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Ceramide Induces Apoptosis and Growth Arrest of Human Glioblastoma Cells by Inhibiting Akt Signaling Pathways

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Abstract

Ceramide is an important lipid mediator of extracellular signals that control various cellular functions, including apoptosis. In this study, we showed that ceramide induced apoptosis in U373MG human glioblastoma cells associated with G1 cell cycle arrest. Treatment of cells with ceramide increased proapoptotic Bax expression and inhibited the expression of antiapoptotic Bcl-2 and Bcl-xL Ceramide also downregulated cyclin E, cyclin D1, cdk 2, and cdk4 which are involved in regulating cell cycle. In addition, ceramide suppressed phosphorylation of Akt, Bad, p70 S6 kinase, and 4E-BP1, suggesting the involvement of Akt/mTOR signaling pathway. Additionally, okadaic acid, an inhibitor of protein phosphatase 2A, partially blocked the ceramide mediated inhibition of phosphorylation of Akt and 4E-BP1. These results suggest that ceramide induces apoptosis in U373MG glioblastoma cells by regulating multiple signaling pathways that involve cell cycle arrest associated with Akt signaling pathway.

Key Words: Akt, 4E-BP1, Glioblastoma cells

INTRODUCTION

Ceramide has long been recognized as a lipid mediator of cell death (Obeid *et al.*, 1993; Hannun, 1996). Many anticancer drugs such as doxorubicin, vincristine, etoposide, and paclitaxel exert their antitumor effect against cancer cells by inducing apoptosis associated with an increase of cellular ceramide (Jaffrezou *et al.*, 1996; Herr *et al.*, 1997; Cabot *et al.*, 1999; Selzner *et al.*, 2001). Aberrant or decreased ceramide signaling has been implicated in contributing to tumor progression and resistance to therapy (Selzner *et al.*, 2001; Senchenkov *et al.*, 2001; Struckhoff *et al.*, 2004).

Ceramide activates proapoptotic mechanisms in response to various stress stimuli, predominantly by acting ceramide-activated phosphatases and kinases, which in turn regulate PKC, Akt, c-Jun, and Bcl-2 family proteins (Hannun and Obeid, 2002). Akt inhibits apoptosis by inactivating proapoptotic proteins such as Bad, procaspase-9, and forkhead and by activating antiapoptotic proteins such as NFκB and cyclic adenosine monophosphate (cAMP)-response element binding protein (Kennedy *et al.*, 1997; Brunet *et al.*, 1999; Wendel *et al.*, 2004). Recently, PI3-Kinase/Akt /mTOR pathways have emerged as an important cancer therapeutic target (Park *et al.*, 2002; Sun *et al.*, 2005; Armengol *et al.*, 2007). p70 S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) are downstream effectors of PI3K/Akt/mTOR and frequently activated in many human cancer cells. 4E-BP1 has been suggested as a prognostic factor in ovarian cancer, in which increased levels of phosphorylated 4E-BP1 are associated with high-grade tumors and a poor prognosis (Wendel *et al.*, 2004; Armengol *et al.*, 2007). Recent studies have shown that inhibition of 4E-BP1 phosphorylation activates apoptosis (Hu *et al.*, 2007; Barnhart *et al.*, 2008). Hypophosphorylated form of 4E-BP1 induces apoptosis through its ability to sequester eIF-4E from a translationally active complex and reducing the rate of protein synthesis.

Malignant gliomas are the most frequent primary brain tumors in adults and the prognosis of patients remains poor (Vescovi *et al.*, 2006; Nandi *et al.*, 2008). Among the deregulated signaling pathways in glioblastoma, aberrant activation of the PI3K/Akt plays a critical role in the tumorigenesis of glioblastoma (Giusssani *et al.*, 2009). It has previously shown that ceramide induced apoptosis associated with inhibition of PI3-Kinase/Akt pathway in certain cancer cells including glioblastoma cells (Kim *et al.*, 2008; Giusssani *et al.*, 2009). However, the mechanism by which ceramide regulates Akt signaling

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pathways in glioblastoma cells have not been elucidated yet. In this study, we demonstrated that ceramide induces apoptosis and cell cycle arrest in U373MG human glioblastoma cells through regulation of Akt and its downstream targets, 4E-BP1 and S6K. Moreover, our results show that PP2A plays an important role in mediating ceramide-induced inactivation of Akt-mediated signaling pathway.

MATERIALS AND METHODS

Materials

Fetal bovine serum was purchased from Welgene (Daegu, Korea), and ECL kit from GE Healthcare (Piscataway, NJ, USA). Antibodies to 4E-BP1 and phospho-70S6K (Thr 389) were from Cell Signaling Technology (Danvers, MA, USA), and antibodies to cyclin D1, cyclin E, CDK2, CDK4, p4E-BP1, Bcl-2, Bcl-xL, Bax, Bad, phospho-Bad (Ser136), Akt, phospho-Akt (Ser473), or GAPDH and HRP-conjugated secondary antibody were Santa Cruz Biotechnology (Santa Cruz, CA, USA). C_6-cermide was purchased from Sigma (St. Louis, MO, USA).

