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Sphingosylphosphorylcholine induces mitochondria-mediated apoptosis in neuro 2a cells: Involvement of protein kinase C

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We demonstrate that sphingosylphosphorylcholine-mediated cell death involves the activation of different protein kinase C isozymes in different manners. Treating cells with sphingosylphosphorylcholine resulted in activation of protein kinase C δ, which is necessary, together with elevation of Ca^{2+}, for sphingosylphosphorylcholine-induced apoptosis. A rapid translocation from cytosol to membrane, and a proteolytic protein kinase C δ cleavage was found, probably due to activation of caspase-3, to give a catalytically active fragment involved in cellular apoptosis. Moreover, sphingosylphosphorylcholine also induced translocation of protein kinase C ζ, resulting in an anti-apoptotic effect. To explore whether a mitochondrial pathway is involved in sphingosylphosphorylcholine-induced apoptosis, we analyzed the effect of sphingosylphosphorylcholine on cytochrome c release and caspase-3 activity. We must point out that the sphingolipid caused an increase of cytochrome c release from mitochondria to cytosol concomitantly with an increase of caspase-3 activity. Furthermore, a translocation of Bax was found, after sphingosylphosphorylcholine treatment.

Key words: Sphingosylphosphorylcholine, apoptosis, protein kinase C, neuroblastoma neuro 2a cells

INTRODUCTION

Sphingolipids such as ceramide and sphingosine, have emerged as novel intracellular signal mediators (Hannun 1996). Sphingolipids may be involved in cell signalling *via* agonist-induced activation of the sphingomyelin cycle, which results in the generation of ceramide, sphingosine, and other sphingolipid metabolites. The activation of this pathway is involved in signal transduction processes with specific agonists, and ceramide production is involved in cell death and growth inhibition (Sawai and Hannun 1999). Numerous studies suggest that sphingosylphosphorylcholine (lysosphingomyelin, SPC) may have a mediator function similar to other lysophospholipids, although rather little is known about the exact cellular targets of this sphingolipid (Meyer et al. 2002). In this sense, we must point out that SPC has been implicated in a number of biological processes, including proliferation, growth inhibition and angiogenesis (Meyer et al. 2002). SPC has been reported to stimulate DNA synthesis and proliferation in a variety of cell types (Desai and Spiegel 1991, Sun et al. 1996). In contrast, SPC can also inhibit the growth of various cell types, especially that of tumor cells (Xu 2002). Taking into account all these data, it is likely that SPC regulates cell proliferation positively or negatively depending on cell types.

Many of the actions of SPC are shared by the structurally related sphingosine-1-phosphate (S1P). It is logical to consider that the two lipids have common mechanisms and sites of action. These lipids bind to G-protein-coupled receptors (GPCRs) (Hla et al. 2001). SPC receptors are ubiquitously expressed, and several SPC receptors have been cloned (Ignatov et al. 2003). The expression of some of these receptors and some effects of SPC in neuroblastoma have been described (Young et al. 2000).

It is well known that signalling cascades eventually leading to apoptotic cell death may utilize two differ-

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ent, extrinsic or death receptor-associated, and intrinsic or mitochondrial pathways (Schimmer et al. 2001). The mitochondrial pathway is the main route of apoptotic signals initiated by cell stress resulting from interaction of chemotherapeutic agents with specific cellular agents. Initiator and effector caspases are critical participants in both apoptotic pathways. Extensive evidence indicates that during apoptosis, the outer mitochondrial membrane becomes permeable to intermembrane space proteins, including cytochrome c (Green and Reed 1998). Once released, cytochrome c is able to activate procaspase 9 (Li et al 1997). The activation of this initiator caspase then leads to the proteolytic activation of caspase 3, that is responsible for biochemical and morphological changes characteristic of apoptosis. Permeabilization of the outer mitochondrial membrane is modulated by proapoptotic proteins such as Bax and antiapoptotic proteins such as Bcl (Polster and Fiskum 2004). In neurons, the relocalization of Bax from cytosol to the mitochondria represents one of the first steps in cellular apoptosis (Pulcha et al. 1999).

It has been reported that lysosphingolipids induce apoptosis in different cells types, including neuro 2a cells (Sueyoshi et al. 2001). In this case, a caspase 3-independent signalling pathway is involved in some lysosphingolipid-induced apoptosis, but a similar mechanism has not been described for SPC in this cell type. Recently, a caspase 3-dependent mechanism has been proposed for apoptosis induced by a stereoisomer of SPC in human adipose tissue-derived mesenchymal stem cells (Jeon et al. 2005a).

On the other hand, evidence is accumulating that enzymes of protein kinase C (PKC) family are essential elements in the control of apoptosis. Thus, PKC α isoform has been shown to act as pro- or anti-apoptotic agents, depending on cell type, whereas PKC δ isoform is involved in the execution phase of apoptosis (Buchner 2000). In addition, PKC ζ has emerged as a potent negative regulator of apoptosis induced by different death receptors (Grunicke et al. 2003). Recently, the involvement of PKC on SPC actions has been demonstrated. Thus, the addition of SPC to the thyroid FRO cells, which express the putative SPC receptors GPR_4 and OGR_1, induces a rapid and transient elevation of calcium concentration. This increase of intracellular calcium is dependent on PKC activation (Afrasiabi et al. 2006).

In the present study, we demonstrated that SPC induced cell death through a mitochondrial signalling pathway in neuro 2a cells, and also the involvement of PKC isozymes in the SPC-induced apoptosis. We also demonstrated, for the first time, the protective role of PKC ζ in the action of SPC.

