Ceramide Induces Cell Death through an ERK-dependent Mitochondrial Apoptotic Pathway in Renal Epithelial Cells

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Ceramide induces cell death in a variety of cell types however, the underlying molecular mechanisms related to renal epithelial cells remain unclear. The present study was undertaken to determine the role of extracellular signal-regulated protein kinase (ERK) in ceramide-induced cell death in renal epithelial cells. An established renal proximal tubular cell line of opossum kidney (OK) cells was used for this research. Ceramide induced apoptotic cell death in these cells. Western blot analysis showed that ceramide induced activation of ERK. The ERK activation and cell death induced by ceramide were prevented by the ERK inhibitor PD98059. Ceramide caused cytochrome C release from mitochondria into the cytosol as well as activation of caspase-3. Both effects were prevented by PD98059. The ceramide-induced cell death was also prevented by a caspase inhibitor. These results suggest that ceramide induces cell death through an ERK-dependent mitochondrial apoptotic pathway in OK cells.

I. INTRODUCTION

Ceramide, a metabolite of sphingolipids, is generated by degradation of sphingomyelin through the action of sphingomyelinases and condensation of sphinganine or sphingosine and fatty acyl-CoA through the enzyme, ceramide synthase (Pettus *et al*, 2002). Ceramide has been implicated as having an important role in the cell signaling pathway involved in cell growth, proliferation, and apoptosis, as well as other cell responses (Kolesnick and Kronke, 1998; Luberto and Hannun, 1999). Many inducers of apoptosis, such as TNFα, Fas ligand, serum deprivation, γ-radiation, chemotherapeutic agents, and ischemia/reperfusion, regulate one or more enzymes of

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ceramide metabolism leading to its accumulation (Pena *et al*, 1997 Dbaibo and Hannun, 1998; Pettus *et al*, 2002).

The extracellular signal-regulated kinase (ERK) is a mitogen-activated protein kinase subfamily involved in the regulation of various cellular responses, such as cell-proliferation, differentiation, and apoptosis (Cross *et al*, 2000; Pearson *et al*, 2001). Although it has been generally accepted that ERK activation delivers survival signals (Xia *et al*, 1995; Cobb, 1999), several studies have shown involvement of ERK activation in cell death induced by various stimuli (Bhat and Zhang, 1999; Choi *et al*, 2004; Kim *et al*, 2005; Lee *et al*, 2005; Kim *et al*, 2006). Ceramide has been reported to activate ERK in astrocytes (Blazquez *et al*., 2000; Kim *et al*, 2005; Oh *et al*, 2006). However, it is unclear whether ERK activation is involved in ceramide-induced cell death in renal epithelial cells.

This study was undertaken to clarify the role of ERK activation in ceramide-induced cell death in renal

epithelial cells. Our results show that ceramide induces cell death through a caspase-dependent mechanism involving ERK activation and cytochrome C release.

II. MATERIALS AND METHODS

1. Chemicals

Ceramide, propidium iodide, and Hoechst 33258 were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Tween 20, PD98059, and DEVD-CHO were purchased from Calbiochem (California, USA). Antibodies of phospho-ERK and β-actin were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-cytochrome C monoclonal antibody and horseradish peroxidase conjugated secondary antibody were purchased from PharMingen (San Diego, CA, USA). All other chemicals were of the highest commercial grade available.

2. OK cell culture

OK cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 10 cm^2 culture dishes (Costar, Cambridge, MA, USA). The cells were grown in Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12, Sigma Chemical Co.) containing 5% fetal bovine serum at 37℃ in a 95% air: 5% CO_2 incubator. When the cultures reached confluence, a subculture was prepared using a 0.02% EDTA: 0.05% trypsin solution. The cells were grown on tissue culture plates in DMEM/F12 medium containing 10% fetal bovine serum. All experiments started 3~4 days after plating when a confluent monolayer culture was achieved. Cells were exposed to ceramide in serum-free media.

