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Bax-dependent apoptosis induced by ceramide in HL-60 cells

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Abstract Ceramide is an important lipid messenger involved in mediating a variety of cell functions including apoptosis. In this study, we show that antisense bax inhibits cytochrome *c* release, poly(ADP-ribose)polymerase cleavage and cell death induced by ceramide in HL-60 cells. In addition, ceramide induces translocation of Bax to mitochondria. The addition of the broad spectrum caspase inhibitor zVAD-fmk prevented ceramide-induced apoptotic cell death but did not inhibit translocation of Bax and mitochondrial cytochrome *c* release. Furthermore, ceramide inhibits the expression of the antiapoptotic protein Bcl-xL with an increase in the ratio of Bax to Bcl-xL. These data provide direct evidence that Bax plays an important role in regulating ceramide-induced apoptosis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ceramide; Bax; Bcl-xL; Apoptosis; Caspase

1. Introduction

Ceramide is an important lipid messenger involved in mediating a variety of cell functions including apoptosis, cell cycle arrest and cell senescence [1–3]. Apoptosis induced by a variety of inducers such as tumor necrosis factor-α (TNF-α), Fas ligation and chemotherapeutic agents and environmental stresses is associated with the hydrolysis of sphingomyelin accompanied by the accumulation of ceramide [4–7]. Moreover, exogenous cell permeable ceramide and endogenous ceramide generated by sphingomyelinase activation specifically induce apoptosis in many different cell types. Ceramide is therefore considered to be a common mediator of apoptotic mechanisms. However, signal transduction pathways mediating ceramide-induced apoptosis are largely unknown. Present knowledge indicates that a ceramide-mediated apoptotic pathway includes cytochrome *c* release and the activation of several caspases, cleavage of specific substrates by caspase which lead to DNA fragmentation [8–12]. But how the caspase activation and cytochrome *c* release occur during ceramide-induced apoptosis is not clear.

Apoptotic stimuli such as activation of cell surface receptors or environmental stress can induce cytochrome *c* release from mitochondria. Once released, cytochrome *c* binds to Apaf-1 and activates caspase-9 in the presence of dATP [13–15]. The activated caspase-9 leads to the activation of downstream effector caspase, such as caspase-3, which cleaves a number of cellular proteins to execute cell death. It has recently been proposed that in receptor-mediated apoptosis, Bid, activated by caspase-8, is translocated to the mitochondria and induces the release of cytochrome *c*, whereas in chemical-induced apoptosis, cytochrome *c* release is caspase-independent and is not mediated by cleavage of Bid [16–18].

Bax is a proapoptotic members of the Bcl-2 family that resides in the cytosol and translocates to mitochondria upon induction of apoptosis [19–21]. Recently, Bax has been shown to induce cytochrome *c* release and caspase activation in vivo and in vitro [20,22]. In contrast, antiapoptotic Bcl-2 and Bcl-xL can block cytochrome *c* release in cells undergoing apoptosis [22–24]. The antiapoptotic Bcl-2 family reside on the outer mitochondrial membrane and can inhibit apoptosis by many mechanisms such as homo- or heterodimerization with other family members, maintenance of normal mitochondrial membrane resulting in the prevention of cytochrome *c* release and subsequent caspase activation. Recent studies have shown that Bcl-xL abolishes apoptosis, caspase-3 activity, and release of cytochrome *c* induced by ceramide [12,25]. At present, it is still not clear how ceramide acts on mitochondria.

In this report, we examined pathways downstream of ceramide, with particular focus on the ability of Bax to induce the release of cytochrome *c* and apoptosis, and we evaluated the relationships between mitochondrial dysfunction and caspase activation. By using a specific bax antisense oligonucleotide, we demonstrate the important functional role of Bax in ceramide-induced apoptosis. We show that antisense bax inhibits cytochrome *c* release, poly(ADP-ribose)polymerase (PARP) cleavage and cell death. In addition, ceramide induces translocation of Bax to mitochondria and increases the ratio of Bax to Bcl-xL. Our findings suggest that Bax plays an important role in regulating the apoptotic process upstream of cytochrome *c* release induced by ceramide.

