyl-D-serine as described above. After the back-exchange procedure, the cells were photographed under fluorescence microscopy using a Zwiss Axioplan microscope equipped with Planneofluar 100× objective.

RESULTS AND DISCUSSION

Exogenously Added Phosphatidylserine Induces Apoptosis in CHO-K1 Cells—Treatment of CHO-K1 cells with phosphatidylserine (PS) caused cell death in a dose-dependent manner (Fig. 1A). This cytotoxic effect was specific for PS and other phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA), had no effect on cell viability. It is rather unlikely that PS-induced cell death was due to lysophosphatidylserine (lyso-PS), as either a contaminant of the PS preparation or the result of conversion of PS by phospholipase A_2, since even an higher concentration of lyso-PS was required to induce cell death (Fig. 2A). The PS-treated cells showed different morphological features from those treated with lyso-PS: cells incubated with lyso-PS underwent extensive membrane fragmentation and swelling, while those incubated with PS became round and underwent a dramatic reduction of cellular volume while maintaining the membrane containment of cellular contents (Fig. 2B). PS-treatment induced chromatin condensation and segregation (Fig. 3A) and an extensive DNA fragmentation with a pattern characteristic

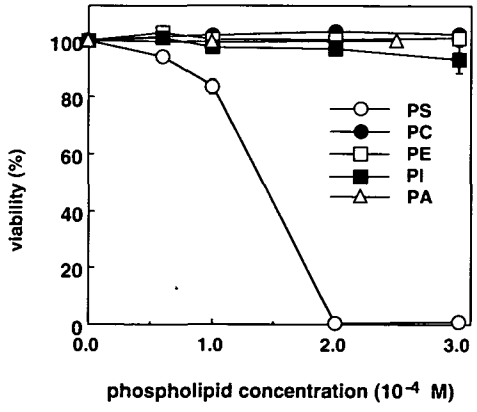

Fig. 1. **Effects of exogenous phospholipids on viability of CHO-K1 cells.** Cells were plated in 96-well plates at 1×10^4 cells/well in 0.1 ml of medium. After 2 days, cells were incubated with various concentrations of phospholipid vesicles for 24 h at 37°C. Media were then changed to a medium containing 300 μg/ml XTT reagent, and the cells were incubated for an additional 4 h. Viable cell numbers were estimated as described in "MATERIALS AND METHODS." Data are means ± SD ($n=3$). m, PS; l, PC; q, PE; n, PI; Δ, PA.

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of internucleosomal fragmentation on agarose gel electrophoresis (Fig. 3B). Neither chromatin condensation nor DNA fragmentation was observed in lyso-PS treated cells (Fig. 3B). Furthermore, PS-induced cell death was effectively inhibited by 100 μM ionic zinc or a Ca^{2+}-chelater, such as 400 μM ethyleneglycol-bis(B-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA), both of which are known to inhibit endonuclease (data not shown) (*32*). These observations indicate that PS induced apoptosis in CHO-K1 cells. The apoptosis-inducing activity of PS was also observed in other cell lines, such as Balb 3T3 (mouse fibroblast cell line) and GOTO (human neuroblastoma cell line) (data not shown).

Structure-Activity Relationship in the Apoptosis Inducing Activity—To explore the molecular mechanisms involved in apoptosis, we first examined the structural requirements for the PS-induced apoptosis. As shown in Fig. 4, the PS-induced apoptosis was highly specific for PS. Synthetic PS analogs such as 1,2-diacyl-*sn*-glycero-3-phospho-D-serine (D-PS), 1,2-diacyl-*sn*-glycero-3-phospho-homoserine (P-H-S), 1,2-diacyl-*sn*-glycero-3-phospho-serine methyl esther (PS-O-Me), and 2,3-diacyl-*sn*-glycero-1-phospho-L-serine (*sn*-2,3-PS) failed to induce the apoptosis (Fig. 4). Endo *et al.* (*27*) have shown that exogenous PS is internalized mainly by a protein called flippase and that the transbilayer movement of PS on the plasma membrane is an important step in the cytotoxic action of PS. This observation raised the possibility that flippase on plasma membranes may selectively internalize PS, while other phospholipids including the synthetic PS analogs, may not be internalized as effectively as PS. To examine this possibility, we studied the uptake of fluorescence-labeled PS analogs of PS (apoptotic) and D-PS (non-apoptotic). Figure 5A shows that fluorescence-labeled PS and D-PS were taken up equally and then transfered to intracellular membranes (Fig. 5B), suggesting that the PS-specific induction of apoptosis was not the result of its specific internalization. These results suggested that certain molecules which may recognize the stereo-specific configuration of PS are involved in the apoptotic process triggered by PS.