3. Measurement of cell viability and cell death

Cell viability was evaluated using a MTT assay (Denizot and Lang, 1986). After washing the cells, culture medium containing 0.5 mg/mL of MTT was added to each well. The cells were incubated for 2 hours at 37℃. The supernatant was removed and the formed formazan crystals in viable cells were solubilized with 0.11 mL of dimethyl sulfoxide. A 0.1 mL aliquot of each sample was then transferred to a 96-well plate and the absorbance of each well was measured at 550 nm with an ELISA Reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany). Data was expressed as a percentage of control measured in the absence of MF. Unless otherwise stated, the cells were exposed to 50 μM MF for 48 hours. Test reagents were added to the medium 30 minutes before MF exposure.

Cell death was estimated by counting the cell numbers using a trypan blue exclusion assay. The cells were harvested using 0.025% trypsin and incubated with 4% trypan blue solution. Viable and nonviable cells were counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable.

4. Measurement of apoptosis

a) Cytochemical staining

Cells were grown in 6-well plates after treatment with stimuli. The cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 hour at 4℃ then stained with 10 μM Hoechst 33258 for 15 minutes at 37℃. Cells were then washed twice with PBS and examined by confocal microscopy (LSM510, ZEISS, Germany).

b) Annexin V staining

Phosphotidylserine exposure on the outer layer of the cell membrane was measured using annexin V-fluorescein isothiocyanate (FITC) binding. Cells were harvested and washed with cold PBS, incubated for 15 minutes with annexin V-FITC and propidium iodide, then analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

5. Western blot analysis

Cells were harvested at various times after ceramide

treatment and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl [pH 7.4]). Cell debris was removed by centrifugation at 10,000 g for 10 minutes at 4℃. The resulting supernatants were resolved on 10% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were treated with 5% non-fat dry milk at room temperature for 30 minutes and incubated with rabbit polyclonal antibodies against specific phosphorylated forms of ERK and β-actin. The membranes were washed and incubated with the respective secondary antibodies conjugated with 5% non-fat dry milk. Signals were visualized using enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

6. Measurement of cytochrome C release

Cells were harvested and washed twice with PBS. The cells were incubated with extraction buffer (10 mM Hepes, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 0.05% digitonin, and 1 mM phenylmethylsulfonyl fluoride) at 4℃ for 10 minutes, then centrifuged at 100,000 g for 10 minutes at 4℃ The supernatant represented the cytosolic protein. The pellet was disrupted or in the lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl [pH 7.4], and protease inhibitors). The cytosolic protein was loaded onto 15% SDS-PAGE. The gel was transferred to nitrocellulose paper, which was treated with 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween-20 (TBST), and probed with anti-cytochrome C monoclonal antibody, followed by horseradish peroxidase-conjugated secondary antibody. Bands were visualized by chemiluminescence using an ECL kit (Amersham, Buckinghamshire, UK).

7. Measurement of caspase activity

Caspase-3 activity was measured with a caspase-3 colorimetric assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cell suspensions were centrifuged at 250 g; the supernatant was gently removed; and the cell pellet was lysed in cell lysis buffer at 4°C for 10 minutes. The cell lysate was then incubated with the caspase-3 colorimetric substrate DEVD-pNA at 37°C for 1 hour. The cleavage of the peptide was quantified spectrophotometrically at a wavelength of 405 nm.

8. Statistical analysis

The data are expressed as means±SEM and the significance of the difference between 2 groups was evaluated by t-test. Multiple group comparisons were done using one-way analysis of variance followed by the Tukey *post hoc* test. A probability level of 0.05 was used to establish significance.

III. RESULTS

Apoptosis by ceramide in OK cells

Ceramide induced loss of cell viability depending on time and concentration (Fig. 1). Ceramide also induced cell death in a time-dependent manner with patterns similar to cell viability loss (Fig. 2). To determine if ceramide caused apoptotic cell death, cells were stained with the fluorescence dye Hoechst 33258. The cells treated with ceramide exhibited DNA fragmentation, a typical morphological feature of apoptosis (Fig. 3A). Annexin-V binding assay also demonstrated that 22.92% of cells exposed to ceramide for 24 hours were apoptotic (Fig. 3B right lower and upper quadrants). To ascertain whether ceramide-induced apoptosis occurred in a time-dependent fashion, cells were exposed to ceramide for various times. Significant apoptosis was present after 12 hours of treatment and increased up to 48 hours (Fig. 3C).

