Inhibition of Akt Kinase by Cell-permeable Ceramide and Its Implications for Ceramide-induced Apoptosis*

Honglin Zhou‡, Scott A. Summers§, Morris J. Birnbaum§, and Randall N. Pittman‡¶

From the ‡Department of Pharmacology, §Howard Hughes Medical Institute, and Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Ceramide is an important lipid messenger involved in mediating a variety of cell functions including apoptosis. However, mechanisms responsible for ceramide-induced apoptosis remain unclear. We investigated the possibility that ceramide may decrease antiapoptotic signaling in cells by inhibiting Akt kinase activity. Our data show that C2-ceramide induces apoptosis in HMN1 motor neuron cells and decreases both basal and insulin- or serum-stimulated Akt kinase activity 65–70%. These results are consistent with decreased Akt kinase activity being involved in the apoptotic effects of ceramide. This possibility is further supported by studies showing that constitutively active Akt kinase decreases C2-ceramide-induced death of HMN1 cells as well as COS-7 cells. Decreased Akt activity is not due to ceramide activating the ceramide-activated protein phosphatase or to a direct inhibition of Akt kinase by ceramide, suggesting that ceramide acts upstream of Akt kinase to decrease its activity. Treating cells with C2-ceramide does not affect phosphorylation of insulin receptor substrate-1, interactions between insulin receptor substrate-1 and p85, or insulin-stimulated phosphatidylinositol 3-kinase activity, suggesting that the effects of C2-ceramide on Akt kinase are not mediated through modulating phosphatidylinositol 3-kinase. In sum, our results suggest that inhibition of the key antiapoptotic kinase, Akt, may play an important role in ceramide-induced apoptosis.

Apoptosis is an evolutionarily conserved form of cell death critical for tissue homeostasis. In the nervous system, apoptosis is required for normal development (1, 2) and has been implicated in the pathogenesis of various neurodegenerative conditions such as Alzheimer’s disease, Huntington’s disease, stroke, head trauma, and neuronal death following spinal cord injury (3–8). Although cellular mechanisms underlying apoptosis remain unclear, a number of recent studies suggest that activating sphingomyelinase and subsequent generation of ceramide play an important role in regulating apoptosis in many systems (9–11). Apoptosis induced by a variety of initiators such as tumor necrosis factor-α, Fas ligand, daunorubicin, removal of growth factors, UV irradiation, and x-irradiation is associated with increased ceramide production (11–15), while a strong correlation exists between production of ceramide and subsequent cell death (12, 15). Moreover, cell-permeable ceramide analogues mimic these agents and induce apoptosis in many different cell types including monoblastic leukemia cells, endothelial cells, fibroblasts, pheochromocytoma cells, and oligodendrocytes (9, 12, 16–18). These observations are consistent with ceramide being a lipid second messenger that functions as part of a ubiquitous signaling system to link cell surface receptors and environmental stresses to the apoptotic pathway. However, signal transduction pathways mediating ceramide-induced apoptosis are largely unknown except for a recent study showing that the proapoptotic stress-activated protein kinase/c-Jun N-terminal kinase is important for ceramide-induced apoptosis (12).

Considerable evidence suggests that apoptosis is the consequence of disrupting the balance of antiapoptotic and proapoptotic signaling within the cell. For instance, Bcl-2 and Bax, both members of the Bcl-2 family, have opposite effects on apoptosis, and the effect of each on apoptosis can be overcome by overexpressing the other (19–21). In addition, several studies suggest that a balance between signal transduction pathways controls apoptosis. Effector kinases having a proapoptotic role include stress-activated protein kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinases (22, 23), whereas effector kinases having an antiapoptotic role include the serine/threonine kinases extracellular signal-regulated kinases 1 and 2 and Akt (22, 24–27). Support for an antiapoptotic role of extracellular signal-regulated kinases 1 and 2 and Akt comes from studies showing that growth factors such as insulin, insulin-like growth factor-1, and nerve growth factor that block apoptosis activate these kinases and that increasing kinase activity by expressing either constitutively active forms of the kinases or their upstream regulators blocks apoptosis (22, 24–27).