2. Materials and methods

2.1. Materials

C_6-ceramide was obtained from Sigma. Lipofectamine was obtained from Life Technologies. Fetal bovine serum was from Gibco BRL, ECL kit from Amersham Pharmacia, caspase-3, 8, 9 substrates from Biomol, and Hoechst 33258 from Molecular Probes. Antibody to cytochrome *c* was from Pharmingen. Antibodies to Bax, Bcl-2, Bcl-xL and HRP-conjugated secondary antibody were from Santa Cruz Biotechnology. zVAD-fmk was from Enzyme System Products.

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Abbreviations: TNF-α, tumor necrosis factor-α; zVAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethyl ketone; DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide; PARP, poly(ADP-ribose)polymerase

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2.2. Cell viability and internucleosomal DNA fragmentation

HL-60 cells were washed with serum-free RPMI. Ceramide, zVAD-fmk or vehicle was diluted into serum-free RPMI at the indicated concentrations. Cell viability and DNA fragmentation were analyzed as described previously [26]. For C_6-ceramide-induced apoptosis, HL-60 cells were maintained in serum-free RPMI for 24 h before experiments. Staining nuclei with Hoechst 33258 was performed as described previously [26].

2.3. Oligonucleotide treatment

HL-60 cells were cultured at 5×10^5 cells per ml in the presence or absence of ceramide and/or Bax antisense oligodeoxynucleotides for the indicated times in complete culture medium (RPMI 1640 supplemented with 10% fetal bovine serum). Bax antisense and scrambled oligodeoxynucleotides with a natural phosphodiester backbone were synthesized by Bioneer (Chungbuk, South Korea). Sequences used were according to previously published work [27]: antisense Bax, 5′-TCG ATC CTG GAT GAA ACC CT-3′ and 5′-TCC CCC CCC ATT CGC CCT GC-3′ and scrambled Bax, 5′-TCA GTC CTG GTA GAA CAC CT-3′ and 5′-CTC CCC CCA CTT CGC CTC GC-3′. Inhibition of Bax protein expression was achieved by using a mixture of the two antisense molecules, both at a final concentration of 1 μM.

2.4. Subcellular fractionation

The basic methodology for the preparation of mitochondria and cytosol fractions was modified from a previous report [28]. Briefly, HL-60 cells (3×10^6) at the end of the treatment were harvested and washed with ice-cold PBS. Cells were resuspended in 500 μl of buffer A (20 mM HEPES–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol) containing 250 mM sucrose and a mixture of protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, 1 μg/ml chymostatin). To lyse the cells, the cell suspension was passed five times through a 26-gauge needle fitted to a syringe. Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at $1000\times g$ at 4°C for 10 min. The resulting supernatant was subjected to $10\,000\times g$ centrifugation at 4°C for 20 min. The pellet fraction (i.e. mitochondria) was first washed with the above buffer A containing sucrose and then solubilized in 50 μl of TNC buffer (10 mM Tris–acetate, pH 8.0, 0.5% NP-40, 5 mM $CaCl_2$). The supernatant was recentrifuged at $100\,000\times g$ (4°C, 1 h) to generate cytosol.

2.5. Western blot analysis

Cells were solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Insoluble materials were removed by centrifugation at $10\,000\times g$ for 10 min. Extracted proteins (50 μg/well) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% polyacrylamide gels, and were electrophoretically transferred onto Immobilon-P membrane. Blocking was performed in Tris-buffered saline containing 5% skimmed milk powder and 0.1% Tween-20. The membranes were probed with antibodies against PARP, cytochrome *c*, Bcl-2, Bax, Bcl-xL or actin. Detection was performed with ECL system. Protein content was determined with the Bradford method using bovine serum albumin as a standard.

