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ARTICLES

PKB/Akt Inhibits Ceramide-Induced Apoptosis in Neuroblastoma Cells by Blocking Apoptosis-Inducing Factor (AIF) Translocation

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Abstract Ceramide is a sphingolipid that is abundant in the plasma membrane of neuronal cells and is thought to have regulatory roles in cell differentiation and cell death. Ceramide is known to induce apoptosis in a variety of different cell types, whereas the physiological significance of gangliosides, another class of sphingolipids, in these processes is still unclear. We examined the mechanisms of ceramide-induced cell death using a human neuroblastoma cell line. Treatment of the human neuroblastoma cell line SH-SY5Y with ceramide induced dephosphorylation of the PKB/Akt kinase and subsequent mitochondrial dysfunction. In addition, ceramide-induced neuronal cell death was not completely blocked by inhibition of caspase activity. This incomplete inhibition appeared to be attributable to the translocation of apoptosis-inducing factor to the nucleus. Furthermore, overexpression of active PKB/Akt or Bcl-2 successfully blocked ceramide-induced neuronal cell death through inhibition of the translocation of apoptosis-inducing factor. J. Cell. Biochem. 102: 1160–1170, 2007.

Key words: apoptosis; apoptosis-inducing factor (AIF); ceramide; neuroblastoma; neuronal cell death; PKB/Akt

Ceramide, an evolutionarily conserved sphingolipid, is a lipid messenger that has functions and actions similar to those of diacylglycerol. It has been implicated as a second messenger in the regulation of cell cycle arrest, cell differentiation, and the induction of programmed cell death [Obeid et al., 1993]. The intracellular level of ceramide is controlled by the de novo synthesis of ceramide and the hydrolysis of sphingomyelin by sphingomyelinases. A number of extracellular agents that can act as inducers of ceramide formation have been identified, including 1,25-dihydroxyvitamin D_3, tumor necrosis factor-α, nerve growth factor, endotoxin, interferon-γ, interleukin-1, Fas ligands, CD28, dexamethasone, retinoic acid, progesterone, chemotherapeutic agents, ionizing irradiation, and heat [Hannun, 1996; Ito et al., 1999; Goswami and Dawson, 2000; Hannun and Obeid, 2002]. The ceramide concentration is also elevated in cells infected with human immunodeficiency virus and in senescent fibroblasts [Hannun, 1996]. Apoptotic neuronal cell death involving the ceramide pathway has been implicated in the pathology of head injury, spinal cord injury, and cerebral ischemia, as well as in chronic neurodegenerative disorders such as epilepsy, Alzheimer's disease, and Huntington's disease [Bredesen, 2000; Eldadah and Faden, 2000; Toman et al., 2002]. However, the cellular mechanisms

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underlying ceramide-induced apoptosis remain unclear.

Human neuroblastoma SH-SY5Y cells, which may be considered neuroblasts at various stages of maturation, become committed to apoptosis upon exposure to ceramide [Biedler et al., 1973]. Although apoptosis is an important event during normal developmental processes [Oppenheim, 1991; Ross, 1996], it also occurs in adults during the late stages of numerous neurodegenerative disorders of the central nervous system [Johnson, 1994; Pollard et al., 1994; Hickenbottom et al., 1999]. Apoptosis has also been reported to occur in peripheral nerve diseases [Matthews et al., 1997; Russell et al., 1998; Delaney et al., 1999; van Golen et al., 2000] and in neuropathies induced by treatment with anticancer drugs [Gill and Windebank, 1998]. The SH-SY5Y cell line has previously been used to examine some aspects of anticancer drug toxicity [Colombaioni et al., 2002] and to elucidate the mechanisms of diabetic neuropathy [Matthews et al., 1997; van Golen et al., 2000]. The upregulation of ceramide levels has been observed in cultured rat cerebellar granule cells (CGC) and in cortical neurons, in association with apoptosis induced by withdrawal of trophic support or treatment with etoposide [Toman et al., 2002]. It has also been shown that serum deprivation causes an increase in ceramide levels with concomitant apoptosis in the neuronal cell line HN9.10e [Colombaioni et al., 2002].

The administration of cell-permeable ceramide analogs or sphingomyelinase causes neuronal cell death in various neuronal culture systems such as CGC, cortical neurons, motor neuron cells, and hippocampal neurons [Pelled et al., 2002]. The activation of caspase-3 and the nuclear fragmentation in ceramide-treated cells provide further evidence in support of a role for ceramide in neuronal apoptosis. In addition, the intrinsic caspase-9 pathway, but not the extrinsic caspase-8 pathway, has been shown to be involved in ceramide-induced neuronal apoptosis [Movsesyan et al., 2002]. Ceramide treatment initiates a cascade of biochemical alterations associated with the cell death of primary cortical neuronal cells in a rat system. These changes include dephosphorylation and inactivation of protein kinase B (PKB)/Akt, which is followed by dephosphorylation of pro-apoptotic regulators such as BAD, forkhead transcription factors, and glycogen synthase kinase 3-β (GSK-3β); depolarization and permeabilization of mitochondria; and release of cytochrome C into the cytosol [Goswami et al., 1999; Ito et al., 1999; Stoica et al., 2003]. It has been speculated that Akt inhibits apoptosis by maintaining Bcl-xL function and preventing cytochrome *C* release from the mitochondria. However, a direct effect of Akt in regulating cytochrome C translocation during apoptosis has not been shown. Therefore, although the inhibition of apoptosis by Akt independent of cytochrome *C* release remains to be determined [Zhou et al., 2000], these findings demonstrate the importance of the mitochondria-dependent intrinsic pathway in ceramide-induced neuronal apoptosis [Ito et al., 1999; Kim et al., 2002; Stoica et al., 2003].