Since survival and death likely represent a balance between antiapoptotic and proapoptotic signaling, we considered the possibility that the apoptotic lipid messenger ceramide may induce apoptosis by increasing proapoptotic signaling, by decreasing antiapoptotic signaling, or both. To explore the possibility that ceramide may decrease antiapoptotic signaling, we developed a model system for ceramide-induced apoptosis using the neuroblastoma-motor neuron cell line, HMN1 (28). Our data show that ceramide decreases activity of the antiapoptotic kinase, Akt, and induces apoptosis in HMN1 cells. Moreover, a constitutively active form of Akt inhibits ceramide-induced apoptosis in both HMN1 and COS-7 cells. Our data are consistent with ceramide inducing apoptosis by regulating multiple signaling pathways that involve decreasing antiapoptotic signals in addition to increasing proapoptotic signals.

EXPERIMENTAL PROCEDURES

Materials—C2-ceramide, C2-dihydroceramide, C2-ceramide, and MOWIOL were obtained from Calbiochem. LipofectAMINE, G418, and

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† To whom correspondence should be addressed: Dept. of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6084, Tel.: 215-898-9736; Fax: 215-573-2236; E-mail: pittman@pharm.med.upenn.edu.

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streptavidin/horseradish peroxidase were obtained from Life Technologies, Inc. Fetal bovine serum was from HyClone (Logan, UT), okadaic acid from RBI (Natick, MA), and Hoechst 33342 from Molecular Probes, Inc. (Eugene, OR). Polyvinylidene difluoride transfer membrane, ECL substrate, and [γ-32P]ATP were from NEN Life Science Products. Sheep polyclonal antibody to Akt was from Upstate (Lake Placid, NY). Rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA). pCR3.1 vector was from Invitrogen (San Diego, CA). Antibody to hemagglutinin (12CA5) was a generous gift from Dr. Jeffery Field. Rabbit polyclonal antibody 255 to Akt has been previously described (25). Bovine serum albumin was from Calbiochem (La Jolla, CA). Immunolocalization of Akt was performed using a mouse monoclonal antibody to Akt (S.Activation) (Cell Signaling Technology, Beverly, MA) and a Cy3-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). Rabbit polyclonal antibody 255 to Akt has been previously described (25).

Cell Culture, Expression Constructs, and Transfections—HMN1 cells (28) and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Two days after reaching 90% confluence, cells were treated with 70% ethanol, air-dried, and dissolved in 1 mM EDTA, 10 mM Tris, 1% SDS, 10% glycerol, and 2 mM β-mercaptoethanol. DNA was then extracted and washed. The reaction was started by adding 50 μl of 100 mM MgCl2, 10 μl of phosphatidylserine (2 μg/μl) and 10 μl of 400 μM ATP containing 20 μCi [γ-32P]ATP to each pellet. After 10 min at 22 °C, the reaction was stopped by the addition of 20 μl of 8 M HCl and 160 μl of CHCl3/methanol (1:1). Samples were centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography plate that had been coated with potassium oxalate. Thin layer chromatography plates were developed in CHCl3/CH3OH/H2O/NH4OH (60:47:11.3:2), dried, visualized, and quantified by autoradiography.