Role of ERK activation in ceramide−induced apoptosis

To determine if ERK activation is involved in ceramide-induced apoptosis, cells were exposed to

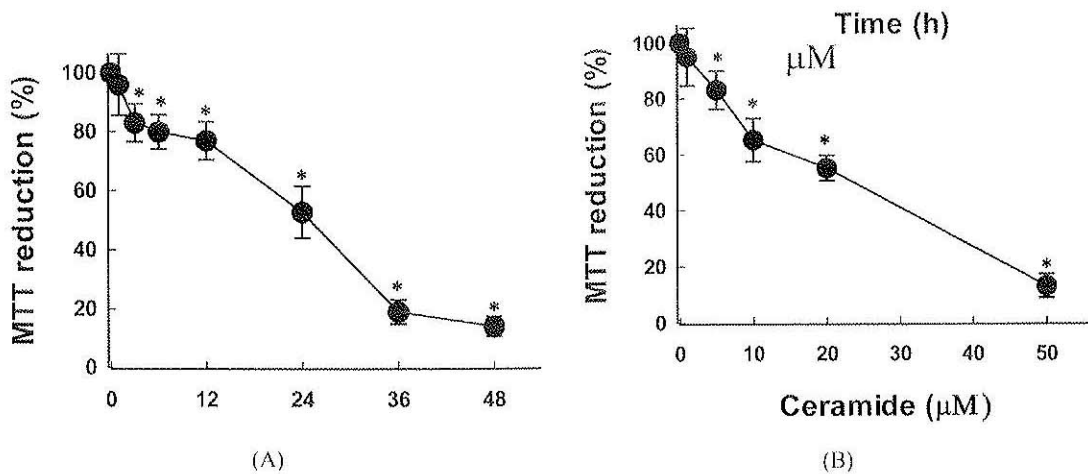(A)   (B)

Fig. 1. Effect of ceramide on cell viability. Cells were exposed to 20 μM ceramide for various times (A) and various concentrations for 24 hours (B). Cell viability was estimated by MTT assay. Data are mean±SEM of 4 independent experiments performed in duplicate. *p<0.05 compared to control.
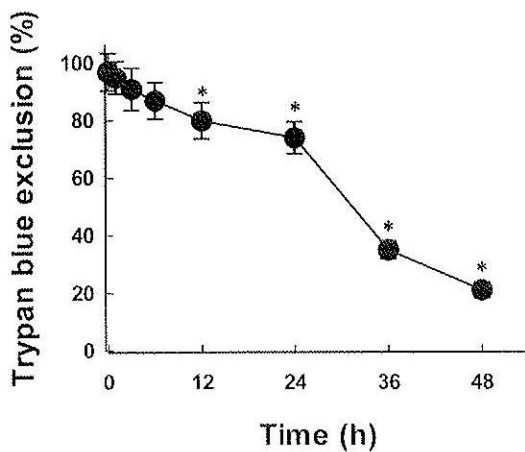

Time (h)

Fig. 2. Effect of ceramide on cell death. Cells were exposed to 20 μM ceramide for various times. Cell death was estimated by trypan blue exclusion. Data are mean±SEM of 4 independent experiments performed in duplicate. *p<0.05 compared to control.

ceramide for various times and phosphorylation levels of ERK were estimated by Western blot analysis using antibody specific to the respective phosphorylated form. ERK was activated after 1 hour of ceramide treatment and the activation increased up to 24 hours (Fig. 4A). Ceramide-induced ERK activation was inhibited by the ERK

inhibitor PD98059 (Fig. 4B), confirming that the bands changed by ceramide are those specific to p-ERK1/2.

Previous studies have shown that ceramide induces apoptosis through a mechanism associated with mito-chondrial cytochrome C release (Cuvillier et al, 2003; Seefelder et al, 2003). Therefore, we examined whether the ceramide-induced cytochrome C release is affected by ERK activation. Cells were exposed to 20 μM ceramide for 24 hours in the presence or absence of 10 μM PD98059. Ceramide caused cytochrome C release from mitochondria into the cytosol and its effect was inhibited by the ERK inhibitor (Fig. 4C).

