Ceramide Promotes Apoptosis in Lung Cancer-Derived A549 Cells by a Mechanism Involving c-Jun NH₂-Terminal Kinase

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ABSTRACT

Ceramide regulates diverse signaling pathways involving cell senescence, the cell cycle, and apoptosis. Ceramide is known to potentely activate a number of stress-regulated enzymes, including the c-Jun NH₂-terminal kinase (JNK). Although ceramide promotes apoptosis in human lung cancer-derived A549 cells, a role for JNK in this process is unknown. Here, we report that ceramide promotes apoptosis in A549 cells by a mechanism involving JNK. The JNK inhibitor SP600125 proved effective at protecting cells from the lethal effects of ceramide. To understand which JNK-mediated pathway may be involved, a number of JNK target proteins were examined, including the transcription factor, c-Jun, and the apoptotic regulatory proteins Bcl-X and Bim. A549 cells exhibited basal levels of phosphorylated c-Jun in nuclear fractions, revealing that active c-Jun is present in these cells. Ceramide was found to inhibit c-Jun phosphorylation, suggesting that JNK-mediated phosphorylation of c-Jun is not likely involved in ceramide-induced apoptosis. Ceramide did not promote Bcl-X phosphorylation. On the other hand, ceramide promoted phosphorylation of Bim and induced translocation of active JNK from the nucleus to the cytoplasm and mitochondrial fraction. Ceramide-mediated changes in localization of JNK were consistent with the observed changes in phosphorylation status of c-Jun and Bim. Furthermore, ceramide promoted Bim translocation to the mitochondria. Mitochondrial localization of Bim has been shown recently to promote apoptosis. These results suggest that JNK may participate in ceramide-induced apoptosis in A549 cells by a mechanism involving Bim.

INTRODUCTION

The c-Jun NH₂-terminal kinases (JNKs) are a family of enzymes that are commonly activated in response to stress and hence are also known as stress-activated protein kinases (SAPKs; refs. 1–3). At present, three JNK family members have been identified (JNK1, JNK2, and JNK3). The best-studied target of JNK is c-Jun (4–7). JNK phosphorylation of c-Jun at serines 63 and 73 promotes activation of this transcription factor (2, 3). The c-Jun protein has been implicated in both the induction and prevention of apoptosis (8). JNK activation of c-Jun is necessary for apoptosis in myeloid and lymphoid cells, because use of a dominant-negative c-Jun mutant blocks programmed cell death in these cells (5). One possible mechanism to explain how activation of c-Jun may promote cell death involves the induction of the proapoptotic Fas ligand (9). Still, c-Jun has been shown to regulate numerous cell processes, and whether c-Jun activation promotes or prevents programmed cell death is highly dependent on the given cell type (8). Thus, it is not surprising that JNK activation alone is not sufficient to induce apoptosis in many cell types (10).

Early studies to determine the role of JNK in apoptosis have focused on its role as a c-Jun kinase (3–7). Recent studies have identified novel targets for JNK, suggesting that this SAPK likely activates other regulatory pathways in addition to those that involve c-Jun. These JNK targets include many proteins that regulate apoptosis, such as BCL2 (11–13), Bcl-xL (14), and Bim (15). JNK appears to play a key role in the intrinsic (i.e., mitochondria-mediated) apoptotic pathway but is not required in the extrinsic (i.e., death receptor mediated) apoptotic pathway, because cells derived from jnk¹/¹ jnk²/² mice were resistant to killing by intrinsic pathway-mediated pathway stress challenges (e.g., UV), but these cells were still sensitive to Fas (16). Furthermore, JNK is required for the activation of proapoptotic Bax, cytochrome c release, and caspase activation during UV-induced apoptosis in mouse embryonic fibroblasts (17). These studies suggest a strong link between stress activation of JNK and activation of the apoptosome via the mitochondria. However, activation of JNK does not always result in cell death. Studies in human lung cancer-derived cell lines indicate that JNK plays a prosurvival role in these cells, and the enzyme contributes to oncogenic transformation (18–20).