RESULTS

C3- ceramide Induces Apoptosis in HMG1 Cells—The cell-permeable ceramide analogue, C3- ceramide, is toxic to HMG1 cells, with 90% of the cells dying by 5 h following exposure to 50 μM C3- ceramide (Fig. 1A). Cell death is first detected 1–2 h after exposure (based on trypan blue exclusion; data not shown) but is not significant until 3 h (Fig. 1A). C3-ceramide shows a dose-dependent toxicity, with 30% of the cells dying by 5 h in the presence of 10 μM C3- ceramide (Fig. 1B). Over an 18-h period, this same concentration of ceramide kills more than 95% of the cells (data not shown). The biologically inactive ceramide analogue, C3-dihydroceramide, does not have toxic effects at the times or concentrations tested (Fig. 1). When viewed using phase contrast microscopy, cells treated with ethanol vehicle or C3-dihydroceramide maintain normal morphology, whereas cells treated with C3- ceramide are rounded and shrunk and often have membrane blebs (Fig. 2, A–C). These are characteristic morphological features of apoptosis; therefore, experiments were performed to determine if additional features of apoptosis were present in ceramide-treated HMG1 cells. Chromatin has a characteristic condensed and fragmented appearance following Hoechst 3342 staining of cells treated with C3- ceramide (Fig. 2E), while chromatin in cells treated with C3-dihydroceramide has a normal homogeneous appearance (Fig. 2D). Consistent with the time course of ceramide-induced toxicity (Fig. 1A), DNA laddering is not prominent until 3 h after treatment of cells with 50 μM C3- ceramide and is not present in cells treated with 50 μM C3-dihydroceramide (Fig. 2F). Therefore, characteristic features of apoptosis including cell shrinkage, membrane blebs, DNA laddering, and chromatin condensation are present in cells treated with anti-Akt antibody and protein A beads for 2 h at 4 °C. Precipitates were washed three times with lysis buffer and two times with rinse buffer (20 mM HEPES, pH 7.2, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol). Precipitates were then incubated in 40 μl of kinase reaction buffer (20 mM HEPES, pH 7.2, 10 mM MgCl2, 10 mM MnCl2, 1 mM dithiothreitol, 0.2 mM EGTA, 20 μM ATP, 5 μg of myelin basic protein (MBP), 10 μCi of [γ-32P]ATP (3000 Ci/mmol), and 2 μg of protein kinase inhibitor (Sigma)) at 30 °C for 30 min. Reactions were stopped by adding 3× Laemmli buffer and boiling for 3 min. Proteins were resolved on 12% SDS-polyacrylamide gels, and phosphorylation was quantified using a Molecular Dynamics PhosphorImager.

For immunoblotting Akt precipitates, samples were boiled in 3× Laemmli buffer for 3 min. Proteins were then resolved in 10% SDS-polyacrylamide gels, electroblotted onto polyvinylidene difluoride transfer membrane, incubated with sheep anti-Akt antibody, and visualized by ECL.

In Vitro PI 3-Kinase Assay—In vitro phosphorylation of phosphatidylinositol was carried out in the immunocomplexes as described (31). Log phase HMN1 cells were made quiescent by growing in serum-free DMEM medium for 24 h. Cells were then incubated with 30 μM C2-ceramide or 30 μM C2-dihydroceramide for 30 min and then stimulated with 10 μg/ml insulin for 10 min. Cells were then washed once in ice-cold phosphate-buffered saline and twice with buffer A (20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 100 μM Na3VO4). The cells were solubilized in 1 ml of buffer A containing 1% Nonidet P-40 and 10% glycerol, and soluble cell lysates were collected after centrifuging at 13,000 × g for 10 min. Cell lysates were normalized for protein concentration using bovine serum albumin as a standard. Equal amounts of cell extracts were incubated with anti-IRS-1 antibody and protein A beads for 2 h at 4 °C. Immunocomplexes were then precipitated and washed. The reaction was started by adding 10 μl of 100 mM MgCl2, 10 μl of phosphatidylinositol (2 μg/μl) and 10 μl of 440 μM ATP containing 20 μCi [γ-32P]ATP to each pellet. After 10 min at 22 °C, the reaction was stopped by the addition of 20 μl of 8 M HCl and 160 μl of CHCl3/methanol (1:1). Samples were centrifuged, and the lower organic phase was removed and applied to a silica gel thin-layer chromatography plate that had been coated with potassium oxalate. Thin layer chromatography plates were developed in CHCl3/CH3OH/H2O/NH4OH (60:47:11.3:2), dried, visualized, and quantified by autoradiography.