METHODS

Materials

Escherichia coli diacylglycerol kinase, caspase substrate Ac-DEVD-pNA, myristoilated PKC ζ pseudosubstrate peptide (PS-ζ), bisindolylmaleimide (BSM) and rottlerin were obtained from Calbiochem (Darmstadt, Germany). Anti-PKC and anti-Bax antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SYBR* Green PCR master mix and RT-PCR kit were obtained from Applied Biosystems (Foster City, CA, USA). SPC, MTT, Dulbecco's modified Eagle's medium and other chemical required were from SIGMA (St. Louis, MO, USA).

Cell culture and treatments

Neuro 2a cells were maintained in 44% Dulbecco's modified Eagle's medium, 44% F-12 Ham medium, 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine (Invitrogen), in a humidified incubator with 5% CO_2 at 37°C. SPC was dissolved in ethanol at 10 mM. The cells were incubated for the desired time periods with the chosen SPC concentration. Control cells were treated with ethanol.

Cell viability assay

Cell viability was determined by MTT assay as previously described (Alley et al. 1988). Thus, 20 µl of the stock solution (5 mg/ml MTT) was added to each well of a 96-well plate. The plates were incubated at 37°C for 4 h, and the formazan granules generated by the live cells were dissolved in isopropyl alcohol acid (0.5% of HCl concentrate in isopropyl alcohol) and absorbance at 570 nm was monitored by using a PowerWave$_x$ microplate spectrophotometer (Bio-Tek Instruments, Unc. Winooski, VT, USA).

DNA fragmentation assay

Treated and untreated cells (1×10^6) were washed twice in PBS. Cell lysis was performed in a buffer containing 10 mmol/l TRIS, 10 mmol/l EDTA and

0.5 % Triton X-100. Afterwards, proteinase K (1 mg/ml) was added and lysate was incubated for 60 min at 37°C. It was then heated at 70°C for 10 min followed by addition of RNAase (200 μg/ml) and another one hour incubation at 37°C. Samples were subsequently transferred to 2% agarose gel and run with 40 V at 3 h. DNA fragments were visualized by UV transluminator by ethidium bromide staining.

Flow cytometric analysis

The percentage of apoptotic cells (the intactness of the cell membrane, affinity for annexin V-FITC and devoid of PI staining) and necrotic cells (the cell membrane looses its integrity, the cell becomes PI staining) were analyzed by flow cytometry using an annexin V-FITC/PI kit (ApoTarget, BioSource International, Inc., CA, USA) following the manufacturer's instructions.

Preparation of subcellular fractions

The cellular fractionation was carried out as previously described (Miguel et al. 2005)**.** The cytosol, membrane and mitochondrial fractions were prepared at 4°C. All media used were supplemented with: 1% v/v 2-mercaptoethanol, 0.1 mM sodium metavanadate, 20 mM sodium fluoride, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, immediately before use. Cells were resuspended in a buffer containing: 20 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, and 0.5 mM EGTA, and lysed with 50 strokes of a Dounce homogenizer using a B type pestle. Cell lysis was confirmed by microscopy. The lysate was centrifuged at 800 × g for 10 min at 4°C. The pellet was discarded, and the supernatant was used to isolate cytosolic and particulate fractions, as described below: the 800 × g supernatant was centrifuged at 100 000 × g for 60 min at 4°C, the supernatant obtained was cytosolic fraction. The pellet was resuspended in buffer containing: 20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA and 1% Nonidet P-40 and was then agitated at 4°C for 120 min and centrifuged at 100 000 × g for 60 min at 4°C. The supernatant obtained was the particulate fraction. The mitochondrial fraction was assayed for the marker enzyme cytochrome c reductase as described (Sottocasa et al. 1967).The mitochondrial fraction was obtained as described (Wang et al. 2006). Briefly, 15 × 10^6 cells were homogenated in 300 μl of lysis buffer containing: 250 mM sucrose, 50 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF). After centrifugation at 800 × g for 15 min, the supernatant was centrifuged at 7 500 × g for 20 min at 4°C. The resulting pellet was washed twice in lysis buffer, and then dissolved in hypotonic buffer containing: 10 mM HEPES, pH 7.9, 1 mM PMSF. After incubation on ice for 30 minutes, the suspension was sonicated and recentrifuged at 13 000 × g for 30 min. The soluble mitochondrial proteins were stored at −80°C until use. The mitochondrial fraction was assayed for the marker enzyme cytochrome c reductase as described (Sottocasa et al. 1967).

Western blotting

Immunoblot analysis was carried out in each of the four isolated fractions, homogenate, cytosolic, membranes and nuclei as previously described (Calcerrada et al. 2002). Equal amounts of protein (100 μg) from each fraction were resolved by SDS-PAGE and then transferred onto nitrocellulose membranes. After blocking nonspecific sites, the membranes were incubated with the primary antibody overnight at 4°C. The membranes were then incubated for 2 h at room temperature with horseradish peroxidase-linked secondary antibody. Bound antibodies were visualized using Enhanced Chemiluminescence's Western blotting system (ECL, Amersham Biosciences, UK).

Caspase-3 activity

The caspase 3 activity was measured by the method of Troyano and others (2003). After treatment, cells (2 × 10^6) were resuspended in 100 μl of lysis buffer (50 mM Tris-HCl pH 7.5, NP-40 03% (v/v), DTT 1 mM) and kept on ice for 30 min. The lysate was centrifuged at 13 800 × g for 15 min at 4°C. Aliquots of 40 μg of proteins were added to 96-well microtiter plates with 100 μl of assay buffer (100 mM HEPES, pH 7.4), 10% w/v sucrose, 5 mM DTT, 6% NP-40, 0.1% CHAPS) and 10 μl of caspase substrate (final concentration 200 μM). Samples were prepared in triplicate and incubated at 37°C. Cleavage was monitored colorimetrically at 405 nm in a microtiterplate reader and recorded at 1 h intervals for 180 min. The substrate used for caspase-3 was Ac-DEVD-pNA.