Cells treated with ceramide may undergo programmed cell death, become growth arrested, or, in rare cases, become stimulated to proliferate. The diversity of biological responses of cells to ceramide reflects the complexity of the role of this sphingolipid as a second signal molecule (21–23). Ceramide treatment of A549 cells promotes apoptosis in a caspase-dependent process (24). Chalfant et al. (25, 26) have discovered that ceramide activates protein phosphatase 1 (PP1) dephosphorylation of serine/arginine-rich proteins, resulting in alternative splicing of at least two critical apoptotic regulators (Bcl-xL and caspase 9). Although JNK is known to be regulated by ceramide, the exact role for this enzyme in ceramide-induced cell death in A549 cells is unknown. The goal of the present study was to determine whether a role for JNK exists in ceramide-induced apoptosis in A549 cells. Results suggest that JNK participates in ceramide-mediated apoptosis by a process that may involve the apoptotic regulatory protein Bim.

MATERIALS AND METHODS

Reagents. All of the reagents used were purchased from commercial sources unless otherwise stated.

Cell Lines. A549 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 1% nonessential amino acids and 10% fetal bovine serum at 37°C in 5% CO₂.

Analysis of Cell Viability and Apoptosis. Cells were treated with 50 μmol/L C6-ceramide (BioMol, Plymouth Meeting, PA) or 50 μmol/L C6-dihydoceramide (BioMol) for 24 hours. Where appropriate, cells were pretreated for 1 hour with 1 μmol/L SP600125 (Calbiochem, La Jolla, CA) or 10 μmol/L SB203580 (Calbiochem). Cell viability was measured by trypan blue dye exclusion, and apoptosis was analyzed by cell morphology. To observe cell morphology changes, cells were transferred to slides using a cytocentrifuge, fixed, and stained robotically with 20% May-Grumwald-Giemsa composite stain using an Aerospray Slide Stainer as per the manufacturer’s recommendations (Wescor, Logan, UT). Cells were observed by light microscopy using ×40 magnification.

Cell Fractionation Studies. Subcellular fractionation of cells was performed as described previously (27). Where appropriate, cells were treated with 50 μmol/L C6-ceramide for 3 hours before fractionation. Cells were swelled in ice-cold hypotonic HEPES buffer (10 mmol/L HEPES [pH 7.4], 5
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RESULTS

JNK but not p38 Regulates Ceramide-Induced Apoptosis in A549 Cells. C6-ceramide promotes apoptosis in A549 cells by a caspase-dependent mechanism that can be blocked by overexpression of exogenous BCL2 (24). Because it is not known if SAPK participates in this process in these cells, studies were performed to determine whether JNK or p38 kinase is required for ceramide-induced killing. Pharmacological inhibitors for JNK (SP600125, 1 μmol/L) and p38 kinase (SB203580, 10 μmol/L) were used to determine whether either of these stress signaling kinases can block cell killing by ceramide in A549 cells. SP600125 is a small molecule inhibitor that exhibits high specificity for JNK (28, 29). Cells were pretreated with 1 μmol/L SP600125 or 10 μmol/L SB203580 for 1 hour before addition of 50 μmol/L C6-ceramide for 24 hours as described in Materials and Methods. Although the inactive C6-dihydroceramide analogue (50 μmol/L) had no effect on cell viability, 50 μmol/L C6-ceramide killed approximately half the cells after 24 h (Fig. 1A). Cells treated with C6-ceramide exhibited morphologic features of apoptosis (e.g., nuclear condensation, membrane blebbing, and cellular debris), whereas untreated cells or cells treated with C6-dihydro-

JNK but not p38 protects A549 cells from ceramide-induced killing. In A, A549 cells were treated with 50 μmol/L C6-ceramide for 3 hours as metabolic labeling with 33P-Pi, and Bcl-XL was analyzed by immunoprecipitation and Immunoblotting Analysis. A549 cells were treated with 50 μmol/L C6-ceramide for 3 hours during metabolic labeling with 33P-Pi, and Bcl-XL was analyzed by immunoprecipitation by a method similar to that described for studies with BCL2 (27). Samples were electrophoresed in a 12% acrylamide/0.1% SDS gel, transferred to nitrocellulose, and exposed to Kodak X-Omat film at ~80°C. The same blot was used for Western blotting with anti-Bcl-XL antibody and developed using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Immuno-PCR. A549 cells were grown and treated with 50 μmol/L C6-ceramide for 3 hours in chamber slides. For permeabilization and fixation, cells were treated with paraformaldehyde and methanol. Where appropriate, nuclei were visualized with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR). Cells were washed and blocked with 10% serum PBS, and then primary antibody was added. Antibodies used were phospho-JNK mouse monoclonal sera (Cell Signaling), Bcl-XL, rabbit polyclonal sera (Santa Cruz Biotechnology, Santa Cruz, CA), Bim (Calbiochem), actin (Santa Cruz Biotechnology), and prohibitin (Research Diagnostics, Inc., Flanders, NJ) was performed as described previously (27).