The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; MBP, myelin basic protein; PI, phosphatidylinositol; IRS, insulin receptor substrate.
with C2-ceramide, indicating that C2-ceramide induces apoptosis in HMN1 cells.

C2-ceramide Inhibits Akt Kinase Activity—A previous study reported that ceramide-induced apoptosis is associated with activation of a proapoptotic signal, the stress-activated protein kinase/c-Jun N-terminal kinase (12). However, the possibility that ceramide may also decrease antiapoptotic signaling pathways has not been investigated. Among the known antiapoptotic pathways, the PI 3-kinase pathway has been shown to protect a variety of cells against apoptosis (32–34). In addition, recent studies indicate that the survival-promoting effect of PI 3-kinase is primarily mediated through activation of one of its downstream targets, the serine/threonine kinase, Akt (24–27). Therefore, we investigated the possibility that C2-ceramide may interfere with Akt kinase activation. Endogenous Akt kinase was isolated from HMN1 cells treated with various agents, and its kinase activity was monitored using MBP as substrate. C2-ceramide (but not C2-dihydroceramide) decreases basal Akt kinase activity about 3-fold in serum-starved HMN1 cells (Fig. 3). In addition, C2-ceramide decreases insulin as well as serum-stimulated Akt kinase activity about 3-fold (Fig. 3). Consistent with these data showing a decrease in Akt kinase activity are additional experiments showing that ceramide inhibits the decreased mobility and upward shift of Akt kinase (representing phosphorylation of Akt) seen on SDS-polyacrylamide gels following stimulation with insulin (data not shown). These data indicate that C2-ceramide inhibits insulin- or serum-stimulated Akt kinase activation and therefore interferes with an important antiapoptotic signaling pathway, suggesting that inhibition of Akt kinase activity may be part of the underlying mechanisms responsible for the apoptotic effects of ceramide.

Constitutively Active Akt Kinase Inhibits C2-Ceramide-induced Death of HMN1 and COS-7 Cells—To further address whether the inhibition of Akt kinase activity following C2-ceramide treatment may represent an important aspect of ceramide-induced apoptosis, we examined the effect of increasing Akt kinase activity on ceramide-induced death of HMN1 cells. A constitutively active form of Akt containing an Src myristoylation signal sequence (29) was overexpressed in HMN1 cells followed by exposure of cells to C2-ceramide or C2-dihydroceramide. Overexpressing constitutively active Akt in HMN1 cells decreased ceramide-induced apoptosis by about 50% (Fig. 4), indicating that increased kinase activity is effective in blocking ceramide-induced apoptosis, and thus inhibition of Akt kinase activity may be important for the apoptotic effects of ceramide. To determine whether the protective role of constitutively active Akt against ceramide-induced apoptosis is limited to HMN1 cells or is a more general mechanism for other cell types as well, we examined whether constitutively active Akt kinase is also effective in inhibiting ceramide-induced apoptosis in COS-7 cells. Following exposure of COS-7 cells to C2-ceramide, an obvious protective effect is seen in cultures transfected with the constitutively active Akt compared with cultures transfected with the nonconstitutively active Akt. Whereas most
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**FIG. 3.** C2-ceramide decreases Akt kinase activity in HMN1 cells. A, HMN1 cells were pretreated with the indicated agent for 15 min, and Akt kinase activity was measured following immunoprecipitation using MBP as a substrate for phosphorylation. Lane 1, 0.25% ethanol; lane 2, 30 μM C2-dihydroceramide; lane 3, 30 μM C2-ceramide; lane 4, C2-ceramide plus 20% serum; lane 5, 20% serum; lane 6, 10 μg/ml insulin; lane 7, C2-ceramide plus insulin; lane 8, C2-dihydroceramide plus insulin. Since serum proteins bind ceramide, cells were pretreated with C2-ceramide for 30 min in serum-free medium and then treated with 20% fetal bovine serum for 15 min (lane 4). For all other samples, combined treatments were started at the same time. The bottom blot is a Western blot showing the amount of immunoprecipitated Akt protein in each sample. B, quantitation of substrate (MBP) phosphorylation determined by PhosphorImager analysis. Data represent mean ± S.E. of three independent experiments. C2-ceramide significantly decreases (p < 0.02) basal, serum-stimulated, and insulin-stimulated Akt activity. C2-dihydroceramide has no significant effect on Akt activity.