Quantitative RT-PCR

RT-PCR was performed to study the alteration of PKC ζ expression at the mRNA level. Total RNA was isolated from cells with ULTRASPEC system and 5 μg of RNA from each sample were reverse transcribed to cDNA using reverse transcriptase (Taq Man system). Quantitative PCR analysis was performed using the SYBR* Green system as suggested by the manufacturer. Briefly, reactions were performed in 40 μl with 10 μl of obtained cDNA, 20 μl of SYBR* Green solution and 0.1 μM primers. The sequence of primers used were: mouse pkC ζ sense 5'- CAAGCACTTTTTGTGGAACCC-3', antisense 5'-CATAAGGACACCCAGTGCCC-3'; mouse β-actin sense 5'-AGAGGGAAATCGTGCGTGAC-3', antisense 5'- CAATAGTGATGACCTGGCCGT-3'. The Gen-Bank accession numbers of the nucleotides for the PCR products are: β-actin, NM007393 and PKC ζ, NM008860.The thermal cycle conditions were 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60° for 1 min.

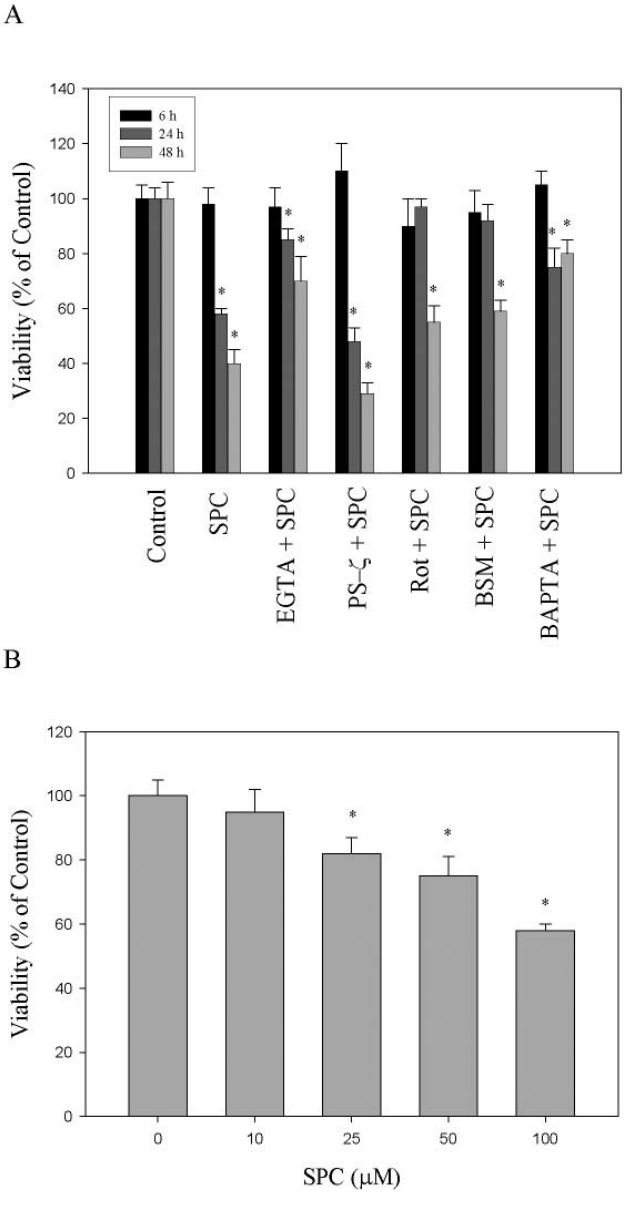

Fig. 1. Effect of exogenous SPC on the viability of neuro 2a cells. (A) Isolated cells were treated with 100 μM SPC alone or in the presence of different inhibitors: 1 mM EGTA, or 20 μM PS-ζ, 10 nM rottlerin (rot), or 10 nM BSM, or 20 μM BAPTA, for the indicated times. (B) Neuro 2a cells were exposed to different SPC concentrations for 24 h. Values are expressed as percentage of control with no additions (100%), and represent means ± SE of at least three separate experiments. *Significantly difference from the control value (P<0.05)

Statistical analysis

Results presented are the mean ± standard error (SE) of at least three separate experiments performed in triplicate. Statistical comparisons were performed using analysis of variance (ANOVA) or Student' t-test. Values of P<0.05 were considered statistically significant.

RESULTS

SPC-induced death neuro 2 a cells

In this study, we investigated the mechanism of SPC-induced cell death in neuro 2a neuroblastoma cells, and the involvement of activation of PKC isozymes. Cells were treated with SPC for 6, 24 or 48 hours and the viability of the cells was measured by MTT. Figure 1A shows the capacity of 100 μM SPC to attenuate the viability after 24 h of treatment to 40%. Treatments as long as 48 h caused significant cell death (nearly 60%), due probably to necrosis of the cells. It has been described that SPC has opposite actions on cell viability in human adipose tissue-derived mesenchymal stem cells (Jeon et al. 2005a). Thus, SPC in a dose of 5 μM increased viability. However, concentrations higher that 10 μM SPC attenuated this effect in this cell type. In our experiments, the effect of SPC was dose-related (from 25 μM to 100 μM) after 24 h of treatment, and SPC concentrations lower than 25 μM did not cause any

effect (Fig. 1B). We also analyzed the role of calcium on SPC-induced cell death. Figure 1A shows that EGTA or BAPTA treatment prevented the SPC effect. These results could indicate that both external and internal calcium are necessary to SPC-induced cell death in neuro 2a neuroblastoma cells. However, the permeabilizing effect of the high SPC concentration used, or the activation of specific calcium channels by SPC as previously reported by us (Miguel et al. 2001) must be taking into account in order to have a more precise interpretation of our results.