Nishijima *et al.* showed the PS content in CHO-K1 cells to be strictly regulated and that exogenously added PS was incorporated into cells and utilized for membrane biogenesis, resulting in the suppression of endogenous PS formation (*34*). Analysis of cellular phospholipid composition revealed the PS content to have increased to three times the control level in the PS-treated apoptotic cells (Table I), implying that the aberrant increase in cellular PS content may cause malregulation of cellular signaling pathways, leading to apoptosis of the cell. Recently, Bratton *et al.* showed that PS is translocated to the outer leaflet of plasma membrane in a calcium-dependent manner when apoptosis is induced (*44*). It is presently unknown whether the accumulation of PS in the outer leaflet leads to apoptosis.

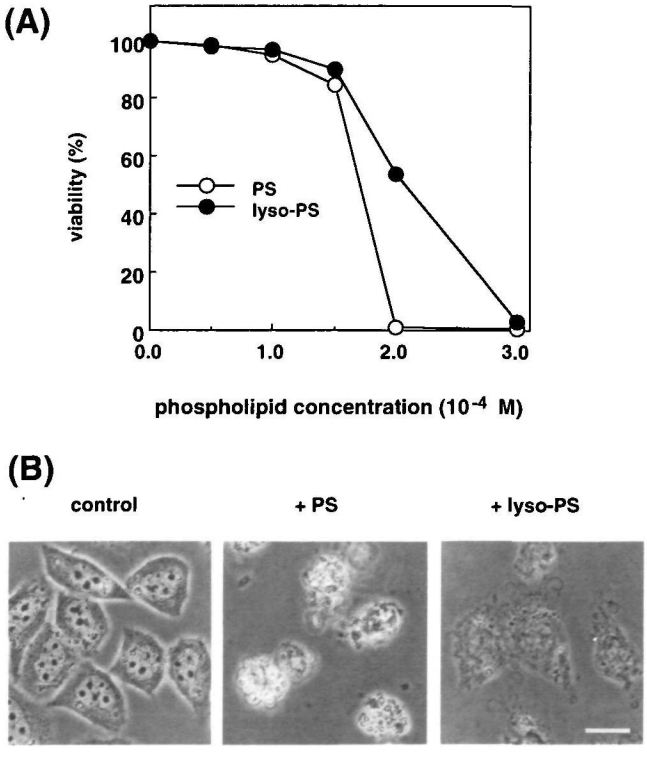

Fig. 2. **Effects of lyso-PS on viability and morphological features of CHO-K1 cells.** (A) Cells were plated in 96-well plates at 1×10^4 cells/well in 0.1 ml of medium. After 2 days, cells were incubated with various concentrations of phospholipid suspensions for 24 h at 37°C. Media were then changed to a medium containing 300 μg/ml XTT reagent, and the cells were incubated for an additional 4 h. Viable cell numbers were estimated as described in "MATERIALS AND METHODS." Data are the means±SD ($n=3$). m, PS; l, lyso-PS. (B) Semiconfluent cells, growing on a glass coverslip, were incubated with 200 μM PS or lyso-PS for 24 h and photographed. Bar represents 10 μm.