Statistical analysis was performed using standard r test analysis with Sigma Stat computer software (SSPS, Chicago, IL).

Ceramide Promotes JNK Translocation from the Nucleus to the Cytoplasm and Non-Nuclear Membranes. The mechanism by which JNK might regulate apoptosis is not clear. Recent studies indicate the importance of mitochondrial JNK in the apoptotic process (17). JNK activation and translocation to mitochondria in response to stress challenges have been observed in a number of human leukemia cell lines (30). To determine whether ceramide could affect JNK subcellular compartmentalization, subcellular localization experiments were performed. Subcellular fractions containing heavy membrane (which contain mitochondrial membranes), light membrane (which contain endoplasmic reticulum, Golgi, and light membranes), nucleus, and cytosolic supernatant were isolated from untreated A549 cells and cells treated with 10 μmol/L C6-ceramide for 3 hours as described in Materials and Methods. As shown in Fig. 1B, SB203580 was effective at blocking p38 activation in these cells. These data suggest a role for JNK but not p38 in ceramide-mediated apoptosis in A549 cells.

Ceramide appeared healthy (data not shown). As shown in Fig. 1A, inactivation of the JNK kinase by SP600125 significantly protects A549 cells from cell death (P < 0.001). In contrast to the effects with the JNK inhibitor, suppression of p38 with SB203580 did not significantly protect A549 cells from ceramide (P > 0.05; Fig. 1A). To rule out the possibility that our SB203580 drug stock was not active, we treated A549 cells with 10 μmol/L SB 203580 for 24 hours and examined p38 activity as indicated by phosphorylation status. As shown in Fig. 1B, SB203580 was effective at blocking p38 activation in these cells. These data suggest a role for JNK but not p38 in ceramide-mediated apoptosis in A549 cells.

Western Blotting. Western blotting using antibodies to JNK and phospho-JNK (Cell Signaling, Beverly, MA), c-Jun and phospho-c-Jun (Cell Signaling), BCL2 (DAKO, Carpinteria, CA), Bcl-XL (Santa Cruz Biotechnology, Santa Cruz, CA), Bim (Calbiochem), actin (Santa Cruz Biotechnology), and prohibitin (Research Diagnostics, Inc., Flanders, NJ) was performed as described previously (27).

Fig. 1. Inhibition of JNK but not p38 protects A549 cells from ceramide-induced killing. In A, A549 cells were treated with 50 μmol/L C6-Dihydroceramide (C6-dihydro, 1 μmol/L JNK-specific inhibitor SP600125, 10 μmol/L p38-specific inhibitor SB203580, and 50 μmol/L C6-ceramide and combination of 1 μmol/L SP600125 + 50 μmol/L C6-ceramide or 10 μmol/L SB203580 + 50 μmol/L C6-ceramide for 24 hours. Cells receiving both ceramide and an inhibitor were pretreated with the inhibitor for 1 hour before ceramide addition. Cell survival was accessed by trypan blue dye exclusion assay. Bars, mean ± SE from three separate experiments. In B, protein lysate (20 μg) from untreated A549 cells or cells treated with 10 μmol/L SB203580 for 24 hours was used in Western blot analysis using phospho-p38, p38, and actin anti-sera.
is localized in mitochondrial and nuclear fractions, was used as a control (31). After ceramide treatment, a significant amount of JNK was translocated from the nuclear fraction to the heavy membrane fraction containing mitochondrial membranes. Moreover, phosphorylated JNK is detected in all of the membrane fractions after ceramide treatment, whereas levels of JNK are greatly reduced below the level of detection for the antibody against the total protein. Consistent with loss of JNK from the nucleus, levels of total JNK in the light membrane and cytosolic fractions were translocated from the nuclear fraction to the heavy membrane fraction containing mitochondrial membranes. Moreover, phosphorylated JNK is detected in all of the membrane fractions after ceramide treatment but not in the nuclear fraction in untreated cells (Fig. 2A).