2.6. Measurement of caspase activity

Cell lysates were incubated with the colorimetric substrates: DEVD-pNA (caspase-3) or IETD-pNA (caspase-8) to measure caspase activity according to the protocol suggested by the manufacturer. Reactions were assembled in microtiter plate wells by adding 160 μl of buffer B (100 mM HEPES (pH 7.5), 20% (v/v) glycerol, 5 mM DTT, and 0.5 mM EDTA) containing 100 μM substrate to wells containing 50 μg of cytosolic protein in 40 μl of buffer A. Plates were incubated at 37°C for 1 h. Release of free pNA, which absorbs at 405 nm, was monitored continuously.

3. Results

3.1. Cell death, but not cytochrome c release is prevented by zVAD-fmk in ceramide-treated HL-60 cells

To elucidate the role of caspases in ceramide-induced apoptosis, HL-60 cells were treated with caspase inhibitor, and we investigate the effects of apoptotic signaling events, including cytochrome *c* release during ceramide-induced apoptosis.

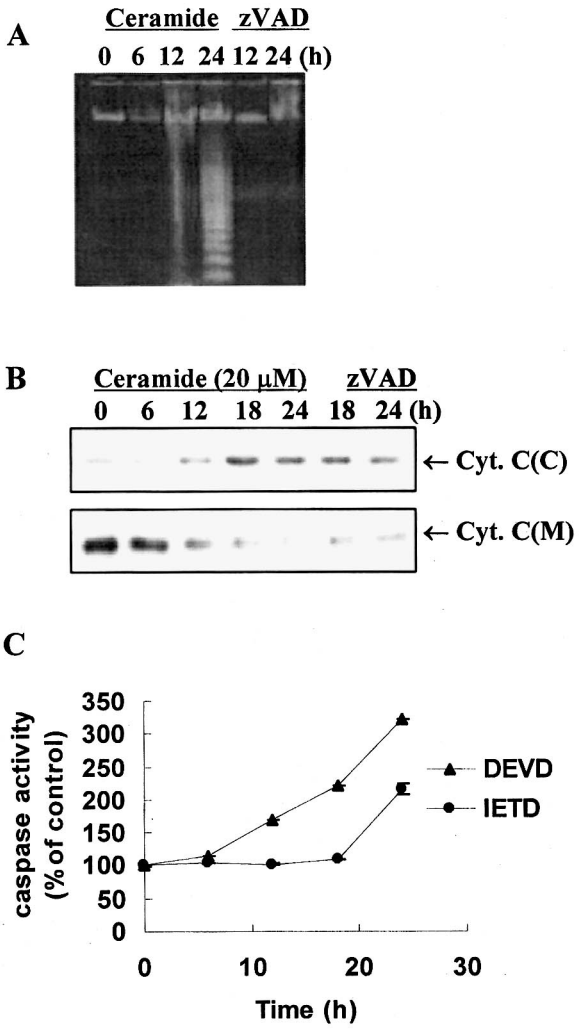

Fig. 1. Cell death, but not cytochrome *c* release is prevented by zVAD-fmk in ceramide-treated HL-60 cells. HL-60 cells were treated with 20 μM C_6-ceramide in the presence or absence of zVAD-fmk (50 μM). A: DNA extracted from the cells was subjected to conventional agarose gel electrophoresis. B: Cytosolic and mitochondrial fractions were prepared at the indicated times, separated by 12% SDS–PAGE, and immunoblotted with a mouse anti-cytochrome *c* antibody. C: At the indicated times, samples of cell extracts were assessed for their ability to hydrolyze the peptides DEVD-pNA and IETD-pNA. Results shown are representatives of three separate experiments.