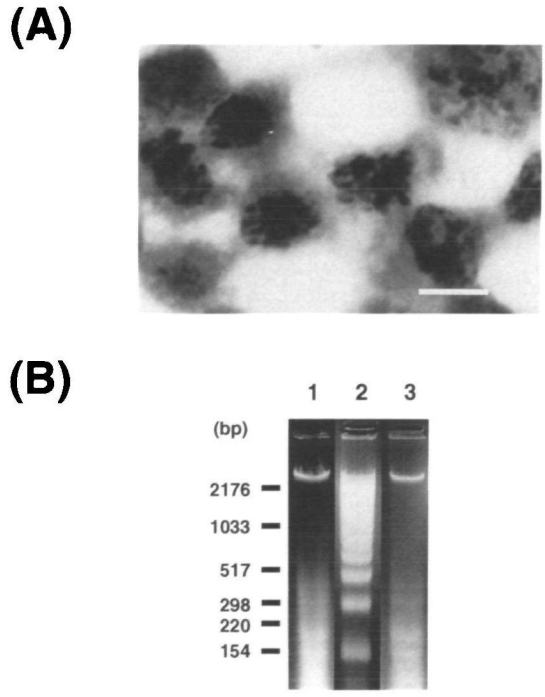

Fig. 3. **Induction of apoptosis by PS in CHO-K1 cells.** (A) Morphological features of nuclei in PS-treated cells. Cells were incubated with 200 μM of PS vesicles for 12 h at 37°C. The cell nuclei were stained with Giemsa reagent. Bar represents 10 μm. (B) DNA fragmentation. Chromosomal DNA was prepared from untreated CHO-K1 cells (lane 1), and cells incubated 200 μM PS (lane 2) or with 200 μM lyso-PS for 12 h (lane 3). DNA was separated by electrophoresis in a 1.8% agarose gel.

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It is well known that PS is an essential cofactor for protein kinase C (PKC) activation and that PKC recognizes the stereo-specific configuration of the serine residue of PS (*35-37*). Our preliminary results showed that PKC inhibitors such as H-7 (*38*) and calphostin C (*39*) slightly enhanced PS-induced apoptois, while an activator of PKC, phorbol 12-myristate 13-acetate (*40*), inhibited apoptosis, suggesting that PKC inactivation rather than activation is involved in the apoptotic process. Ceramide is a cruicial mediator of apoptosis induced by various ligands such as tumor necrosis factor α,γ-interferon, interleukin-1, and Fas activation. This ceramide-mediated apoptosis was shown to be inhibited by the simultaneous additon of PKC activators (*41, 42*), implying that PS activates the ceramide-mediated apoptotic pathway. However, the inhibi-

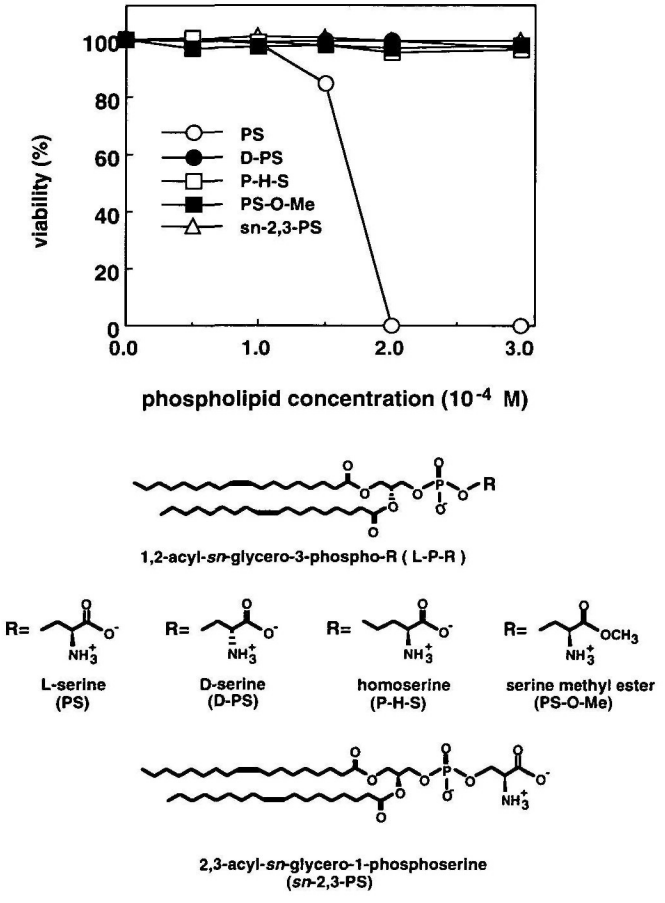

Fig. 4. **Effects of PS analogs on cell viability.** Cells were plated out in 96-well plates at 1×10^4 cells/well in 0.1 ml of medium. After 2 days, cells were incubated with various concentrations of PS analogs for 24 h at 37°C. Media were then changed to a medium containing 300 μg/ml XTT reagent, and the cells were incubated for an additional 4 h. Viable cell numbers were estimated as described in "MATERIALS AND METHODS." Data are means±SD ($n=3$). m, 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (PS); l, 1,2-diacyl-*sn*-glycero-3-phospho-D-serine (D-PS); q, 1,2-diacyl-*sn*-glycero-3-phospho-homoserine (P-H-S); n, 1,2-diacyl-*sn*-glycero-3-phospho-serine methyl ether (PS-O-Me); Δ, 2,3-diacyl-*sn*-glycero-1-phospho-L-serine (*sn*-2,3-PS).