Ceramide Promotes JNK Translocation and Phosphorylation in A549 Cells

To determine how ceramide affected colocalization of JNK with Bcl-XL and Bim, immunofluorescence microscopy was performed. A mouse monoclonal antibody against phospho-JNK and rabbit polyclonal antibodies against Bim and Bcl-XL were used so that cells could be simultaneously stained and colocalization of JNK with Bcl-XL and Bim could be examined (Fig. 4). Staining for phospho-JNK appears red, and JNK target proteins appear green when observed using a fluorescent microscope. Untreated cells display robust nuclear staining with anti-phospho-JNK antibody. There is little, if any, detection of phospho-JNK staining in the cytoplasmic and non-nuclear membranes in untreated cells (Fig. 4). However, after ceramide treatment, nuclear staining with phospho-JNK is diffuse, and the cytoplasm becomes visible with a punctate pattern of staining, indicating migration to the cytoplasm and small organelles. In untreated cells, Bcl-XL is observed around the nuclear region of the cell. The merged image of untreated cells stained with Bcl-XL and JNK antibodies indicates colocalization of the proteins as observed by bright white/yellow punctate staining (Fig. 4). Cells treated with ceramide, however, do not exhibit significant colocalization of Bcl-XL and JNK as demonstrated by the absence of the white/yellow punctate staining pattern. Consistent with the promotion of JNK phosphorylation, ceramide promoted colocalization of Bim and JNK (Fig. 4). The ability of ceramide to suppress c-Jun phosphorylation while promoting Bim phosphorylation suggests that ceramide might affect substrate targeting by altering subcellular localization of the kinase.

Materials and Methods

Cell cultures were treated with 50 μmol/L C6-ceramide for 2 hours, where appropriate. Western blot analysis using 10 μg of protein from each subcellular fraction was performed using anti-sera to JNK, phospho-JNK, phospho-c-Jun, and prohibitin. Lanes, heavy (HM), light (LM), cytosolic (CYT), nuclear (NUC) membranes. In B, A549 cells that were untreated or treated with 50 μmol/L C6-ceramide for 3 hours were fixed, and then goat phospho-c-Jun polyclonal antibody was added. Fluorescent conjugated secondary antibody was used to visualize protein (green). Cells were counterstained with 4',6-diamidino-2-phenylindole (blue) to identify nuclei. Image analysis was performed as described in Materials and Methods.

Ceramide Promotes JNK Phosphorylation of Bim but not Bcl-XL

Because ceramide-activated JNK does not target c-Jun in A549 cells, the question remained whether ceramide would have on phosphorylation of other JNK substrates. A number of newly described JNK targets (e.g., BCL2, Bcl-XL, and Bim) have been identified recently (11–15). Although Bc/II is a JNK target, Bc/II was not examined, because little, if any, of the protein is detected in A549 cells (data not shown). A metabolic radiolabeling experiment was performed to determine whether Bcl-XL was phosphorylated in A549 cells in response to ceramide (Fig. 3A). A549 cells were treated with 50 μmol/L C6-ceramide for 3 hours during metabolic labeling with 32P-Pi, and the phosphorylation status of Bcl-XL was examined after immunoprecipitation. Western analysis demonstrates that roughly equivalent levels of Bcl-XL protein were immunoprecipitated from control and C6-ceramide-treated samples. As shown in Fig. 3A, A549 cells actually exhibit basal levels of Bcl-XL phosphorylation. Ceramide, however, did not augment Bcl-XL phosphorylation levels as determined by metabolic labeling. This result suggests that Bcl-XL phosphorylation, which is associated with loss of antiapoptotic function (14), is not involved in ceramide-induced apoptosis in A549 cells.

Recent studies have revealed that JNK phosphorylation of Bim can be visualized by changes in electrophoretic mobility (15, 17). The electrophoretic migration of Bim is retarded when the protein is phosphorylated by JNK. Western analysis of Bim was performed using protein lysates from untreated cells and cells treated with 50 μmol/L C6-ceramide for 3 hours. As shown in Fig. 3B, C6-ceramide promotes Bim phosphorylation. This finding suggests that Bim may be an important JNK target in ceramide-induced apoptosis in A549 cells.