Here, we examine the mechanisms of ceramide-induced cell death using SH-SY5Y human neuroblastoma cells. We show that ceramide treatment induces changes in Akt activity and in the mitochondrial membrane potential. Our data indicate that the ceramide signaling pathway involves the inactivation of Akt and the translocation of apoptosis-inducing factor (AIF) to the nucleus. In addition, the inhibition of caspases was not sufficient to block ceramide-induced apoptosis of SH-SY5Y cells, whereas the overexpression of Bcl-2 and active Akt were more effective in blocking ceramide-induced cell death. In summary, our results indicate that ceramide treatment causes neuronal cell death through the inactivation of the Akt pathway and the translocation of AIF from the mitochondria and that the blockage of such mitochondrial changes reduces ceramide-induced apoptosis.

MATERIALS AND METHODS

Cells and Reagents

SH-SY5Y human neuroblastoma cells and Bcl-2-transfected SH-SY5Y cells were cultured routinely in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, and 100 U/ml each of penicillin and streptomycin. The cells were detached using trypsin–EDTA solution (Sigma). Cell-permeable, biologically active ceramide, C2-ceramide (*N*-acetyl-D-sphingosine; Sigma), was purchased and dissolved in dimethyl sulfoxide (DMSO). The antibodies to Akt and GSK-3β were from Santa Cruz Biotechnology (Santa Cruz, CA); the antibody to

AIF was from Sigma; the antibodies to phospho-Akt (Ser473), Bad, and phospho-GSK-3β (Ser9) were from Cell Signaling Technology (Beverly, MA); and the antibody to phospho-Bad (Ser136) was from Upstate Biotechnology (Charlottesville, VA). Horseradish peroxidase-conjugated anti-rabbit IgG antiserum was obtained from DAKO (Carpinteria, CA). The anti-goat IgG antiserum was purchased from DiNonA Inc. (Seoul, Korea). The caspase inhibitors, z-VAD-fmk, z-IETD-fmk, and z-LEHD-fmk, were obtained from Calbiochem (La Jolla, CA). The XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) and *N*-methyl dibenzopyrazine methyl sulfate (PMS), used to make the XTT–PMS cytotoxicity detection reagent, and the Hoechst 33258 were from Sigma.

Cell Viability Assays

Cell viability was measured using the LDH release assay [Sinensky et al., 1995] or the XTT dye-reduction assay [Fujiwara et al., 2003]. LDH release was measured with the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, San Luis Obispo, CA) according to the manufacturer's protocol. Briefly, the cells were grown in tissue-culture grade, 96-well, flat-bottom microtiter plates in 100 μl of culture medium per well. Relative absorbance was measured at 490 nm at Ceres 9000 plate reader (Bio-Tek instrument, Winooski, VT). Background LDH release determined in intact control cultures was subtracted from all experimental values. For XTT–PMS assay, XTT (1 mg/ml) was dissolved in warm medium (without phenol red), and 1.25 mM PMS was prepared in PBS. The cells were grown in tissue-culture grade, 96-well, flat-bottom microtiter plates in 100 μl culture medium per well. After incubation for the indicated periods, 50 μl of the XTT–PMS mixture (final XTT concentration, 0.3 mg/ml) was added to each well. The microtiter plates were incubated for 4 h at 37°C, and the production of the formazan product was determined by measuring the absorbance at 492 and 690 nm on a microtiter plate reader. All experiments were performed in triplicate.

Treatment with Caspase Inhibitors

Caspase inhibitors (20 μM of the pan-caspase inhibitor z-VAD-fmk or 20 μM of the caspase-9 inhibitor z-LEHD-fmk) were added to the culture media of control SH-SY5Y and SH-SY5Y cells expressing Bcl-2, before treatment with several concentrations of C2-ceramide to evaluate the modulating effect of the inhibitors on ceramide-induced cell death. The cells were harvested after 24 h, and the extent of cell death was analyzed.

Western Blot Analysis

Cells treated with C2-ceramide for various times were harvested with cell scrapers and placed on ice. The samples were washed once with ice-cold PBS, and the cell pellets were resuspended in lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10 mM NaF, 10 mM iodoacetamine, 200 μM Na_3VO_4, 25 mM NaF, and 1% NP-40), incubated on ice for 30 min, and centrifuged. The resulting postnuclear supernatants were loaded onto 10–15% SDS-PAGE gels, separated by electrophoresis, and transferred to polyvinylidene difluoride Immobilon membranes (Millipore, Bedford, MA). The membranes were probed with specific primary antibodies, and the immune-complexes were detected by using appropriate horseradish peroxidase-linked secondary antibodies. The specific bands were visualized using the ECL system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunofluorescence Staining and Confocal Analysis

The translocation of AIF from the mitochondria to the nucleus during apoptosis was analyzed by confocal microscopy, as described previously [Pardo et al., 2001]. In brief, the cells were allowed to attach to coverslips overnight and then were treated with ceramide. The cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. The coverslips were then washed once with PBS and placed onto a drop of 0.1% saponin in PBS for 20 min. The coverslips were then incubated with anti-AIF antiserum (1:500) in 0.1% saponin for 30 min at room temperature in a humidified chamber. The coverslips were washed twice with 0.1% saponin in PBS and incubated with anti-rabbit IgG antibody labeled with Cy3 (1:500; Molecular Probes, Eugene, OR). The cells were washed twice with PBS, and the nuclei were stained by incubation with Hoechst 33258 (0.5 μg/ml) in 0.1% saponin for 30 min. Finally, the coverslips were washed with PBS and mounted onto glass slides using a drop of Mowiol mounting medium

(Calbiochem, Madrid, Spain) and observed under a confocal microscope (Radiance 2000; Bio-Rad Laboratories, Hercules, CA).