To determine whether ERK activation plays a critical role in ceramide-induced apoptosis, cells were exposed to ceramide in the presence or absence of the ERK inhibitor and cell viability was estimated by MTT assay. Ceramide caused cell death and its effect was prevented by PD98059 (Fig. 5).

Role of caspase activation in ceramide-induced apoptosis

Cytochrome C release leads to the activation of caspase cascades in the cytosol, known as key executioners of
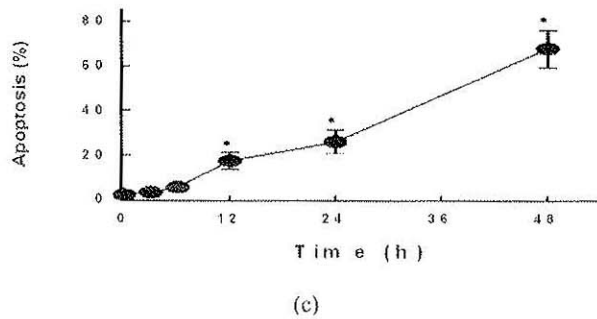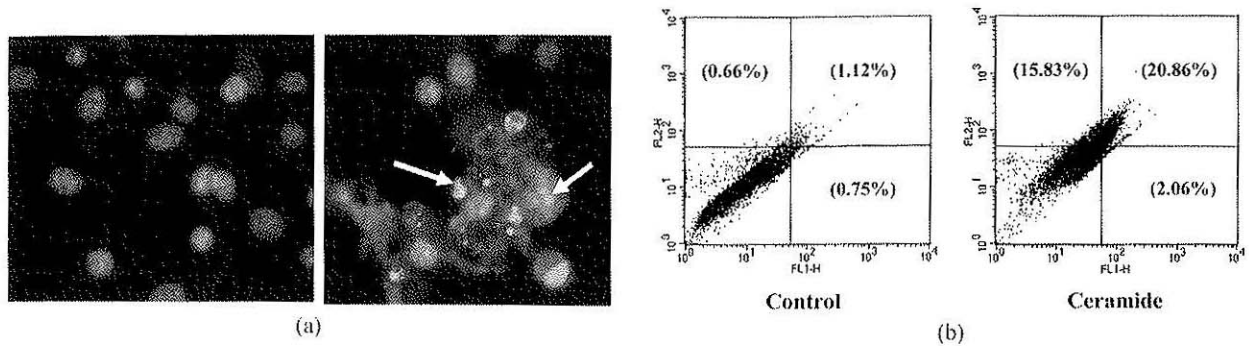
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(a)

Control   Ceramide

(b)

(c)

Fig. 3. Effect of ceramide on apoptosis. Cells were exposed to 20 μM ceramide for 24 hours (A and B) and various times (C). Apoptosis was estimated by Hoechst 33258 staining (A) and annexin-V binding assay (B and C). In annexin-V binding assay, early and late apoptotic cells are shown in right lower and right upper quadrants, respectively. Data in (C) are mean±SEM of 4 independent experiments performed in duplicate. *p <0.05 compared to control.
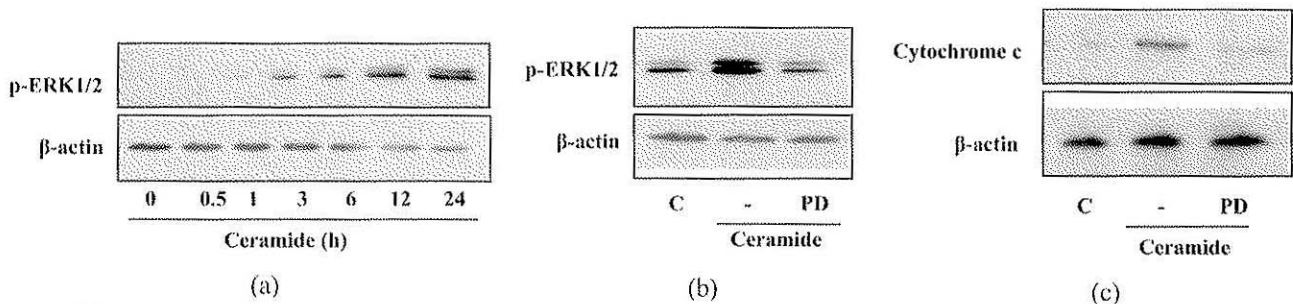