**FIG. 4.** Constitutively active Akt decreases C2-ceramide-induced apoptosis of HMN1 cells. HMN1 cells were transfected with an RSV β-galactosidase vector (gray bars), RSV β-galactosidase vector plus an empty pCR3.1 vector (striped bars), or RSV β-galactosidase vector plus pCR3.1 myr-Akt-HA (constitutively active Akt; stippled bars). Cells were treated with 30 μM C2-dihydroceramide or C2-ceramide for 5 h, and cell survival was determined by cell counts of healthy and apoptotic transfected cells. Expression of transfected hemagglutinin-tagged Akt protein was confirmed by immunofluorescence and immunoblotting (not shown). Data represent mean ± S.E. for three independent experiments. *, p < 0.05 compared with other C2-ceramide groups.

control cells (data not shown) or cells transfected with the nonconstitutively active Akt (Fig. 5A, b) are rounded, shrunken, or detached from the dish and floating in the presence of C2-ceramide, many cells transfected with constitutively active Akt are flattened and have a normal appearance in the presence of C2-ceramide (Fig. 5A, d). In control groups, C2-dihydroceramide is not toxic, and cells maintain a normal appearance in both groups (Fig. 5A, a and c). Quantitation of β-galactosidase-positive cells indicates that constitutively active Akt significantly protects COS-7 cells from ceramide-induced death (Fig. 5B). Furthermore, membrane targeting of Akt kinase results in kinase activation in the absence of growth factor stimulation, and C2-ceramide does not inhibit the activity of membrane-targeted Akt kinase (Fig. 5C).

**Decreased Akt Kinase Activity Is Not Due to a Direct Inhibition by Ceramide or to Ceramide-activated Protein Phosphatase 2A-like Phosphatase**—The possibility that C2-ceramide may have a direct effect on Akt kinase was tested by determining the effect of C2-ceramide on Akt immunoprecipitated from control or insulin-stimulated HMN1 cells. In these *in vitro* immunocomplex kinase assays, the addition of C2-ceramide does not affect basal or insulin-stimulated Akt kinase activity, indicating that C2-ceramide does not inhibit Akt kinase activity directly (Fig. 6A). Previous studies have shown that Akt kinase is primarily activated through phosphorylation (29) and inactivated through dephosphorylation by a protein phosphatase 2A (35, 36). Since it has been reported that ceramide stimulates ceramide-activated protein phosphatase, a protein phosphatase 2A-like phosphatase (37), we investigated the possibility that ceramide may decrease Akt activity by activating a protein phosphatase 2A phosphatase to promote Akt inactivation. Treating HMN1 cells with okadaic acid, which effectively blocks protein phosphatase 2A activity in these cells, does not reverse the effects of C2-ceramide in inhibiting insulin-stimulated Akt kinase activation (Fig. 6B), suggesting that ceramide is not decreasing Akt activity by activating a protein phosphatase 2A-like phosphatase. Furthermore, the possibility that activation of ceramide-activated protein phosphatase may be involved in ceramide-mediated apoptotic signaling in HMN1 cells was investigated, since recent studies suggest that protein phosphatase activation is involved in apoptosis (38). Okadaic acid treatment does not block C2-ceramide-induced DNA fragmentation in HMN1 cells (Fig. 6C), suggesting that activation of ceramide-activated protein phosphatase is probably not a component of the apoptotic signaling system mediated by ceramide. Therefore, in HMN1 cells ceramide-activated protein phosphatase activation does not appear to be involved in ceramide-induced apoptosis or to be responsible for inhibiting Akt kinase activity.