RESULTS

Caspase Inhibitors do not Completely Block C2-Ceramide-Induced Apoptosis in SH-SY5Y Cells

To assess cell death induced by the administration of C2-ceramide, we measured cell death using both the XTT and LDH methods [Sinensky et al., 1995; Fujiwara et al., 2003]. As expected, the addition of C2-ceramide alone caused apoptotic cell death in cultured human neuroblastoma SH-SY5Y cells in a dose-dependent manner, as shown by the colorimetric XTT assay for the quantification of cell proliferation and viability (Fig. 1A) and by measurement of LDH release (Fig. 1B).

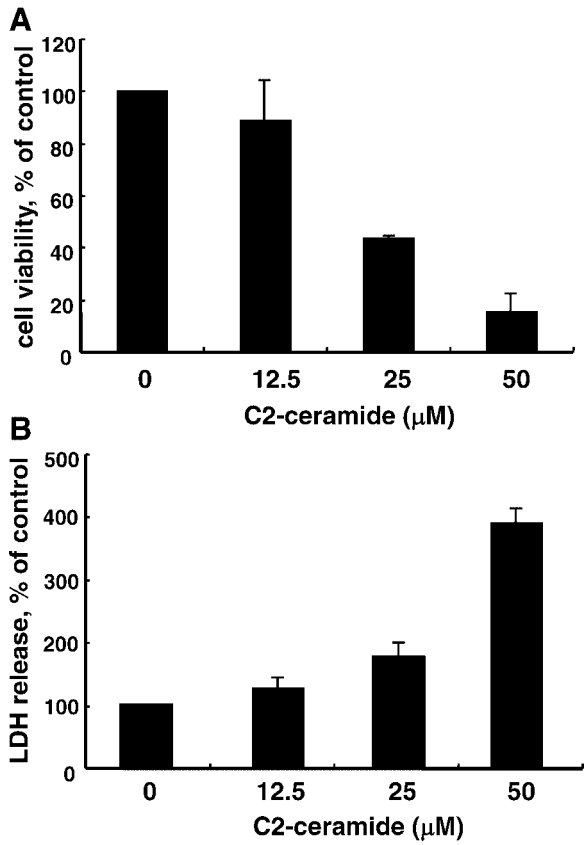

Fig. 1. Treatment with C2-ceramide induced apoptosis in SH-SY5Y human neuroblastoma cells. Cell death was evaluated by measurement of cell proliferation (**A**) or LDH release into the culture media (**B**). The cells were cultured in DMEM containing 10% FBS with or without C2-ceramide at the indicated concentrations. Histograms indicate cell survival fraction (A) and LDH release (B) as percentage of controls ± SD; n = 5 cultures per condition.

It has been demonstrated that neuronal death induced by ceramide may be linked to the caspase-9/caspase-3 regulated intrinsic apoptotic pathway [Ito et al., 1999; Movsesyan et al., 2002]. We therefore examined whether the inhibition of caspases would be sufficient to totally protect against ceramide-induced neuronal cell death by investigating the effect of caspase inhibitors on C2-ceramide-stimulated apoptosis of SH-SY5Y cells and comparing the results with the effect of the inhibitors on staurosporine-induced apoptosis. The addition of the pan-caspase inhibitor z-VAD-fmk or the caspase-9 inhibitor z-LEHD-fmk before the addition of the apoptosis inducers resulted in nearly complete inhibition of staurosporine-induced apoptosis (Fig. 2, right panels), whereas neither inhibitor fully prevented ceramide-induced apoptosis (Fig. 2B, left panels). Therefore, we sought other systems that might be regulated by ceramide signaling in SH-SY5Y cells.

C2-Ceramide-Induced Death of SH-SY5Y Cells is Effectively Inhibited by Bcl-2 Overexpression

As the caspase inhibitors were not able to completely block ceramide-induced cell death in human neuroblastoma cells, we investigated other factors that might be involved, focusing particularly on mitochondrial factors. We overexpressed the anti-apoptotic mitochondrial protein Bcl-2 in SH-SY5Y cells using a retroviral system and examined the effect of Bcl-2 on ceramide-induced apoptosis using the LDH release assay (Fig. 3). The overexpression of Bcl-2 successfully inhibited ceramide-induced apoptosis, and this protective effect was more complete than that of caspase inhibitors (Fig. 3). The combination of Bcl-2 overexpression and treatment with caspase inhibitors slightly augmented the anti-apoptotic effect as compared with that induced by Bcl-2 overexpression alone. Thus, we asked whether ceramide signaling might act through a caspase-independent pathway, in contrast to staurosporine that acts exclusively through caspase-mediated pathways. Although it has been reported that ceramide induces a mitochondria-dependent apoptotic pathway involving caspases in rat cortical neuronal cells [Stoica et al., 2003], we examined mitochondria-dependent, caspase-independent pathways.