Induction of apoptosis by ceramide was confirmed by detecting DNA fragmentation in HL-60 cells. In parallel, cytochrome *c* release and caspase activation were determined. In agreement with other cell lines [11,12], ceramide induced internucleosomal DNA fragmentation, cytochrome *c* release from mitochondria and subsequent activation of caspase-3 (Fig. 1). However, activation of caspase-8 occurred at a late time after ceramide treatment (Fig. 1C). Cells treated with ceramide exhibited activation of caspase-3 after 12 h while caspase-8 was activated at 24 h after ceramide treatment. This observation indicates that both caspases act as downstream caspases. DNA fragmentation induced by ceramide was inhibited by the broad caspase inhibitor benzoyloxycar-

bonyl-VAD-fluoromethylketone (zVAD-fmk) (Fig. 1A), whereas cytochrome *c* release was not affected by zVAD-fmk (Fig. 1B). Present results show that zVAD-fmk has no effect upstream of cytochrome *c* release but blocks cell death, indicating downstream caspases are required for ceramide-mediated cell death in HL-60 cells.

3.2. *Bax is required for cytochrome c release in ceramide-induced apoptosis*

The experiments described above indicate that ceramide-induced cytochrome *c* release in HL-60 cells is caspase-independent. Recent reports have shown that Bax can directly induce cytochrome *c* release from mitochondria without requirement for caspases [22]. Induction of cytochrome *c* release from mitochondria occurs via caspase-8-mediated cleavage of Bid and (or) Bax mitochondrial translocation [17,19,20]. Since ceramide-induced caspase-8 activation was observed after cytochrome *c* release (Fig. 1), we analyzed whether Bax is involved in cytochrome *c* release. To determine if Bax is essential for cytochrome *c* release in ceramide-mediated apoptosis in HL-60 cells, we used Bax antisense oligodeoxynucleotides to specifically decrease intracellular Bax levels. Cells exposed to 1 μM of Bax antisense oligodeoxynucleotides for 24 h expressed markedly reduced Bax protein levels (Fig. 2A). As assessed by trypan blue or Hoechst dye staining, Bax antisense inhibited cell death and prevented nuclear DNA fragmentation (Fig. 2), indicating that Bax is required for mediating apoptosis induced by ceramide. Furthermore, Bax antisense prevented ceramide-induced cytochrome *c* release and PARP cleavage (Fig. 3). These results indicate that Bax promotes apoptotic cell death and induces cytochrome *c* release downstream of ceramide.