(a)

(b)

(c)

Fig. 4. Effect of ceramide on ERK activation (A and B) and cytochrome C release (C). Cells were exposed to 20 μM ceramide for various times (A) and 24 hours (B and C) in the presence or absence of 10 μM PD98059 (PD). ERK activation and cytochrome C release were estimated by Western blot analysis.
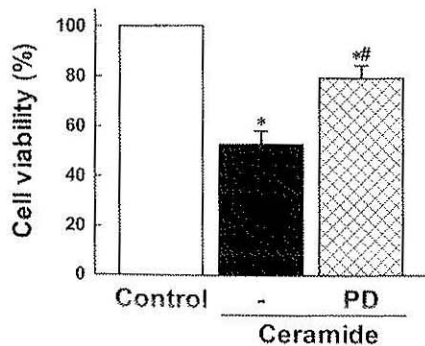

Fig. 5. Effect of ERK inhibitor on ceramide-induced cell death. Cells were exposed to 20 μM ceramide for 24 hours in the presence or absence of 10 μM PD98059 (PD). Cell death was estimated by trypan blue exclusion. Data are mean±SEM of 3 independent experiments performed in duplicate. *p<0.05 compared to control; #p<0.05 compared to ceramide alone.
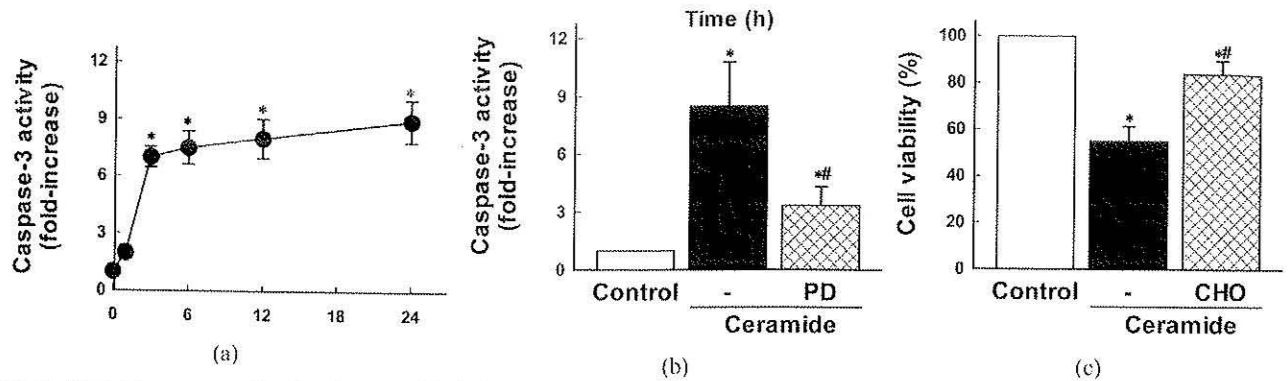
Fig. 6. Role of caspase activation in ceramide-induced cell death. Cells were exposed to 20 μM ceramide for various times (A) for 24 hours (B) in the presence or absence of 10 μM PD98059 (PD). Caspase-3 activation was measured by colorimetric assay kit. Data are mean SEM of 4 independent experiments performed in duplicate. *p<0.05 compared to control; #p<0.05 compared to ceramide alone. (C) Effect of caspase inhibitor on ceramide-induced cell death. Cells were exposed to 20 μM ceramide for 24 hours. Cell viability was estimated by MTT assay. Data are mean±SEM of 4 independent experiments performed in duplicate. *p<0.05 compared to control; #p<0.05 compared to ceramide alone.