Consequently, we investigated the translocation of AIF, which is known to be involved in

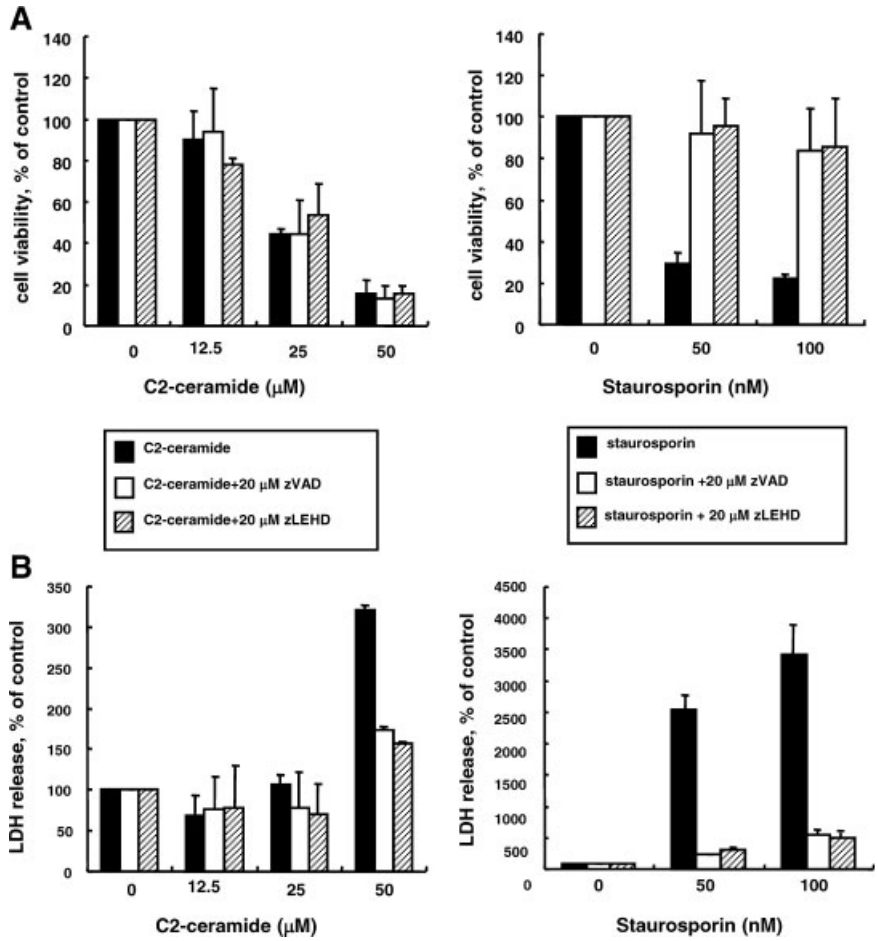

Fig. 2. Caspase inhibitors largely blocked staurosporine-induced (**B**), but not C2-ceramide-induced (**A**), apoptosis of SH-SY5Y cells. A pan-caspase inhibitor (20 μM z-VAD-fmk) or a caspase-9 inhibitor (20 μM z-LEHD-fmk) was applied 2 h prior to the addition of staurosporine or C2-ceramide. The extent of cell death was analyzed after 24 h. Cell proliferation (left panels) and LDH release (right panels) were evaluated as described in Figure 1.

apoptosis and to act in a caspase-independent manner. The SH-SY5Y cells and Bcl-2-expressing SH-SY5Y cells were treated with ceramide and immunostained for AIF using a fluorescent Cy3-tagged secondary antibody (red) and Hoechst 33258 nuclear dye (blue; Fig. 4A and B). Once translocation of AIF to the nucleus occurs, both cytoplasm and the nucleus should be stained by anti-AIF Ab (Fig. 4 left panels) and the color of nuclei should be changed from blue (nuclear dye only) to violet (nuclear dye (blue) plus AIF (red)) in merged pictures (Fig. 4 right panels). After ceramide treatment, in case of wild type SH-SY5Y cells, both cytoplasm and the nuclei were stained by anti-AIF Ab and the color of nuclei was changed from blue to violet suggesting that the AIF protein was translocated to the nuclei of SH-SY5Y cells (Fig. 4A). The overexpression of the anti-apoptotic mitochondrial protein Bcl-2 was able to completely inhibit the translocation of the pro-apoptotic mitochondrial AIF protein to the nucleus as the color of nuclei were still blue after the ceramide treatment (Fig. 4B).

Treatment of Human Neuroblastoma Cells with C2-Ceramide Induces Dephosphorylation of Akt on Ser473, GSK3β on Ser9, and BAD on Ser136

It has been reported that the protective effects of phosphatidylinositol (PI) 3-kinase are mediated primarily by one of the downstream protein kinases, Akt [Franke et al., 1997; Zhou et al., 2000]. The phosphorylation of Akt on the Ser473 residue, which is known to be essential for cell survival, decreased rapidly and markedly following C2-ceramide treatment

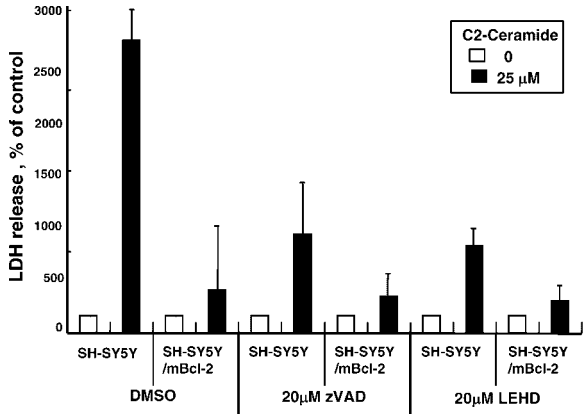

Fig. 3. The effects of Bcl-2 overexpression on ceramide-induced neuroblastoma cell death. The control neuroblastoma cells or cells overexpressing Bcl-2 were pretreated with 20 μM z-VAD-fmk or 20 μM z-LEHD-fmk for 2 h and then treated with 25 μM ceramide. Cell death was measured using the LDH release assay after 24 h.

of SH-SY5Y cells (Fig. 5A), to an extent comparable to that previously observed in a rat model system [Stoica et al., 2003]. As early as 2 h after the application of ceramide, a significant decrease in the level of Akt phosphorylated on Ser473 (phospho-Akt Ser473) was observed, and the decrease lasted up to 24 h, but phospho-Akt Thr308 was not detected in all cases (data not shown). Figure 5 illustrates the effects of C2-ceramide treatment on the dephosphorylation of Akt (Fig. 5A), GSK-3β (Fig. 5B), and Bad (Fig. 5C), which was confirmed by quantitative densitometry. No decrement in the total level of Akt, GSK-3β, or BAD was detectable over comparable time intervals of C2-ceramide treatment (Fig. 5).