On the other hand, taking into account the differential role of PKC δ and ζ isozymes in cell apoptosis in several cell types (Buchner 2000), we also studied the involvement of PKC δ and ζ isoforms on SPC-decreased cell viability. As can be seen in Fig. 1A, pretreatment of cells with BSM, a general PKC inhibitor, or rottlerin, a relatively specific inhibitor of PKC δ, each prevented SPC-induced cell death . However, after treatment with myristoylated PKC ζ pseudosubstrate peptide (PS-ζ), a PKC ζ specific inhibitor (Eichholtz et al. 1993), the effect of SPC in viability cell was slightly increased. The treatment with all these inhibitors alone did not cause any effect (data not shown).

To explore whether SPC induces apoptosis in neuro 2a cells, cells were lysed and DNA was separated by electrophoresis in agarose gel to investigate the characteristic pattern of DNA fragmentation in apoptosis. Results in Fig. 2A show that apoptotic cell death of neuro 2a cells induced by 100 μM SPC was evident after continuous treatment with the sphingolipid for 24 h. In addition, treatment with either PKC ζ or PKC δ inhibitors confirms the pro-apoptotic role for PKC δ and anti-apoptotic role for PKC ζ. SPC-induced apoptosis was confirmed by flow cytometry analysis. Cells were incubated with SPC (from 10 μM to 100 μM) for 24 h and cellular apoptosis and necrosis were measured. As can be seen in Fig. 2B, SPC treatment increased the percentage of apoptosis in a range of 10–25 μM. The apoptotic effect was decreased when the SPC concentration increased to 100 μM concomitantly with cell death by necrosis.

SPC-induced PKC isoform translocation and PKC δ cleavage

One of the possible manifestations of PKC activation is kinase redistribution between the cytosol and membrane. For this reason, immunoblot analysis using

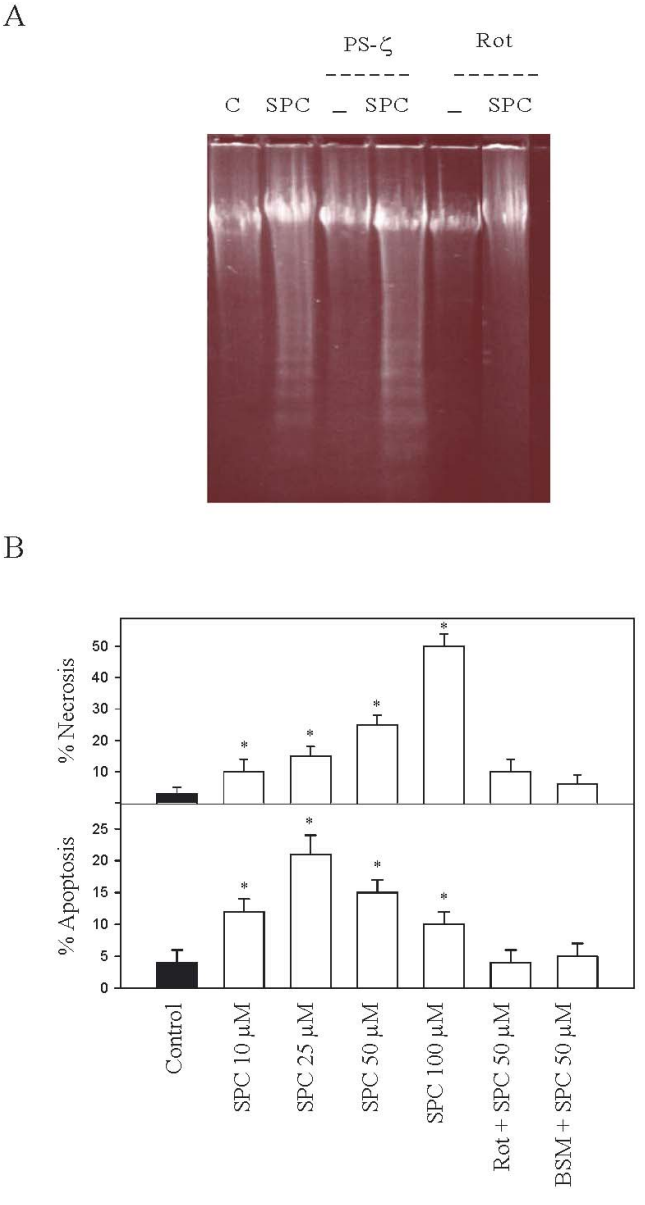

Fig. 2. Effect of SPC on cell apoptosis in neuro 2a cells. (A) Cells were treated with 100 μM SPC in the presence or absence of 20 μM PS-ζ or 10 nM rottlerin for 24 h. Apoptosis was determined by DNA fragmentation assay. These experiments were performed three times, and typical results are shown. (B) The percentages of apoptotic and necrotic cells were analyzed by flow cytometry. PKC inhibitors were added 30 min before SPC treatment. Values are expressed as percentage of control with no additions (100%), and represent means ± SE of at least three separate experiments. *Significantly difference from the control value (P<0.05)