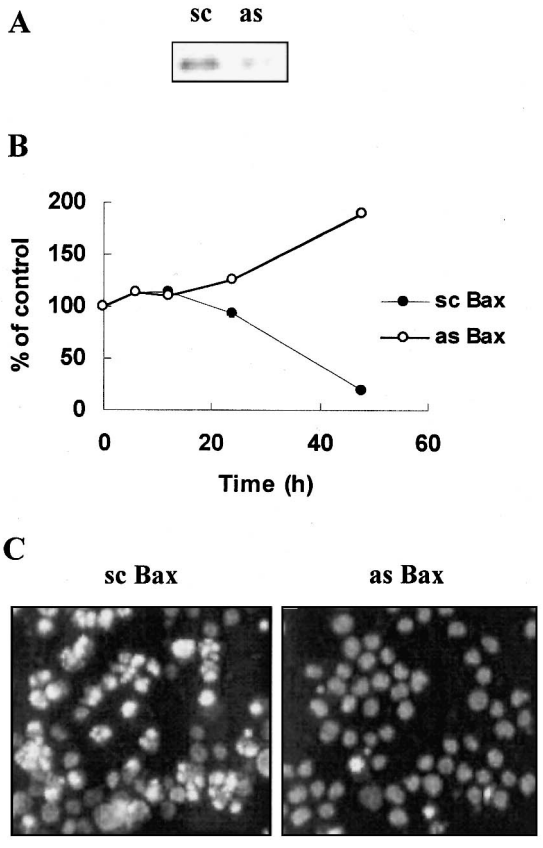

Fig. 2. Bax antisense reduces cell death induced by ceramide. HL-60 cells were incubated with 1 μM of Bax antisense (as) or scrambled oligonucleotides (sc). A: After a recovery period of 24 h, Bax protein levels were determined by Western blot. The Bax band migrates at ~23 kDa. B: After treatment with C_6-ceramide (20 μM), cell viability was determined at the indicated times. C: Fluorescence photomicrographs of Hoechst 33258 staining of HL-60 cells treated with C_6-ceramide (20 μM, 24 h) in the presence of Bax antisense or scrambled oligonucleotides.

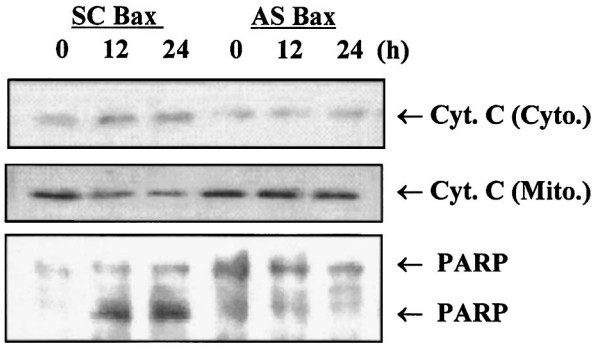

Fig. 3. Bax is required for cytochrome *c* release in ceramide-induced cell death. HL-60 cells were incubated with 1 μM of Bax antisense or scrambled oligonucleotides. After a recovery period of 24 h, cells were treated with C_6-ceramide and collected at the indicated times. Cells were fractionated and equal amounts of cytosolic and mitochondrial protein were loaded, separated by SDS–PAGE, and immunoblotted with an anti-cytochrome *c* antibody or anti-PARP antibody.

3.3. *Ceramide induces downregulation of Bcl-xL protein and alteration of Bax/Bcl-xL ratio*

Bax may play a crucial role in the apoptotic process via a number of different mechanisms. For example, Bcl-2 or Bcl-xL counteracts the effect of Bax by forming heterodimers with it. Previous studies have shown that the ratio between pro-apoptotic and antiapoptotic Bcl-2 families plays a major role in susceptibility of cells to apoptotic stimuli [29,30].

To determine the mechanisms by which ceramide causes Bax-dependent apoptosis, we investigated the expression of antiapoptotic and proapoptotic members of the Bcl-2 family in ceramide-treated cells. As shown in Fig. 4, Bcl-2 level was not changed by ceramide treatment, but the expression of the Bcl-xL protein was substantially reduced whereas the level of

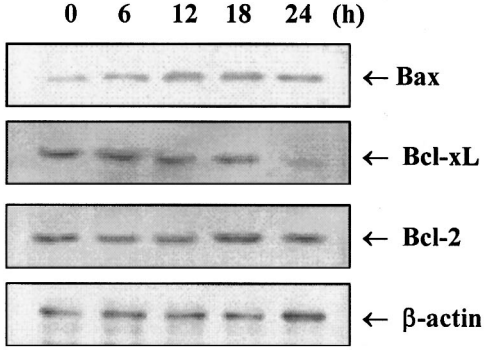

Fig. 4. Ceramide induces alteration in the Bax/Bcl-xL ratio. HL-60 cells were treated with 20 μM C_6-ceramide for the indicated time points. Equal amounts of cell lysates were separated by SDS–PAGE, followed by Western blot with a rabbit anti-Bax antibody, an anti-Bcl-xL antibody, or anti-Bcl-2 antibody.