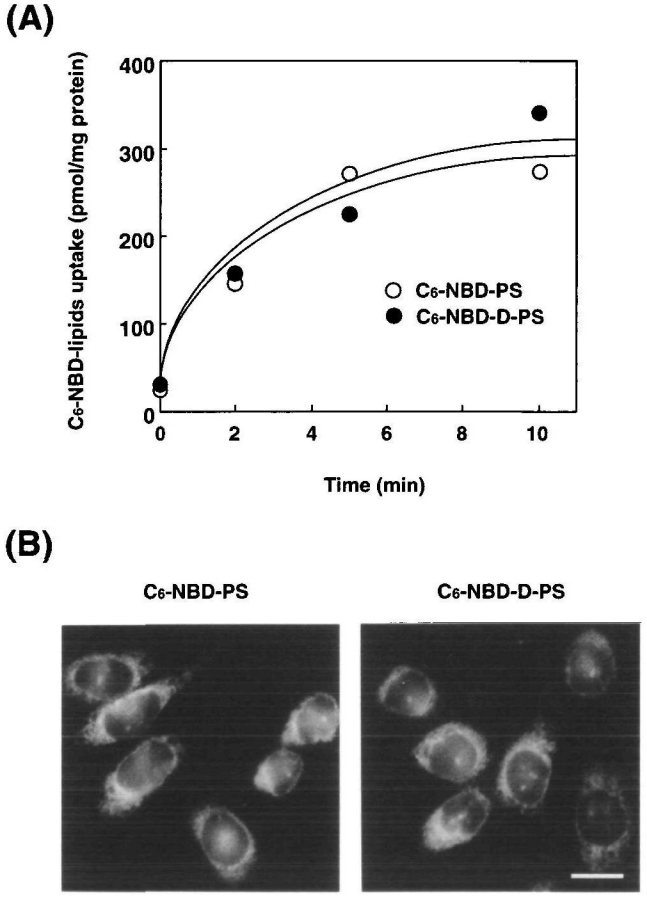

Fig. 5. **Cellular uptake of fluorescent lipids in CHO-K1 cells.** (A) Cells were incubated with 20 μM C6-NBD-PS or C6-NBD-D-PS for 30 min on ice, then washed and incubated at 37°C for various periods. The cells were then washed with cold HBS containing 2% fetal calf serum (FCS) and subjected to back exchange procedure at 4°C. The cells were harvested by scraping, and the lipid fractions were extracted. The amounts of fluorescent lipids were measured as described in "MATERIALS AND METHODS." ($n=2$). (B) Cells were incubated with 3 μM C6-NBD-PS or C6-NBD-D-PS as described above. After the back-exchange procedure, the cells were photographed under fluorescence microscopy using a Zwiss Axioplan microscope equipped with Planneofluar 100×objective. Bar represents 10 μM.

TABLE I. **Phospholipid compositions of CHO-K1 cells grown with or without phosphatidylserine.** Cells were seeded at 1×10^6 cells/100-mm-diameter dish and incubated at 37°C. After 2 days, the medium was replaced to fresh medium with or without 200 μM PS. After an additional 12 h, the cellular phospholipids were extracted and analyzed by two-dimensional thin layer chromatography as described (*34*). To quantitate the individual phospholipids, the phosphate in each spot on a chromatogram was determined chemically (*43*). Values are the mean± SD of three independent experiments. SM, sphingomyelin. Others include phosphatidylglycerol, phosphatidic acid, and cardiolipin.

PS supplement	Percentage of total phospholipids					
	PS	PC	PE	PI	SM	Others
−	7.2±0.6	54.0±0.1	16.1±1.1	8.2±0.1	9.9±1.1	4.6±0.4
+	18.9±0.2	51.0±0.8	19.4±0.7	2.1±0.3	6.4±0.7	2.2±0.2

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tors of interleukin-1 converting enzyme (ICE)-like proteases (Caspase), such as tosyl-L-lysine chloromethyl ketone (TLCK) and tosyl-L-phenylalanine chloromethyl ketone (TPCK) which inhibit ceramide-mediated apoptosis, had no effect on PS-induced apoptosis (data not shown), suggesting that the PS-induced apoptotic pathway is distinct from that mediated by ceramide. Further studies are required to clarify the molecular mechanisms underlying PS-induced apoptosis.