Akt is Involved in the Inhibition of C2-Ceramide-Induced Apoptosis in Human Neuroblastoma Cells

To further examine the role of Akt in apoptosis elicited by ceramide treatment, SH-SY5Y cells were stably transfected with expression vectors encoding a constitutively active Akt (act-Akt) or a dominant negative Akt mutant (DN-Akt). The expression level of Akt in the transfected cells was verified by comparison with that in mock-transfected SH-SY5Y cells (Fig. 6A). The catalytically inactive Akt (K179M, DN-Akt) has been shown to act as a dominant negative blocker of the phosphorylation of anti-apoptotic Akt substrates in several cell types [Kim and Park, 2003]. SH-SY5Y cells transfected with a control plasmid (mock) or with the act-Akt or DN-Akt vectors were treated with C2-ceramide at various concentrations. Ceramide-induced apoptosis was significantly decreased in cells overexpressing act-Akt, but the extent of apoptosis was slightly increased in cells expressing DN-Akt (Fig. 6B and C). These results indicate that changes in Akt activity can change the apoptotic response of SH-SY5Y cells to ceramide treatment.

As the ceramide-induced translocation of AIF was blocked by overexpression of the mitochondrial anti-apoptotic protein Bcl-2 (Figs. 3 and 4) and as Akt functions as a survival signal partly by phosphorylating (and thereby inactivating) the mitochondrial pro-apoptotic protein Bad, we asked whether the function of Akt was related to the translocation of AIF to the nucleus. We treated SH-SY5Y cells expressing act-Akt or DN-Akt with C2-ceramide and examined the translocation of AIF. In mock-transfected SH-SY5Y cells treated with 25 μM C2-ceramide, AIF was clearly translocated from the mitochondria to the nucleus at 12 h as the color of nuclei was changed from blue to violet (Fig. 7A). The overexpression of act-Akt blocked the translocation of AIF (Fig. 7B), whereas the overexpression of DN-Akt did not (Fig. 7C).

DISCUSSION

The apoptotic death of neurons occurs at many different developmental stages and has been implicated in the pathology of head injury, spinal cord injury, and cerebral ischemia, as well as in neurodegenerative disorders [Bredesen, 2000; Eldadah and Faden, 2000]. Ceramide and gangliosides are sphingolipids that are abundant in the plasma membrane of neuronal cells and are thought to have regulatory functions in cellular differentiation and apoptosis [Dawson et al., 1997; Goswami et al., 1999; Stoica et al., 2003]. In rat cortical neurons and SH-SY5Y human neuroblastoma cells, it has been shown that neuronal cell death induced by C2-ceramide may be linked to the caspase-9/caspase-3 intrinsic pathway [Movsesyan et al., 2002]. Recently, Stoica et al. [2003] showed that changes in Akt activity and mitochondrial membrane potential were induced early after ceramide treatment in primary cultures of rat cortical neurons. In human cells, cytochrome C release and caspase activation have been shown to be involved in neuronal cell death [Ito et al., 1999]. Although caspases and mitochondrial