The ability of ceramide to suppress c-Jun phosphorylation while promoting Bim phosphorylation suggests that ceramide might affect substrate targeting by altering subcellular localization of the kinase.
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Ceramide Promotes Translocation of Bim to the Mitochondria.
Recent studies have suggested that Bim migration to the mitochondria is an event associated with apoptosis (15, 17). To determine whether ceramide promoted mitochondrial translocation of Bim, Western analysis was performed using protein lysates from isolated mitochondrial fractions. Heavy membrane fractions were isolated as described in “Materials and Methods” from untreated cells and cells treated with 50 μmol/L C6-ceramide for 3 hours. Although little, if any, Bim is detected in the mitochondria of untreated cells, ceramide promotes Bim translocation to the mitochondria (Fig. 5). This migration of Bim to the mitochondria in response to ceramide suggests that this translocation event may be important in ceramide-induced apoptosis in A549 cells.

DISCUSSION
The mechanism of how ceramide activates JNK is not clear. Ceramide may activate SAPK via Rac-1 (32), PKC ζ (33), or TAK1 (34). It is also not clear how SAPK-activating pathways are triggered in response to short chain ceramides like C6-ceramide. One possibility involves the conversion of exogenous short chain ceramide to endogenous long chain ceramide (35). C16-ceramide failed to effectively kill A549 cells, although the drug was able to potently kill HL60 cells (data not shown). It is possible that C6-ceramide in A549 cells promotes the generation of another metabolite that is responsible for JNK activation (e.g., a sphingosine derivative) or that C16-ceramide fails to penetrate A549 cells to reach critical target regions (i.e., the nucleus). The latter possibility is supported by studies in human kidney proximal tubule cells and HCT116 human colon cancer cells, where it was found that short chain ceramides were toxic and long chain ceramide was not and that exogenous C16-ceramide did not reach intracellular locales, because the molecule mainly accumulated in the light membrane (36, 37). Although the exact mechanism is not clear, a model whereby JNK is activated by C6-ceramide via the production of long chain ceramides is supported by the study demonstrating that exogenous C6-ceramide promotes the production of endogenous C16-ceramide and C24-ceramide in A549 cells (35).

Previous studies have demonstrated that C6-ceramide-induced apoptosis involves caspase activation, although little else is known of the signaling pathways involved in this process (24). Ceramide has been shown to activate the PPI (25, 26) and affect telomerase activity (35, 38) in A549 cells; however, the effect of C6-ceramide on well-characterized ceramide targets, such as the SAPKs or the P2A, in these cells is unknown. Ceramide-induced killing of A549 cells appears to require JNK but does not require the p38 kinase (Fig. 1A). JNK appears activated in untreated cells, although active enzyme appears to be limited to the nucleus (Fig. 2A and Fig. 4). The cells display basal levels of phosphorylated c-Jun, suggesting that this transcription factor may play a prosurvival role in these cells (Fig. 2B).

JNK has been shown to activate either prosurvival or prodeath signaling cascades depending on the mode of how the enzyme itself is regulated (39). The finding that nuclear JNK is normally active in A549 cells whereas ceramide promotes translocation of the enzyme from the nucleus in association with death suggests that there are both prosurvival and prodeath JNK pathways in A549 cells.

Interestingly, A549 cells displayed basal levels of phosphorylated Bcl-XL (Fig. 3A). This finding suggests that phosphorylated Bcl-XL is not detrimental to these cells. Another BCL2 family member, Bim, is one of the more recently identified targets of JNK (15, 17). The observed ceramide-induced phosphorylation of Bim by JNK in A549 cells suggests a mechanism by which JNK might participate in ceramide-mediated apoptosis in these cells. Lei and Davis (15) have demonstrated recently that JNK is a Bim kinase and that phosphorylation of Bim may be sufficient to cause Bax-induced apoptosis. JNK phosphorylation of Bim promotes its release from a cytosolic Dynemin/Myosin V complex (15). The significant increase in cytosolic phosphorylated JNK in response to ceramide in the A549 cells is consistent with the detection of Bim phosphorylation. Furthermore, the ability of ceramide to potently promote Bim translocation to the mitochondria (Fig. 5) suggests this may be an important event in ceramide-induced apoptosis in these cells. Still, the role of JNK in apoptosis is controversial. In some cell types (e.g., fibroblasts, cells derived from various regions of the brain) suppression of JNK promotes chemoresistance (16, 40). Conversely, JNK may play an antiapoptotic role in some cell types.
types, such as in thymocytes and mature T cells (41). The findings reported here suggest that JNK has the capacity to be either prosurvival or proapoptotic depending on conditions. Still, it is tempting to speculate that the subcellular translocation of JNK in response to growth agonist or stress challenge will determine which potential signaling pathways are activated resulting in growth or death, respectively.

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