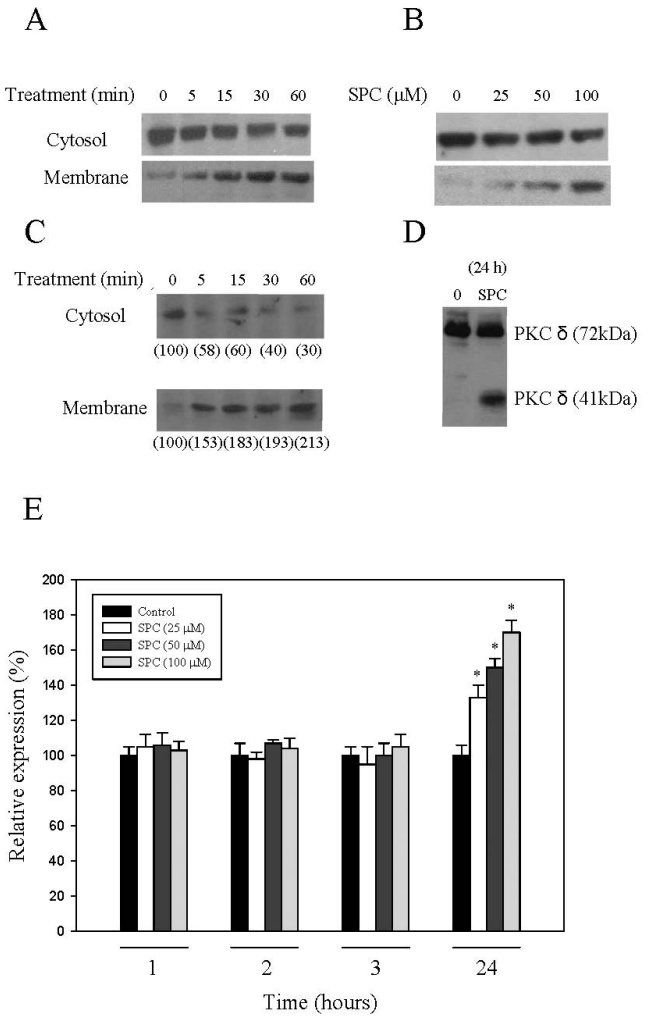

Fig. 3. Intracellular redistribution of PKC isoforms after SPC treatment. The figure shows the relative amounts of PKC ζ and δ in cytosolic and membrane fractions in neuro 2a cells, as determined by subcellular fractionation and immunoblot, using appropriate antibodies. The blots are representative of one of three experiments with similar results. (A) Levels of the PKC ζ in cells treated with 100 μM SPC for different times. (B) Levels of PKC ζ in cells treated with different concentrations of SPC for 60 min. (C) Levels of PKC δ in cells treated with 100 μM SPC for different times. The numbers below the lanes represent the results of the densitometric analysis, relative to control value (100%). (D) PKC δ cleavage in untreated cells and cells treated for 24 h with 100 μM SPC. The figure shows the relative amount of the entire PKC δ isoform (72 kDa) and its derived catalytic fragment (41 kDa). (E) Effect of different concentrations of SPC on PKC ζ expression in neuro 2a cells. Values are expressed as percentage of control with no additions (100%), and represent means ± SE of at least three separate experiments. *Significantly difference from the control value (P<0.05)

specific antibodies against different PKC isoforms were carried out to determine possible changes in intracellular PKC localization in SPC-treated cells. Among the different PKC isoforms, only significant changes in PKC δ and ζ were observed after SPC treatment (Fig. 3A,B,C, and data not shown).

PKC δ is involved in the induction of cell apoptosis in a different manner depending on the apoptotic stimuli. One of the factors that may contribute to the distinct effects of PKC δ on cell apoptosis is the differential pattern of PKC translocation in response to the various apoptotic stimuli. Thus, translocation of PKC δ to cell membrane, mitochondria or nucleus, caused apoptosis in response to UV radiation, oxidative stress or etoposide treatment, respectively (Brodie and Blumberg 2003). In the present study, and taking into account the above data, we have analyzed the translocation of PKC δ to a total particulate fraction, to study its involvement in SPC-induced apoptosis.

PKC translocation in response to SPC treatment was detected at 5 min of treatment, and was maintained for at least 60 min. The effect was SPC concentration-dependent, from 25 μM to 100 μM. The translocation was also time-dependent from 5 min to 60 min. Since it is well known that cells treated with apoptosis-inducing agents undergo proteolytic PKC δ cleavage to give a catalytically active fragment that may contribute to apoptosis (Emoto et al. 1995), we studied the appearance of the characteristic apoptosis-associated 41 kDa fragment in a cellular extract. As can be seen in Fig. 3D, the fragment was detected after 24 h of SPC treatment, concomitantly with the execution of apoptosis (see Fig. 2). Taking into account that, in some experiments, an increase in PKC ζ, but not in PKC δ, was observed in all fractions analyzed, after SPC treatment, experiments using quantitative RT-PCR were performed to analyze the posible increase in PKC ζ expression. Results shown in Fig. 3E indicate that SPC induced, in a concentration-dependent manner, an increase of the PKC ζ expression after 24 h of treatment. It is possible that the sphingolipid evoked an early translocation of the enzyme and increased the PKC isoform synthesis after 24 h of treatment. As indicated above, many of the actions of SPC are shared by the structurally related S1P. In our experimental conditions S1P in a concentration as low as 10 μM caused a higher PKC ζ translocation than did 100 μM SPC (data not shown).

This result suggests that SPC might utilize specific S1P receptors, which exhibit a low affinity for SPC, as described in some cell types (Meyer et al. 2002, Xu 2002).