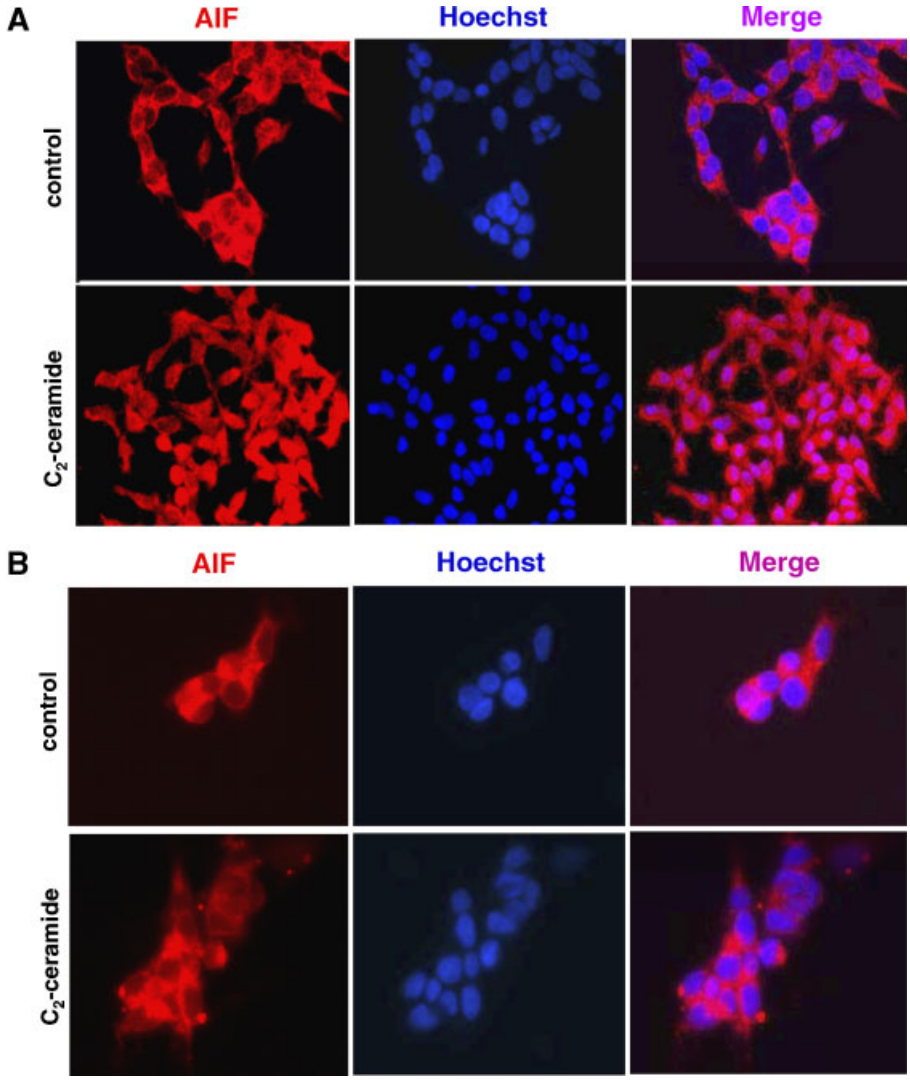

Fig. 4. The C2-ceramide-induced translocation of apoptosis-inducing factor (AIF) into the nucleus was inhibited by Bcl-2 overexpression. SH-SY5Y cells (**A**) and Bcl-2-overexpressing SH-SY5Y cells (**B**) were treated with 25 μM ceramide for 12 h. The cells were then fixed, stained with anti-AIF antibody and Cy3-conjugated secondary antibody (red fluorescence) and with Hoechst 33258 nuclear dye (blue fluorescence), and examined by confocal microscopy. After 12 h of ceramide treatment, the translocation of AIF into the nucleus was observed in SH-SY5Y cells, but not in cells overexpressing Bcl-2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

proteins are known to be candidate targets for ceramide-induced apoptosis, it is not clearly understood how the ceramide signaling cascade functions in human neuronal cells. In addition, Zhou et al. [2000] also demonstrated that Akt activation may inhibit apoptosis independently of cytochrome *C* release. Consequently, we examined apoptosis-related factors that could be the target(s) of ceramide signaling in a human cell system. We had noticed that the inhibition of caspases was not sufficient to completely block ceramide-induced apoptosis in neuronal cells [Toman et al., 2002]. In our system, we also observed that the addition of caspase inhibitors did not completely block ceramide-induced apoptosis, whereas staurosporine-induced apoptosis was totally inhibited by caspase inhibitors (Fig. 2). The observation led us to ask what other mechanisms could be involved in ceramide-mediated neuronal cell death.

Mitochondria have an important role in apoptotic-signaling pathways; severe malfunctions at any level in the cell are eventually translated into the release of apoptogenic factors from the mitochondrial intermembrane space [Daugas et al., 2000; Gurp et al., 2003]. As the Akt protein is known to be involved in

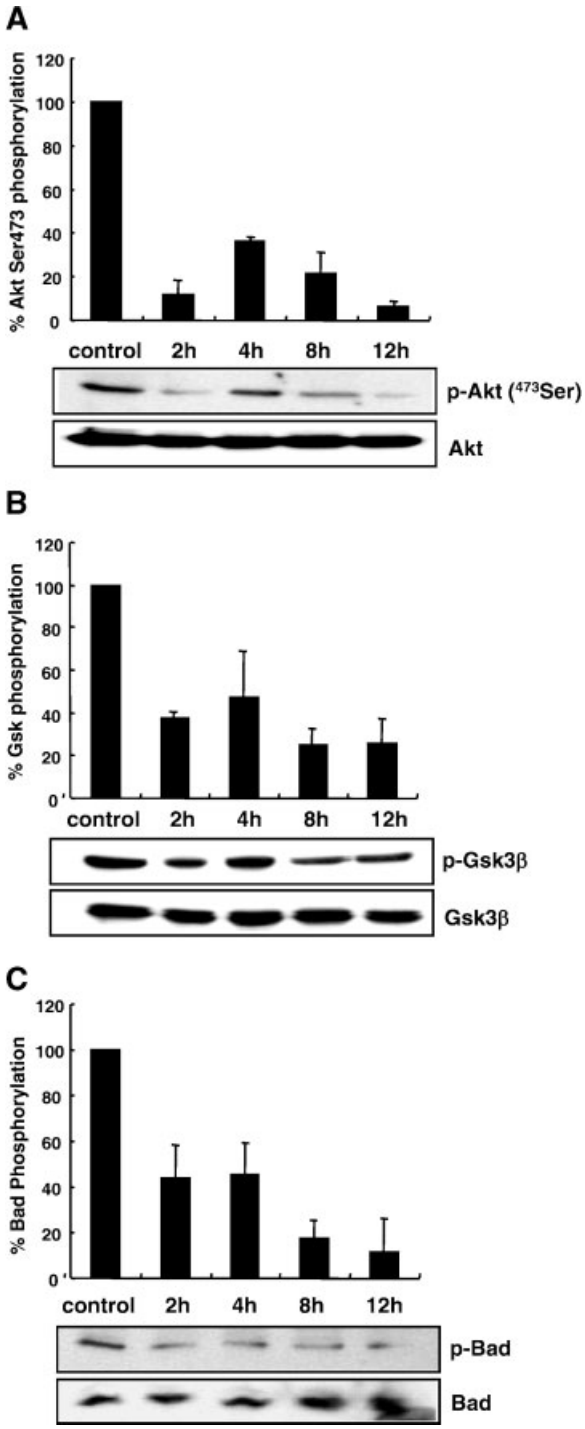

Fig. 5. The effect of C2-ceramide on dephosphorylation of Akt on Ser473 (**A**), Gsk3-β on Ser9 (**B**), and BAD on Ser136 (**C**). Human neuroblastoma (SH-SY5Y) cells were treated with 25 μM of ceramide. At the indicated time points, the cells were harvested and subjected to immunoblot analysis as described in the experimental procedures. Quantitative densitometry confirmed the observations presented in each blot and is shown as histograms. The total levels of Akt, Gsk3, and BAD proteins were not decreased after ceramide treatment.