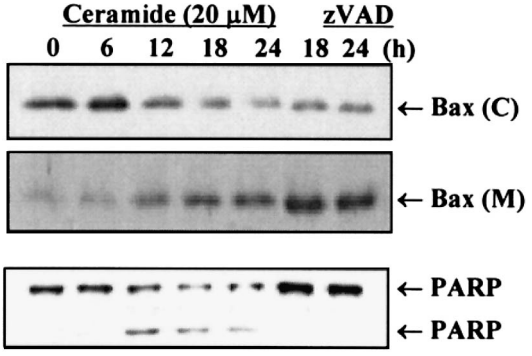

Fig. 5. Ceramide induces caspase-independent Bax translocation from cytosol to mitochondria. HL-60 cells were preincubated with 50 μM of zVAD-fmk for 1 h and then treated with C_6-ceramide (20 μM) for the indicated times. Cells were fractionated and equal amounts of cytosolic and mitochondrial protein were loaded, separated by SDS–PAGE and immunoblotted with an anti-Bax antibody or anti-PARP antibody.

Bax was slightly increased by ceramide. Downregulation of Bcl-xL was detected 24 h after treatment with ceramide (Fig. 4), which is in accordance with the time course of caspase-8 activation (Fig. 1).

3.4. *Ceramide induces caspase-independent alteration in subcellular distribution of Bax*

Since Bax has been shown to induce cytochrome *c* release from mitochondria to the cytosol in conjunction with apoptosis in several cell lines and translocation of Bax to mitochondrial outer membrane is a primary event in triggering mitochondrial function [20], we examined the subcellular distribution of Bax after ceramide treatment of HL-60 cells. As shown in Fig. 5, Bax translocation from the cytosol to the mitochondria occurred within 6 h after treatment with ceramide in HL-60 cells. Bax translocation was accompanied by cytochrome *c* release and PARP cleavage (Figs. 1 and 5). Pretreatment of HL-60 cells with zVAD-fmk did not block Bax mitochondrial translocation (Fig. 5). Therefore, Bax translocation is caspase-independent and downstream caspase is required for cell death in the ceramide-mediated apoptosis.

4. Discussion

Although numerous studies document mitochondria-dependent cell death induced by ceramide, the molecular ordering of ceramide signaling remains unclear. In this study we have shown that Bax mediates mitochondrial cytochrome *c* release, and caspase activation during ceramide-induced apoptosis in HL-60 cells. Of particular interest, ceramide induces subcellular redistribution of Bax, which was associated with cytochrome *c* release from the mitochondria independently of caspase activation. We also found that caspase activation is required for signaling events downstream of mitochondria, such as PARP cleavage and DNA fragmentation in ceramide-induced cell death.

Bax has been known to cause cytochrome *c* release from mitochondria and caspase activation in cell-free extracts and in cells treated with apoptosis-inducing agents [22,24]. In addition, Bax translocates from its predominantly cytoplasmic location to the mitochondria upon apoptosis induction [19,20]. Bcl-2 and Bcl-xL have been shown to inhibit Bax translocation, cytochrome *c* release and caspase activation induced by Fas or other apoptotic inducing agents [20,22,23].