**C2-ceramide Does Not Inhibit Insulin-stimulated PI 3-Kinase Activity**—We next examined whether C2-ceramide might inhibit insulin-stimulated Akt kinase by disrupting upstream signaling that is required for its activation. It is known that binding of insulin to its receptor induces rapid autophosphorylation of the insulin receptor, which promotes the docking of IRS-1 and its phosphorylation at multiple tyrosine residues by the insulin receptor (39). Subsequently, IRS-1 can recruit the p85 regulatory subunit of PI 3-kinase by interacting with the Src homology 2 domain of p85, which leads to activation of PI 3-kinase and downstream activation of Akt kinase. Previous studies have shown that C2-ceramide decreases insulin-stimulated tyrosine phosphorylation of IRS-1 and impairs its subsequent association with the p85 subunit (40). Therefore, we examined the effects of C2-ceramide on insulin-stimulated IRS-1 tyrosine phosphorylation, association between IRS-1 and p85 subunit, and PI 3-kinase activity in HMN1 cells. HMN1 cells were stimulated with insulin in the presence or absence of C2-ceramide, and then tyrosine phosphorylation of IRS-1 was determined by immunoblot with a phosphotyrosine-specific antibody PY20. C2-ceramide only slightly decreased insulin-stimulated IRS-1 tyrosine phosphorylation, but this decrease was not statistically significant (Fig. 7A). As expected, treatment of insulin-stimulated HMN1 cells with the PI 3-kinase inhibitor wortmannin completely inhibited IRS-1 tyrosine phosphorylation (Fig. 7A). Since the decrease in IRS-1 tyrosine phosphorylation was not statistically significant, we next examined whether C2-ceramide affected IRS-1 association with the p85 subunit of PI 3-kinase. IRS-1 tyrosine phosphorylation is required for its association with the p85 subunit of PI 3-kinase (40). To examine this interaction, we determined the amount of IRS-1 tyrosine phosphorylation associated with the p85 subunit of PI 3-kinase. As shown in Fig. 7B, C2-ceramide does not inhibit IRS-1 tyrosine phosphorylation associated with the p85 subunit of PI 3-kinase. Therefore, C2-ceramide does not inhibit IRS-1 tyrosine phosphorylation or its association with the p85 subunit of PI 3-kinase, which is consistent with the results shown in previous sections.
ulated IRS-1 tyrosine phosphorylation (Fig. 7A) but did not decrease insulin-stimulated association between phosphotyrosine protein and p85 as determined by immunoprecipitation with PY20 antibody followed by immunoblotting with p85 antibody (Fig. 7B). In addition, C2-ceramide had no effect on insulin-stimulated association between IRS-1 and p85 as determined by immunoprecipitation with IRS-1 antibody and immunoblotting with p85 antibody (Fig. 7C). Finally, PI 3-kinase activity was assayed in immune complexes prepared with anti-IRS-1 antibody, and it was found that C2-ceramide did not affect insulin-stimulated PI 3-kinase activation in HMN1 cells (Fig. 7, D and E). This observation is consistent with the immunoprecipitation studies showing that C2-ceramide did not affect the amount of p85 associated with IRS-1.

DISCUSSION

The sphingomyelin pathway is a ubiquitous, evolutionarily conserved signaling system initiated by hydrolysis of sphingomyelin to generate the second messenger, ceramide. Generation of endogenous ceramide appears to be part of a general apoptotic response following a variety of cellular insults in a large number of different cell types (10–12). Mechanisms responsible for the apoptotic effects of ceramide are poorly understood; however, ceramide has been shown to activate the
Akt was immunoprecipitated, and the immunocomplex was treated with soluble DNA isolated from HMN1 cells treated for 3 h with 30 μM C2-ceramide. Acid does not block the ceramide-induced decrease in Akt activity. C2-ceramide decreases insulin-stimulated Akt activity and that okadaic acid to block C2-ceramide-decreased Akt kinase activity or for okadaic acid to block C2-ceramide-induced apoptosis.