SPC-induced caspase-3 activation and cytochrome c release

Although the mechanisms by which PKC δ regulates cell apoptosis are currently not defined, a number of downstream targets have been described in different systems. PKC δ translocates to the mitochondria or to the nucleus in response to different apoptotic stimuli (Brodie and Blumberg 2003). Translocation of the enzyme to specific cellular compartments may lead to the regulation of apoptosis by different cellular effects, due to the phosphorylation of key apoptotic proteins and the association of PKC δ with distinct proteins present in these locations. In addition, other studies have shown that PKC δ is cleaved in the third variable region by caspase-3 in the apoptotic response of cells to DNA damage (Jeon et al. 2005b). In this study, we studied the SPC-induced caspase-3 activation. Thus, we analyzed the effect of 100 μM SPC on the enzyme activity after 24 and 48 h of treatment. As can be seen in Fig. 4A, SPC treatment evoked an increase, to nearly 40%, of caspase-3 activity at 24–48 h of treatment, concomitantly with PKC δ cleavage and the execution of apoptosis. In addition, it is noteworthy that the treatment with PS-ζ, a PKC ζ inhibitor, caused an increase rather than a decrease of SPC-induced caspase-3 activity (Fig. 4A), suggesting that the inhibition of PKC ζ, caused an increase of the activation of this caspase, and possibly an increase of cellular apoptosis, as seen in Fig. 2. It is well known that mitochondria play a crucial role in regulating cell death, which is mediated by outer membrane permeabilization. This induces the release of cytochrome c from the mitochondria to the cytosol, leading to activation of a caspase cascade, which is known to be an important step in the apoptotic signalling pathway (Green and Reed 1998). To explore whether the mitochondria-dependent pathway is involved in the SPC-induced apoptosis in neuro 2a cells, we examined the effect of SPC on cytochrome c release into cytosol. The release of cytochrome c from mitochondria to cytosol appeared after 1 h of 100 μM SPC treatment, and continued for up 5 h (Fig. 4B). The cytochrome c

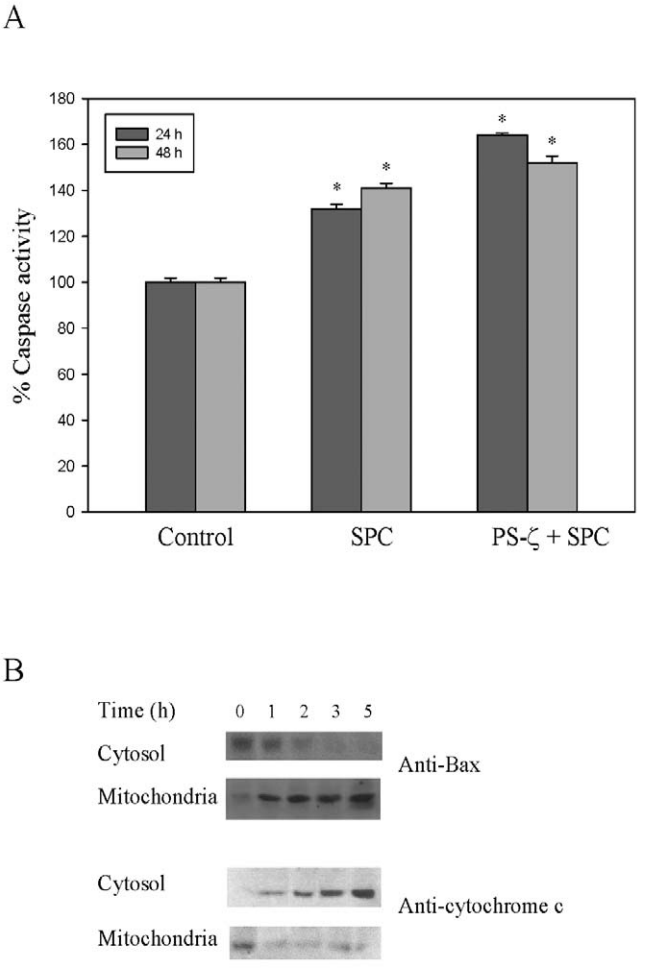

Fig. 4. Effect of SPC on caspase 3 activity in neuro 2a cells. (A) Neuro 2a cells were incubated for 24h or 48 h with 100 μM SPC in the absence or presence of 20 μM PS-ζ and caspase 3 activity was measured. Values are expressed as percentage of control with no additions (100%), and represent means ± SE of at least three separate experiments. (B) Translocation of Bax protein (upper panel) from cytosol to mitochondria and cytochrome c (lower panel) from mitochondria to cytosol by SPC (100 μM) action, as determined by immunoblot using appropriate antibodies. The blots are representative of one of three experiments with similar results.

release was maintained after 24 h of SPC treatment (data not shown). The cytochrome c release is regulated by proapoptotic and antiapoptotic proteins such as Bax and Bcl respectively (Pulcha et al. 1999, Polster and Fiskum 2004). In this study, we also investigated the translocation of Bax from cytosol to membrane. Fig. 4B shows that SPC evoked a translocation of Bax concomitantly with the release of cytochrome c.

DISCUSSION

Although sphingolipids have been proposed as important intracellular signal mediators, involved in various cellular responses, including apoptosis, the mechanisms by which these compounds induce cell death remain to be elucidated. SPC has emerged as an important mediator of cellular signalling. SPC regulates a variety of cellular functions when applied exogenously to intact cells. Although its action on plasma membrane G-protein-coupled receptors has been extensively studied, not much is known about its intracellular site and mechanism of action, or even the identity of its target proteins. SPC has been shown to be a potent mitogen for several cell types, concomitantly with an activation of PKC and MAP kinases and calcium release (Brodie and Blumberg 2003). In this study, we analyzed the viability of cells after SPC treatment, and the involvement of both external and internal Ca^{2+}. In this regard, it has recently been hypothesized that SPC might bind to calmodulin, the ubiquitous intracellular Ca^{2+} sensor, and calmodulin might be an intracellular receptor for SPC (Kovacs and Liliom 2008). For quantitation of cell apoptosis, we used annexin V-PI staining/flow cytometry assay. The results indicate that the sphingolipid derivative induces cell death after 24 h of treatment. We observe after 48 h of treatment an increase in cell death, probably due to a apoptosis-derived necrosis, instead of genuine necrosis as opposed to apoptosis.