Cell culture

U373MG human glioblastoma cells were purchased from Korea Cell Line Bank (Seoul, Korea). U373MG cells were grown in RPMI 1640 medium containing with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cell proliferation assay

Cell proliferation assays were performed by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). U373MG cells were plated in 96-well plates at 1×10^4 cells per well and cultured in the RPMI growth medium. At the indicated time points, the cell numbers in triplicate wells were measured at the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt).

Apoptosis assay

Cells were washed with serum-free RPMI. Ceramide or vehicle (DMSO) was diluted into serum-free RPMI at the indicated concentrations. Cells were maintained in serum-free RPMI for 2 h before experiments. To analyze apoptosis, cells were treated with ceramide for 48 h and resuspended in buffer containing 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, and pelleted by centrifugation. Cell pellets were washed in ice-cold PBS, resuspended in binding buffer, and incubated with FITC-conjugated Annexin V and propidium iodide (Zymed Laboratories, South San Francisco, CA) for 10 min at room temperature in the dark. Cells stained with Annexin V-FITC were washed with the binding buffer. Apoptotic cells were determined using fluorescence microscopy with excitation and emission wavelengths of 488 nm and 518 nm, respectively.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

To measure the $\Delta\Psi_m$ of U373MG cells, the fluorescent probe JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide) was used. JC-1 exists as a monomer at low values of $\Delta\Psi_m$ (green fluorescence), while it forms aggregates at a high $\Delta\Psi_m$ (red fluorescence). Cells (6×10^5) were collected by trypsinization, washed in warm phosphate buffered saline (PBS, pH 7.4) and incubated for 15 min at 37°C with 2 μM JC-1. Cells were pelleted at 1,000 rpm for 5 min, washed in warm PBS, resuspended the pellet with PBS, and analysed by flow cytometric analysis (Becton Dickinson FACScan, USA).

Cell cycle analysis

Cells were pelleted at 1,200 rpm and washed once with 1 ml of ice-cold PBS. The resulting pellets were resuspended in 1 ml of cold PBS, and ethanol (80%), pre-chilled at −20°C, were added with periodic vortexing. The resulting mixture was kept on ice for 60 min, and the cells were permeabilized in 0.5% Triton X-100, 20 μg/ml RNaseA, and 50 μg/ml propidium iodide in PBS. The samples were kept at 37°C for 30 min followed by flow cytometric analysis using the CellQuest program.

Western blot analysis

Proteins (30 μg/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% 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 p4E-BP1, p70 S6K, cyclin D1, cyclin E, CDK2, CDK4, Bcl-2, Bcl-xL, Bax, Bad, phospho-Bad, Akt, phospho-Akt, or GAPDH. Detection was performed with enhanced chemiluminescence (ECL) detection system (GE Healthcare). Protein content was determined with bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as a standard.

RESULTS

Ceramide induces apoptotic cell death in U373MG glioblastoma cells

The ability of ceramide to induce cell death was determined in the U373MG human glioblastoma cells. Cells were treated with different concentrations of ceramide for 24 h or 48 h, and cell viability was determined. Ceramide exerted a concentration-dependent inhibition of cell proliferation (Fig. 1A). Induction of apoptosis by ceramide was analyzed by detection of Annexin V-positive cells. Cells were treated with ceramide for 48 h and Annexin V-positive cells were determined using fluorescence microscopy. As shown in Fig. 1B, Annexin-positive cells were increased by treatment with ceramide, suggesting that a population of the cells underwent apoptotic cell death. To further investigate whether ceramide induces apoptosis, we evaluated reduction of mitochondrial membrane potential ($\Delta\Psi_m$). The opening of the permeabilization transition (PT) pore complex is thought to mediate release of proapoptotic proteins from the mitochondria (Green and Reed, 1998). One of the markers of the opening of PT pore is a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). We used the cell-permeable JC-1 dye to monitor changes in mitochondrial membrane potential after ceramide treatment. In non-apoptotic cells with an intact membrane potential, the JC-1 dye accumulates and forms aggregates with a red fluorescence. In apoptotic and