REFERENCES

1. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-611
2. Bell, R.M. and Burns, D.J. (1991) Lipid activation of protein kinase C. *J. Biol. Chem.* **266**, 4661-4664
3. Igarashi, K., Kaneda, M., Yamaji, A., Saido, T., Kikkawa, U., Ono, Y., Inoue, K., and Umeda, M. (1995) A novel phosphatidylserine-binding peptide motif defined by an anti-idiotypic monoclonal antibody. *J. Biol. Chem.* **270**, 29075-29078
4. Mann, K.G., Jenny, R.J., and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem.* **57**, 915-956
5. Comfurius, P., Smeets, E.F., Willems, G.M., Bevers, E.M., and Zwaal, R.F.A. (1994) Assembly of the prothrombinase complex on the lipid vesicles dependent on the stereochemical configuration of polar headgroup of phosphatidylserine. *Biochemistry* **33**, 10319-10324
6. Stekhoven, F.M.A.H.S., Tijmes, J., Umeda, M., Inoue, K., and De Pont, J.J.H.M. (1994) Monoclonal antibody to phosphatidylserine inhibits Na^+/K^+-ATPase activity. *Biochim. Biophys. Acta* **1194**, 155-165
7. Sakane, F., Yamada, K., Imai, S., and Kanoh, H. (1991) Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation. *J. Biol. Chem.* **266**, 7096-7100
8. Ghosh, S., Xie, W.Q., Quest, A.F.G., Strum, J.C., and Bell, R.M. (1994) The cystein-rich region of raf-1 kinase contains zinc, translocates to liposomes, and is adjacent to a segment that binds GTP-ras. *J. Biol. Chem.* **269**, 10000-10007
9. Tuma, P.L., Stachniak, M.C., and Collins, C.A.J. (1993) Activation of dynamin GTPase by acidic phospholipids and endogenous rat brain vesicles. *J. Biol. Chem.* **268**, 17240-17246
10. Sunshine, C. and McNamee, M.G. (1992) Lipid modulation of nicotinic acetylcholine receptor function: the role of neutral and negatively charged lipids. *Biochim. Biophys. Acta* **1108**, 240-246
11. Gagne, J., Giguere, C., Tocco, G., Ohayon, M., Thompson, R.F., Braudry, M., and Massicotte, G. (1996) Effect of phosphatidylserine on the binding properties of glutamate receptors in brain sections from adult and neonatal rats. *Brain Res.* **740**, 337-345
12. Kuge, O., Nishijima, M., and Akamatsu, Y. (1986) Phosphatidylserine biosynthesis in cultured Chinese hamster ovary cells. *J. Biol. Chem.* **261**, 5790-5794
13. Emoto, K., Kobayashi, T., Yamaji, A., Aizawa, H., Yahara, I., Inoue, K., and Umeda, M. (1996) Redistribution of phosphatidylethanolamine at cleavage furrow of dividing cell during cytokinesis. *Proc. Natl. Acad. Sci. USA* **93**, 12867-12872
14. Zachowski, A. (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem. J.* **294**, 1-14
15. Shroit, A.J. and Zwaal, R.F.A. (1991) Transbilayer movement of phospholipids in red cell and platelet membranes. *Biochim. Biophys. Acta* **1071**, 313-329
16. Devaux, P.F. (1991) Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* **30**, 1163-1173
17. Emoto, K., Toyama-Sorimachi, N., Karasuyama, H., Inoue, K., and Umeda, M. (1997) Exposure of phosphatidylethanolamine on the surface of apoptotic cells. *Exp. Cell Res.* **232**, 430-434
18. Martin, S.J., Reutelingsperger, C.P.M.R., McGahon, A.J., Rader, J.A., van Schie, R.C.A.A., LaFace, D.M., and Green, D.R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of initiating smimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182**, 1545-1556
19. Koopman, G., Reutelingsperger, C.P.M., Kuijten, G.A.M., Keehnen, R.M.J., Pals, S.T., and van Oers, M.H.J. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415-1420
20. Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophage. *J. Immunol.* **148**, 2207-2216
21. Verhoven, B., Schlegel, R.A., and Williamson, P. (1995) Mechanisms of phosphatidylserine exprosure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med.* **182**, 1597-1601
22. Kobayashi, T. and Arakawa, Y. (1991) Transport of exogenous fluorescent phosphatidylserine analogue to the Golgi apparatus in culture fibroblasts. *J. Cell Biol.* **113**, 235-244