One of the most relevant aspects in the regulation of apoptosis is the signalling by different protein kinases, including PKC (Cross et al. 2000)**.** The presence of PKC isozymes in neuro 2a neuroblastoma cells was previously described (Mak et al. 2001). The results obtained in our study indicate that PKC δ and ζ isoforms are involved in SPC-induced cell death in a different manners, suggesting a pro-apoptotic role for PKC δ and anti-apoptotic role for PKC ζ in agreement with data described previously (Grunicke et al. 2003, Urso et al. 2005). In this regard, it has been described that PKC activities may modulate cell death in a different manner depending on the cell model and experimental conditions. Thus, atypical isoforms are anti-apoptotic in their action, whereas PKC δ is usually involved in the promotion of apoptosis (Desai et al. 1993). Moreover, our results are in agreement with those reported previously indicating that both Ca^{2+} and PKC δ activation are necessary along with other SPC actions in several cell types. Thus, SPC generated intracellular ROS through a mechanism depending on Ca^{2+} and activation of PKC δ, and PKC δ, but not conventional PKC isozymes, are specifically involved in SPC-induced phospholipase D activity (Gutcher et al. 2003).

We have reported previously the involvement of PKC δ in apoptosis induction by cadmium ions *via* early membrane translocation, and also possibly through late kinase proteolytic PKC cleavage (Miguel et al. 2005). All these findings may be congruent with the idea that PKC δ activation (translocation and proteolytic cleavage) represent an important pathway for the regulation of apoptosis in several cell types, as proposed by Brodie and Blumberg (2003).

It has been proposed that the redistribution of Bax from cytosol to mitochondria may play a role in neural cell death (de Giorgi et al. 2002). Our results are congruent with the consideration of PKC δ cleavage as a late event, mediated by activation of caspase-3. The importance of the PKC δ catalytic fragment in the apoptotic effect was demonstrated by the finding of overexpression of the PKC δ catalytic domain induced apoptosis whereas no apoptosis was observed in cells overexpressing the full-length PKC δ or a kinase inactive PKC δ fragment (Ghayur et al. 1996).

More work must be done to elucidate the role of this PKC isoform in SPC-induced apoptosis. Since it has been found that tumor cells are more susceptible to apoptosis induced by sphingolipid metabolites than are normal cells (Sweeney et al. 1996), our results represent a potentially useful approach to anti-tumor therapy.

CONCLUSION

Our results indicate that SPC induces apoptosis in neuro 2a cells by a PKC δ-dependent mechanism. In this pathway, mitochondria *via* both intracellular and extracellular Ca^{2+} are involved. In addition, we demonstrated the pro-apoptotic role of PKC ζ in neuro 2a cells.

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REFERENCES

Afrasiabi E, Blom T, Ekokoski E, Tuominen RK, Törnquist K (2006) Sphingosylphosphorylcholine enhances calcium entry in thyroid FRO cells by a mechanism dependent on protein kinase C. Cell Signal 18: 1671–1678.

Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 48: 589–601.

Brodie C, Blumberg PM (2003) Regulation of cell apoptosis by protein kinase C δ. Apoptosis 8: 19–27.

Buchner K (2000) The role of protein kinase C in the regulation of cell growth and in signalling to the cell nucleus. J Cancer Res Clin Oncol 126: 1–11.

Calcerrada MC, Miguel BG, Martin L, Catalán RE, Martínez AM (2002) Involvement of phosphatidylinositol 3-kinase in nuclear translocation of protein kinase C zeta induced by C2-ceramide in rat hepatocytes. FEBS Lett 514: 361–365.

Cross TG, Scheel-Toellner D , N.V. Henriquez NV , Deacon E, Salmon M, Lord JM (2000) Serine/threonine protein kinases and apoptosis. Exp Cell Res 256: 34–41.

de Giorgi F, Lartigue L, Bauer MK, Schubert A, Grimm S, Hanson GT, Remington SJ, Youle RJ, Ichas F (2002) The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. FASEB J 16: 607–609.

Desai NN, Carlson RO, Mattie ME, Olivera A, Buckley NE, Seki T, Brooker G, Spiegel S (1993) Signaling pathways sphingosylphosphorylcholine-mediated mitogenesis in Swiss 3T3 fibroblasts. J Cell Biol 21: 1385–1395.

Desai NN, Spiegel S (1991) Sphingosylphosphorylcholine is a remarkably potent mitogen for a variety of cell lines. Biochem Biophys Res Commun 181: 361–366.

Eichholtz T, deBont DB, DeWidt J, Liskamp RM, Ploegh HL (1993) A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. J Biol Chem 268: 982–986.

Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R, Kufe D (1995) Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. EMBO J 14: 6148–6156.

Ghayur T, Hugunin M, Talanian RV, Ratnofsky S, Quinlan C, Emoto Y, Pandey P, Datta R, Kharbanda S, Allen H, Kamen RR, Wong W, Kufe D (1996) Proteolytic activation of protein kinase C δ by an ICE/CED 3-like protease induces characteristics of apoptosis. J Exp Med 184: 2399–2404.