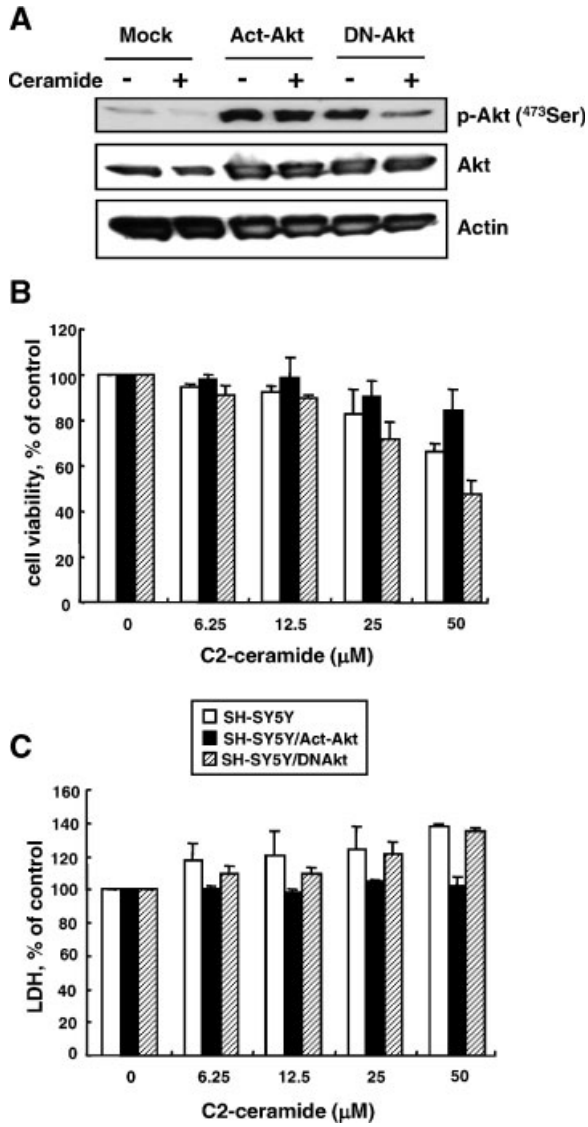

Fig. 6. Akt is involved in the inhibition of ceramide-induced apoptosis. SH-SY5Y cells expressing active Akt (act-Akt) or dominant negative Akt (DN-Akt) were incubated for 4 h with ceramide (25 μM) and were compared with control cells (Mock). (**A**) The protein extracts were prepared and subjected to SDS-PAGE, and Western blots were performed using anti-phospho-Akt (Ser473) antibody and anti-total Akt antibody. Anti-actin antibody was used as a loading control. Cell death was evaluated by measuring cell proliferation (**B**) or LDH release (**C**) as described in Figure 1.

apoptosis through the dephosphorylation the bcl-2 family member Bad [Goswami et al., 1999; Stoica et al., 2003] after Akt inactivation, we focused on mitochondrial factors that are released after apoptotic signals and that act independently of caspase. In order to determine whether anti-apoptotic mitochondrial proteins could block ceramide-induced cell death, we

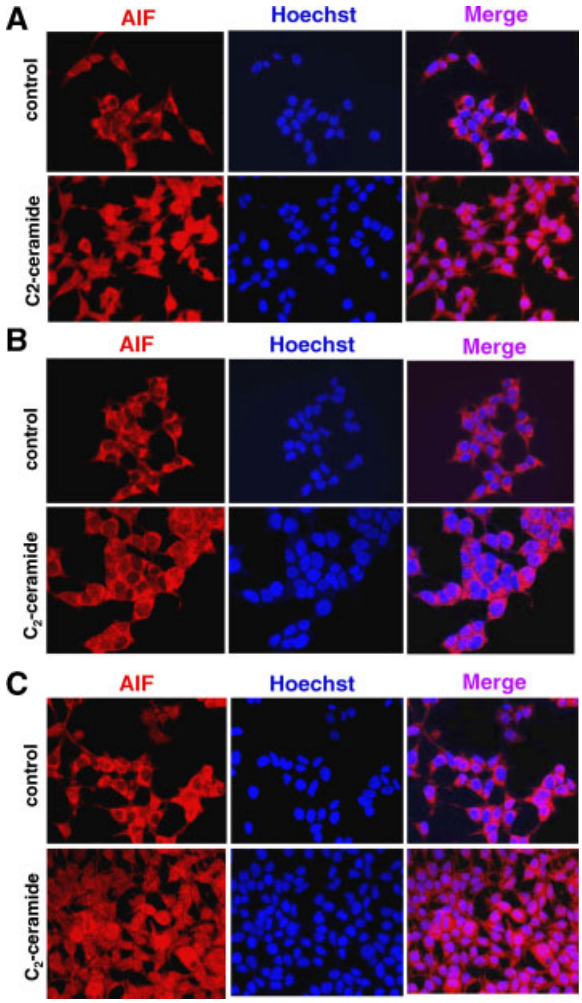

Fig. 7. Ceramide-induced apoptosis occurs through the translocation of apoptosis-inducing factor (AIF) into the nucleus, and Akt expression blocks AIF translocation. Mock-transfected (**A**), act-Akt-expressing (**B**), and DN-Akt-expressing (**C**) SH-SY5Y cells were treated with 25 μM ceramide for 12 h. The cells were then fixed, immunostained for AIF (red fluorescence), stained with Hoechst 33258 (blue fluorescence), and analyzed by confocal microscopy. After 12 h of ceramide treatment, AIF translocation into the nucleus was evident in the mock-transfected (**A**) and DN-Akt-expressing (**C**) SH-SY5Y cells, but not in the act-Akt-expressing cells (**B**). Note the co-localization of AIF and Hoechst 33258 in the nuclei of ceramide-treated cells (orchid fluorescence on merged images). All photographs were taken at the same magnification. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