Fig. 6. Inability of C2-ceramide to directly inhibit Akt kinase activity or for okadaic acid to block C2-ceramide-decreased Akt kinase activity or C2-ceramide-induced apoptosis. A, HMN1 cells were grown in serum-free medium for 20 h and then treated with medium or medium containing 10 μg/ml insulin for 15 min. Endogenous Akt was immunoprecipitated, and the immunocomplex was treated with vehicle or 30 μM C2-ceramide. Akt kinase activity was determined following incubation for 20 min at 30 °C using MBP as a substrate. B, HMN1 cells were treated with 10 μg/ml insulin plus 30 μM C2-ceramide (lane 1), 10 μg/ml insulin (lane 2), 10 μg/ml insulin plus 100 nM okadaic acid (lane 3), or 30 μM C2-ceramide plus 10 μg/ml insulin plus 100 nM okadaic acid (lane 4) for 15 min, and Akt kinase activity was determined following immunoprecipitation and immunocomplex kinase assay by substrate (MBP) phosphorylation. C, Akt kinase activity was determined following incubation for 20 min at 30 °C using MBP as a substrate. Akt kinase activity was determined following incubation for 20 min at 30 °C using MBP as a substrate. Akt kinase activity was determined following incubation for 20 min at 30 °C using MBP as a substrate.

Proapoptotic stress-activated protein kinase/c-Jun N-terminal kinase stress-activated signaling pathway (12). The present study was initiated to test the hypothesis that ceramide may also decrease signaling of antiapoptotic pathways. Although antiapoptotic pathways have not been extensively characterized, the antiapoptotic effects of the PI 3-kinase pathway have been well documented (32–34). Recent studies have identified the serine/threonine kinase, Akt, as a key downstream effector of PI 3-kinase that blocks apoptosis in a variety of cell types (24–27). Our data indicate that ceramide inhibits Akt kinase activity and therefore decreases the activity of a major survival/antiapoptotic pathway. Furthermore, overexpression of constitutively active Akt in either HMN1 or COS-7 cells partially inhibits ceramide-induced apoptosis. Therefore, our work along with that of others (12) supports the view that ceramide may induce apoptosis by decreasing signaling of antiapoptotic pathways as well as increasing signaling of proapoptotic pathways.

Although biochemical mechanisms underlying cellular effects of ceramide have not been thoroughly characterized, a number of direct targets of ceramide have been identified. One well defined target is a ceramide-activated protein kinase (41), which is a member of the proline-directed serine/threonine protein kinase family and was recently shown to be identical to the kinase suppressor of Ras (42). Another target of ceramide is ceramide-activated protein phosphatase, a member of the protein phosphatase 2A family (37). Recent studies suggest that ceramide may also directly activate protein kinase C isoform ξ (43, 44). In addition, the putative guanine nucleotide exchange factor, Vav, a potential activator of Ras and related proteins in hematopoietic cells, may serve as a direct effector of ceramide (45). Although a number of obvious possibilities exist, it remains to be determined how ceramide decreases Akt activity. Akt is activated after cells are exposed to insulin or other growth factors, and its activation occurs via a pathway that includes PI 3-kinase. Activation of PI 3-kinase produces PI 3,4,5-trisphosphate, which can bind to the pleckstrin homology domain of Akt and a number of other signaling molecules. Subsequently, Akt itself is phosphorylated at Thr308 and Ser473 and activated by two different protein kinases, 3-phosphoinositide-dependent protein kinase 1 and 3-phosphoinositide-dependent protein kinase 2 (46). Following its activation, Akt is inactivated by dephosphorylation, which is mediated by protein phosphatase 2A-like phosphatase. Based on our finding that the protein phosphatase 2A phosphatase inhibitor, okadaic acid, does not reverse the effect of ceramide on Akt, it seems likely that ceramide inhibits activation (phosphorylation) of Akt rather than promoting Akt inactivation (dephosphorylation). In addition, since our studies show that C2-ceramide does not inhibit insulin-stimulated PI 3-kinase activation or interactions between IRS-1 and p85, it is likely that C2-ceramide does not inhibit Akt activation through modulating PI 3-kinase activation. In vitro studies also show that C2-ceramide does not have a direct effect on Akt kinase activity as determined by an immunocomplex kinase assay. These observations suggest that the regulation of Akt kinase by C2-ceramide appears to occur upstream of Akt and may involve intermediate regulators of Akt downstream of PI 3-kinase or other pathways independent of PI 3-kinase signaling. Understanding the site of action is clearly important and is the focus of ongoing studies.