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1312.

Gutcher I, Webb PR, Anderson NG (2003) The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci 60: 1061–1070.

Grunicke HH, Spitaler M, Mwanjewe J, Schwaiger W, Jenny M, Ueberall F (2003) Regulation of cell survival by atypical protein kinase C isozymes. Adv Enzyme Regul 43: 213–228.

Hannun YA (1996) Functions of ceramide in coordinating cellular to stress. Science 274: 1855–1859.

Hla T, Lee MJ, Ancellin N, Paik JH, Kluk MJ (2001). Lysophospholipids-receptor revelations. Science 294: 875–878.

Ignatov I, Lintze Jl, Hermans-Borgmeyer HJ, Kreienkamp HJ, Joost P, Thomsen S, Methner A, Schaller HC (2003) Role of the G-protein-coupled receptor GPR12 as high-affinity receptor for sphingosylphosphorylcholine and its expression and function in brain development. J Neurosci 23: 907–914.

Jeon ES, Kang YJ, Song HY, Im D-S, Kim HS, Ryu SH, Kim YK, Kim JH (2005a) Sphingosylphosphorylcholine generates reactive oxygen species through calcium-, protein kinase Cδ- and phospholipase D-dependent pathways. Cell Signal 17: 777–787.

Jeon RS, Kang YJ, Song HY, Woo JS, Jung JS, Kim YK, Kim JH (2005b) Role of MEK-ERK pathway in sphingosylphosphorylcholine-induced cell death in human adipose tissue-derived mesenchymal stem cells. Biochim Biophys Acta 1734: 25–33.

Kovacs R., Liliom K (2008) Sphingosylphosphorylcholine as a novel calmodulin inhibitor. Biochem J 410: 427–437

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad H, Alnemri ES, Wang X (1997) Cytochrome c and dATP dependent formation of Apof-1/caspase-9 complex initiate an apoptotic protease cascade. Cell 91: 479–489.

Mak NK, Lung HL, Wong RN, Leung HW, Tsang HY, Leung KN (2001) Expression of protein kinase C isoforms in euxanthone-induced differentiation of neuroblastoma cells. Planta Med 67: 400–405.

Meyer HD, Himmel HM, Jakobs KH (2002) Sphingosylphosphorylcholine-biological functions and mechanisms of action. Biochim Biophys Acta 1582: 78–89.

Miguel BG, Calcerrada MC, Catalan RE, Martinez AM (2001) Sphingolipid derivatives modulate intracellular Ca^{2+} in rat synaptosomes. Acta Neurobiol Exp (Wars) 61: 113–117

Miguel BG, Rodriguez ME, Aller P, Martínez AM, Mata F (2005) Regulation of cadmium-induced apoptosis by PKCδ in U937 human promonocytic cells. Biochim Biophys Acta 1743: 215–222.

Polster BM, Fiskum G (2004) Mitochondrial mechanism of neural cell apoptosis. J Neurochem 90: 1281–1289.

Pulcha GV, Deshmukh M, Johnson EMJ (1999) Bax translocation is a crucial event in neuronal apoptosis: regulation by neuroprotectans BCL-2 and caspases. J Neurochem 19: 7476–7485.

Sawai H, Hannun YA (1999) Ceramide and sphingomyelinases in the regulation of stress responses. Chem Phys Lipids 92: 141–147.

Schimmer AD, Hedley DW, Penn LZ, Minden MD (2001) Receptor- and mitochondrial-mediated apoptosis in acute leukemia: a translational view. Blood 98: 3541–3553.

Sottocasa GL, Kuylenstierna B, Ernster L, Bergstrand A (1967) An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J Cell Biol 32: 415–438.

Sueyoshi N, Maehara T, Ito M (2001) Apoptosis of neuro 2a cells induced by lysosphingolipids with naturally occurring stereochemical configurations. J Lipid Res 42: 1197–1202.

Sun L, Xu L, Henry FA, Spiegel S, Nielsen TB (1996) A new wound healing agent--sphingosylphosphorylcholine. J Invest Dermatol 106: 232–237.

Sweeney EA, Sakakura C, Shirahama T, Masamune A, Ohta H, S. Hakomori S, Igarashi Y (1996) Sphingosine and its methylated derivative N,N-dimethylsphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. Int J Cancer 66: 358–366

Troyano A, Sancho P, Fernández C, de Blas E, Bernardi P, Aller P (2003) The selection between apoptosis and necrosis is differentially regulated in hydrogen peroxide-treated and glutathione-depleted human promonocytic cells. Cell Death Differ 10: 89–98.

Urso L, Muscella A, Calabriso N, Ciccarese A, Fanizzi FP, Migoni D, Di Jeso B, Storelli C, Marsigliante S (2005) Differential functions of PKC-δ and PKC-ζ in cisplatin response of normal and transformed thyroid cells. Biochem Biophys Res Commun 337: 297–305.

Wang L, Kinnear C, Hammel JM, Zhu W, Hua Z, Mi W, Caldarone CA (2006) Preservation of mitochondrial structure and function after cardioplegic arrest in the neonate using a selective mitochondrial KATP channel opener. Ann Thorac Surg 81: 1817–1823.

Xu Y (2002) Sphingosylphosphorylcholine and lysophosphatidylcholine: G protein-coupled receptors and receptor-mediated signal transduction. Biochim Biophys Acta 1582: 81–88.

Young KW, Bootman MD, Channing DR, Lipp P, Maycox PR, Meakin J, Challiss RA, Nahorski SR (2000) Lysophosphatidic acid-induced Ca2+ mobilization requires intracellular sphingosine 1-phosphate production. J Biol Chem 275: 38532–38539.