overexpressed Bcl-2 in SH-SY5Y cells. Bcl-2 was able to inhibit ceramide-induced apoptosis in human neuroblastoma cells to a greater extent than were caspase inhibitors (Fig. 3), suggesting that ceramide-induced apoptosis occurs primarily through mitochondria-related apoptotic pathways. Cytochrome C release has been shown to be possibly involved in ceramide-induced apoptosis in SK-N-SH cells [Ito et al., 1999]. In addition, ceramide signaling has been shown to involve dephosphorylation of Akt, which may regulate apoptosis independently of cytochrome *C* regulation [Zhou et al., 2000; Powell et al., 2003]. These observations strongly imply that Akt may regulate apoptosis not only through cytochrome *C* release but also through other mitochondrial factors.

Among the mitochondrial factors known to be involved in apoptosis, AIF appears to be highly conserved evolutionarily and is normally confined to the mitochondrial intermembrane space [Candé et al., 2002; Gurp et al., 2003]. Mature AIF is known to translocate to the nucleus in response to apoptogenic stimuli, and the overexpression of AIF induces peripheral chromatin condensation, dissipation of the mitochondrial transmembrane potential, and high-molecular-weight (50 kb) DNA fragmentation [Candé et al., 2002; Yu et al., 2002]. However, the role of AIF in ceramide-induced neuronal cell death in human or murine cells has not yet been investigated. We therefore examined whether AIF is the target of the ceramide-induced proapoptotic cascade and found that the AIF was translocated to the nucleus of human neuroblastoma cells after ceramide treatment (Figs. 4 and 7). These observations imply that ceramide treatment of human neuronal cells induces the inactivation of Akt, followed by mitochondrial membrane depolarization and the translocation of AIF to the nucleus, leading to cell death. This finding strongly suggests that ceramide-induced neuronal cell death occurs not only through the activation of caspase-3 and -9 but also through the translocation of AIF to the nucleus independently of caspase activation.

Although Akt and its substrates have been shown to be dephosphorylated in rat cells after treatment with ceramide, and this dephosphorylation resulted in a failure of survival signals resulting in apoptosis [Stoica et al., 2003], the role of Akt in human neuronal cell death has not been previously investigated. We therefore evaluated the function of Akt in ceramide-induced apoptosis in human neuroblastoma cells. We found that the phosphorylation of Akt and its substrates, Gsk3β and Bad, was decreased after ceramide treatment (Fig. 5) and that the overexpression of act-Akt, but not DN-Akt, was able to rescue the neuronal cells from

apoptosis (Fig. 6). As shown in Figure 5, in cases of p-Akt and p-Gsk3β, the densities were slightly increased at 4 h and then decreased again. As Stoica et al. [2003] showed that in ceramide induced neuronal apoptosis the degrees of p-Akt and p-FKHR were slightly increased after 6 h (p-Akt) or 4 h (p-FKHR) incubation and then decreased again, we reproducibly observed this phenomenon in p-Akt and p-Gsk cases at 4h incubation but we do not have a clue why this occurs. Given that the pro-apoptotic mitochondrial protein Bad was activated (i.e., dephosphorylated) by ceramide signaling, we examined mitochondrial factors that may induce neuronal cell death independently of the caspase-dependent pathway. As mentioned above, the overexpression of the anti-apoptotic protein Bcl-2 successfully blocked ceramide-induced apoptosis in human neuronal cells to a greater extent than did caspase inhibitors (Fig. 3). Although cytochrome *C* is released in a caspase-dependent manner after ceramide treatment of the human neuronal cell line SK-N-SH [Ito et al., 1999], no direct effect of Akt on the regulation of cytochrome *C* translocation during apoptosis has been demonstrated; thus the role of Akt in the modulation of apoptosis independently of cytochrome release remained to be examined [Zhou et al., 2000]. We focused on the pro-apoptotic protein AIF because it resides in the intermembrane space of mitochondria and its translocation to the nucleus after apoptogenic signals is caspase independent. We confirmed that AIF is translocated to the nucleus during ceramide-induced neural cell death in a human cell system (Figs. 4 and 7) and that this translocation is inhibited by the overexpression of active Akt, implying that AIF translocation occurs downstream of the dephosphorylation of Akt (Figs. 4 and 7).

In the present study, we examined the mechanisms of ceramide-induced cell death using SH-SY5Y human neuroblastoma cells. We showed that ceramide treatment induced changes in Akt activity and in the mitochondrial membrane potential. Among apoptosis-related mitochondrial factors, the ceramide signaling pathway involves the inactivation of Akt and the translocation of apoptosis-inducing factor (AIF) to the nucleus in SH-SY5Y cells. The inhibition of caspases was not sufficient to block ceramide-induced apoptosis of SH-SY5Y cells, whereas the overexpression of Bcl-2 and active Akt were more effective in blocking ceramide-induced cell death. Overall, our results indicate that ceramide treatment causes neuronal cell death through the inactivation of the Akt pathway, resulting in mitochondrial dysfunction, and the translocation of AIF to the nucleus and subsequent neuronal cell death.

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