Several recent reports have shown that overexpressing Bcl-2 or Bcl-xL inhibits ceramide accumulation during apoptosis induced by chemotherapeutic agents, irradiation, or hypoxia. In contrast, Bax had no effect on ceramide formation during etoposide-induced apoptosis, but enhanced etoposide-induced apoptosis through acceleration of cytochrome *c* release and caspases activation [8,12,25]. These results indicate that Bax may act downstream or independent of ceramide to directly activate the release of cytochrome *c*. To clarify the role of Bax in the regulation of ceramide-induced apoptosis, we used Bax antisense oligodeoxynucleotides to decrease intracellular Bax levels. We demonstrated that treatment of HL-60 cells with Bax antisense prevented ceramide-induced apoptosis, cytochrome *c* release and PARP cleavage. Our data suggest that Bax acts downstream of ceramide to induce cytochrome *c* release, providing direct evidence for a role of Bax in the apoptotic pathway mediated by ceramide. The mechanism by which ceramide causes Bax-dependent apoptosis has not yet been determined. Recent reports suggest that alterations in the ratio between proapoptotic and antiapoptotic members of the Bcl-2 family, rather than the absolute expression level of any single Bcl-2 member, can determine apoptotic sensitivity [29,30], which would interfere with the availability and translocation of the Bax protein from the cytoplasm to the mitochondria. It was also reported that overexpression of Bcl-2 or Bcl-xL protected against ceramide-induced apoptosis [25]. Previously, we reported ceramide increased Bax/Bcl-2 ratio in HL-60 cells [31]. Here, we observed decreased Bcl-xL expression with an increase in the Bax/Bcl-xL ratio in ceramide-treated HL-60 cells. Therefore, it is suggested that the effect of Bax on ceramide-mediated apoptosis may be related to the decreased levels of proapoptotic members of the Bcl-2 family, thereby weakening the death-protecting signaling during apoptosis. Since Bcl-xL and Bax act antagonistically in the regulation of apoptosis, the ratio of Bax and Bcl-xL protein levels is important for cells undergoing apoptosis. Recent data suggest that ceramide could signal mitochondrial apoptosis by inhibiting the protein kinase Akt [26,32], which phosphorylates Bad. Phosphorylation of Bad via growth factor receptor signaling and the Akt kinase releases Bcl-xL to target mitochondria. Thus, inhibition of Akt by ceramide leads to inhibition of antiapoptotic protein Bcl-xL by Bad. Based on these observations, it is postulated that ceramide may induce apoptosis by increasing proapoptotic signaling and decreasing antiapoptotic signaling, leading to disruption of the balance of antiapoptotic and proapoptotic signaling within the cell.

In this study, we detected caspase-independent mitochondrial Bax translocation and cytosolic release of cytochrome *c*, and observed caspase-dependent PARP cleavage and DNA fragmentation by ceramide, indicating downstream caspase is required for ceramide-induced apoptosis. These findings are similar to reports that caspase inhibitors had no effect on Bax-induced cytochrome *c* release, but prevented cleavage of nuclear substrates and DNA fragmentation [22]. In addition to activation of caspase-3 in ceramide-treated cells, caspase-8 activation was also observed. Caspase-8 has been shown to cleave Bid and the cleaved Bid is reported to be more efficient for triggering the oligomerization and translocation of Bax into mitochondrial membrane [33]. Many re-

ports indicate that ceramide formation in response to various death triggers is mediated by caspase-8 activation [8,9,12]. These results indicate that caspase-8 is positioned upstream of ceramide or between ceramide and Bax in the apoptotic signaling pathway. However, we observed caspase-8 activation in response to ceramide occurred after caspase-3 activation implying that caspase-8 acts as a downstream caspase in ceramide-induced apoptosis. This discrepancy may be explained by the differential timing of caspase-8 activation between receptor-mediated and non-receptor-induced apoptosis. It is demonstrated that caspase-8 is the most upstream caspase for the induction of receptor-mediated apoptosis, but may be activated downstream of cytochrome *c* release in non-receptor forms of apoptosis [34]. It is also reported that Bcl-xL blocked TNF-α-induced caspase-8 activation [35]. When comparing the time course for activation of caspase-8 with expression of Bcl-xL protein, it is suggested that decreases in Bcl-xL levels could trigger caspase-8 activation downstream of mitochondria.

In summary, ceramide mediates apoptosis of HL-60 cells through mitochondrial signaling which involves translocation of Bax to mitochondria where it promotes the release of cytochrome *c*. Our results contribute to the ordering of events during ceramide-induced apoptosis, by demonstrating that Bax is responsible for cytochrome *c* release and caspase-3 activation. In addition, Bax translocation is independent of caspase activation and precedes cytochrome *c* release from the mitochondria. Further studies will be required to identify the specific signals that induce mitochondrial Bax translocation by ceramide.

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