23. Kobayashi, T. and Pagano, R.E. (1988) ATP-dependent fusion of liposomes with the Golgi apparatus of perforated cells. *Cell* **55**, 797-805
24. Martin, O.C. and Pagano, R.E. (1987) Transbilayer movement of fluorescent analogs of phosphatidylserine and phosphatidylethanolamine at the plasma membrane of culture cells: Evidence for a protein-mediated and ATP-dependent process(es). *J. Biol. Chem.* **262**, 5890-5898
25. Fukasawa, M., Adachi, H., Tsujimoto, M., Arai, H., and Inoue, K. (1996) SRBI, a class B scavenger receptor, recognizes both negatively charged liposomes and apoptotic cells. *Exp. Cell Res.* **222**, 246-250
26. Fukasawa, M., Hirota, K., Adachi, H., Mimura, K., Murakami-Murofushi, K., Tsujimoto, M., Arai, H., and Inoue, K. (1995) Chinese hamster ovary cells expressing a novel type of acetylated low density lipoprotein receptor. *J. Biol. Chem.* **270**, 1921-1927
27. Endo, T.A., Kobayashi, T., and Ohki, K. (1996) A Chinese hamster ovary mutant resistant to phosphatidylserine is defective in transbilayer movement of cell surface phosphatidylserine. *Exp. Cell Res.* **228**, 341-346
28. Confurius, P. and Zwaal, R.F.A. (1977) The enzymatic synthesis of phosphatidylserine and purification by CM-cellulose column chromatography. *Biochim. Biophys. Acta* **488**, 36-42
29. Dummond, D.C. and Daleke, D.L. (1995) Synthesis and characterization of *N*-acylated, pH-sensitive 'caged' aminophospholipids. *Chem. Phys. Lipids* **75**, 27-41
30. Sellins, K.S. and Cohen, J.J. (1987) Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.* **139**, 3199-3206
31. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917
32. Gaido, M.L. and Cidlowski, J.A. (1991) Identification, purification, and characterization of a calcium-dependent endonuclease (NUC 18) from apoptotic rat thymocytes. *J. Biol. Chem.* **266**, 18580-18585
33. Shiokawa, D., Ohyama, H., Yamada, T., Takahashi, K., and Tanuma, S. (1994) Identification of an endonuclease responsible for apoptosis in rat thymocytes. *Eur. J. Biochem.* **226**, 23-30
34. Nishijima, M., Kuge, O., and Akamatsu, Y. (1986) Phosphatidylserine biosynthesis in cultured Chinese hamster overy cells. *J. Biol. Chem.* **261**, 5784-5789
35. Kaibuchi, K., Takai, Y., and Nishizuka, Y. (1981) Cooperative roles of various membrane phospholipids in the activation of calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **256**, 7146-7149
36. Lee, M.E. and Bell, R.M. (1989) Phospholipid functional groups in protein kinase C activation, phorbol ester binding, and binding to mixed micelles. *J. Biol. Chem.* **264**, 14797-14805
37. Orr, J.W. and Newton, A.C. (1992) Interaction of protein kinase C with phosphatidylserine. *Biochemistry* **31**, 4667-4673
38. Kawamoto, S. and Hidaka, H. (1984) 1-(5-Isoquinolinesulfonyl)-

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2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets. *Biochem. Biophys. Res. Commun.* **125**, 258-264
39. Kobayashi, E., Nakano, H., Morimoto, M., and Tamaoki, T. (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159**, 548-553
40. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257**, 7847-7851
41. Ni, R., Tomita, Y., Matsuda, K., Ichihara, A., Ishimura, K., Ogasawara, J., and Nagata, S. (1994) Fas-mediated apoptosis in primary cultured mouse hepatocytes. *Exp. Cell Res.* **215**, 332-337
42. Obeid, L.M., Linardic, C.M., Karolak, L.A., and Hannun, Y.A. (1993) Programmed cell death induced by ceramide. *Science* **259**, 1769-1771
43. Zhou, X. and Arthur, G. (1992) Improved procedures for determination of lipid phosphorus by malachite green. *J. Lipid Res.* **33**, 1233-1236
44. Bratton, D.L., Fadok, V.A., Richter, D.A., Kailey, J.M., Guthrie, L.A., and Henson, P.M. (1997) Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and enhanced by loss of the aminophospholipid translocase. *J. Biol. Chem.* **272**, 26159-26165

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