apoptosis (Cohen, 1997). Caspase-3 is responsible, at least in part, for the proteolytic cleavage of many key proteins, such as poly (ADP-ribose) polymerase. To confirm the involvement of caspase activation in ceramide-induced apoptosis, the effect of ceramide on caspase-3 activation was examined. Caspase-3 was activated by ceramide after 3 hours of treatment, and inhibited by the ERK inhibitor (Fig. 6A and B). Ceramide-induced apoptosis was also prevented by the caspase inhibitor (Fig. 6C), indicating that ceramide induces apoptosis through a caspase-dependent mechanism in OK cells.

IX. DISCUSSION

The present study demonstrates that ceramide induces a loss of cell viability in a time- and dose-dependent manner and these changes were similar to that of cell death as estimated by trypan blue exclusion assay (Figs. 1 and 2). The results of Hoechst 33258 staining and the annexin-V binding assay show that ceramide induces cell death through apoptosis (Fig. 3).

The present study demonstrates that activation of ERK plays an important role in ceramide-induced apoptosis, in contrast to the general view that ERK activation delivers survival signals. Ceramide induced sustained ERK activation after 1 hour of treatment (Fig. 4). The ERK activation and cell death induced by ceramide were inhibited by PD98059, a specific inhibitor of ERK upstream kinase MEK1 (Alessi et al, 1995; Dudley et al, 1995) (Figs. 4B and 5).

These results support findings that activation of ERK is involved in the induction of apoptosis by cisplatin in HeLa cells (Wang et al, 2000) and neuroblastoma cells (Woessmann et al, 2002). The underlying mechanisms responsible for such opposing observations on the role of ERK activation in cells treated with cisplatin are poorly understood, but the kinetics and duration of action may be important factors. For example, in situations where ERK activation enhances survival, the activation occurs rapidly and is more transient (Guyton et al, 1996; Aikawa et al, 1997); whereas in situations where it is apoptotic, the activation tends to be delayed and sustained (Jimenez et al, 1997; Wang et al, 2000). In the present study, the antioxidant N-Acetyl Cysteine inhibited cisplatin-induced activation of ERK (Fig. 5), suggesting that cisplatin-induced activation of ERK is mediated by a lipid peroxidation-dependent mechanism.

An activation of the ERK pathway is thought to

mediate survival signals (Xia *et al*., 1995); however, in the present study, we observed that ERK activation mediated cell death rather than cell survival in ceramide-exposed cells. Previous studies have reported that ERK activation is involved in cell death induced by cisplatin in renal epithelial cells (Nowak, 2002; Kim *et al*, 2005). In our study the ERK inhibitor PD98059 prevented ceramide induced activation of ERK (Fig. 4A and B) as well as ceramide induced cell death (Fig. 5).

Two distinct apoptotic pathways have been proposed in mammalian cells: receptor-mediated and mitochondria-mediated. The receptor-mediated pathway is triggered by an activationof cell death receptors (Fas and tumor necrosis factor) followed by an activation of caspase-8, which in turn cleaves and activates downstream caspase-3 (Wang *et al*, 2000; Woessmann *et al*, 2002; Xia *et al*, 1995). The mitochondrial pathway is initiated by cytochrome C release which promotes the activation of caspase-9 through Apaf-1. The activated caspase-9 then activates downstream caspase-3 (Chandra *et al*, 2002). Previous studies demonstrated that ceramide-induced cell death was associated with cytochrome C release and caspase activation (Cuvillier *et al*, 2003; Seefelder *et al*, 2003); however, whether these events were mediated by ERK activation was not explored in renal epithelial cells. In the present study, ceramide induced cytochrome C release, caspase activation, and subsequent effects were eliminated by ERK inhibitor PD98059 (Figs. 4C and 6B). These results suggest that ERK activation is involved in the mitochondria-dependent apoptotic pathway in ceramide-exposed OK cells.

In conclusion, we demonstrated that ceramide-induced cell death in OK cells is mediated by activation of ERK occurring upstream of cytochrome C release and caspase activation.

Acknowledgment

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