Apoptosis represents a complex cellular response with multiple signaling pathways and regulatory proteins. As with most cellular processes, apoptosis is controlled by both positive and negative regulators and is dependent upon the balance between these opposing forces. A striking example of proapoptotic and antiapoptotic pathways being modulated soon after an apoptotic stimulus is apoptosis of PC12 cells following removal of nerve growth factor (22). Nerve growth factor withdrawal activates stress-activated protein kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and decreases activity of the extracellular signal-regulated kinase 1 and 2 pathway. A similar situation may exist for ceramide-induced cell death where signaling through the proapoptotic stress-activated protein kinase/c-Jun N-terminal kinase pathway is increased by ceramide and inhibiting this pathway blocks death (12), while signaling through the antiapoptotic Akt kinase pathway is inhibited by ceramide and constitutively active Akt blocks death (the present study). The observation that constitutively active Akt kinase only partially inhibits (50%) ceramide-induced HMN1 and COS-7 cell death suggests that
Ceramide may inactivate additional survival signals and/or activate proapoptotic pathways to induce apoptosis. Alternatively, the level of Akt obtained following transfection may not be sufficiently high in all cells to block apoptosis (i.e., the sensitivity for detecting β-galactosidase expression may be greater than the expression level of Akt needed to block death). In sum, our studies indicate that inhibition of Akt kinase activity by ceramide may represent a general mechanism affecting apoptosis in a number of systems. This would be particularly true for cell types that depend on the PI 3-kinase/Akt kinase pathway as a crucial antiapoptotic signal, which include many neuronal cells such as cerebellar granule neurons, sympathetic ganglion neurons, and PC 12 cells. However, our results do not rule out the possibility that ceramide interferes with other pathways to induce apoptosis.

The mechanism by which overexpressing constitutively active Akt blocks ceramide-induced apoptosis in HMN1 and COS-7 cells is currently unknown. The constitutively active form of Akt used in our experiments lacks its phospholipid-binding pleckstrin homology domain but is targeted to the cell membrane via an Src myristoylation sequence added to its amino terminus. Previous studies as well as our study show that targeting Akt to the membrane confers constitutive kinase activity even in the absence of growth factor stimulation. Assuming that the survival promoting effects of myr-Akt (lacking the pleckstrin homology domain) are not dependent on endogenous wild-type Akt, binding of phospholipids or other interactions mediated by the pleckstrin homology domain would not appear necessary for myr-Akt to promote survival in HMN 1 and COS-7 cells as long as activation of Akt is achieved.

A previous study has shown that C2-ceramide decreases insulin-stimulated tyrosine phosphorylation of IRS-1 and dis-
rupts the association between IRS-1 and the p85 subunit of PI 3-kinase in rat hepatoma Fao cells (40). However, it was not shown whether this affected PI 3-kinase activation. In the present study, we find that C2-ceramide weakly decreases insulin-stimulated IRS-1 tyrosine phosphorylation but does not affect the association between IRS-1 and p85, which may indicate that the tyrosine whose phosphorylation state is modestly affected may play an important role in the apoptotic effects of ceramide.

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