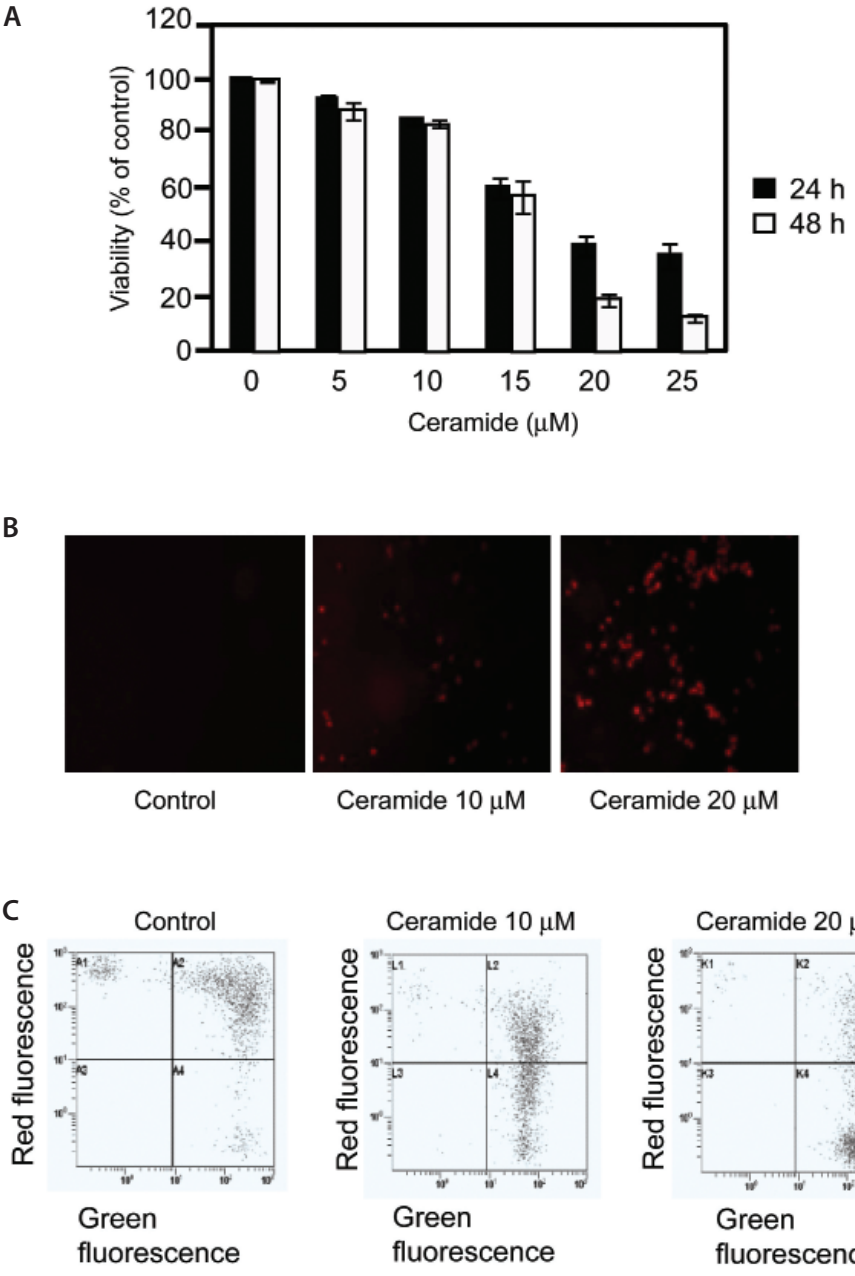

Fig. 1. Ceramide induces apoptotic cell death in U373MG cells. (A) Cells were treated with ceramide at various concentrations for indicated time periods, and the cell viability was detected using Cell Counting Kit-8. Formazan formation was quantified by spectrometry at 450 nm. (B) Cells were incubated with ceramide for 48 hr. After staining cells with Annexin V-FITC, positive cells were analyzed by fluorescence microscopy. (C) Detection of mitochondrial transmembrane potential by JC-1 was analyzed by flow cytometry. Histograms represent the JC-1 fluorescence of normal cells and those treated with ceramide for 48 h.

dead cells, the dye is not actively taken up into the mitochondria and remains in its monomeric form and appears green. Thus, as shown in Fig. 1C, treatment of ceramide (20 μM) resulted in a significant decrease in mitochondrial membrane potential, as compared with control, indicating that mitochondria are important target for ceramide- induced apoptosis in U373MG cells.

Ceramide regulates the expression of Bcl-2 family proteins

To elucidate the mechanism by which ceramide induces apoptosis in U373 MG glioblastoma cells, we investigated the expression of antiapoptotic and proapoptotic Bcl-2 proteins in ceramide-treated cells. As shown in Fig. 2, the expressions of antiapoptotic Bcl-2 and Bcl-xL proteins were reduced whereas the level of proapoptotic Bax was slightly increased by the treatment with ceramide.

Ceramide induces G1-phase cell cycle arrest and regulated expression of cell cycle regulatory proteins

To analyze whether ceramide-induced inhibition of cell proliferation is accompanied by alterations in cell cycle distribution, cell cycle distribution was analyzed by flow cytometry. Ceramide treatment (20 M) caused 55% of the cells to be in G0/G1 phase compared to 45% in the control cells after 24 h exposure (Fig. 3A). To investigate the mechanism by which ceramide induces G1 arrest in U373MG cells, we examined the expression of proteins that participate in the regulation of the cell cycle. Ceramide induced G1 arrest by a simultaneous decrease in Cdk2, Cdk4, cyclins D1 and E detected by West-

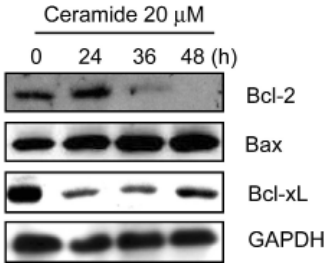

Fig. 2. Ceramide regulates expression of Bcl-2 family proteins in U373MG cells. Cells were treated for the indicated times with 20 μM ceramide and immunoblotted with anti-Bcl-2, anti-Bax, anti-BCl-xL, or anti-GAPDH.

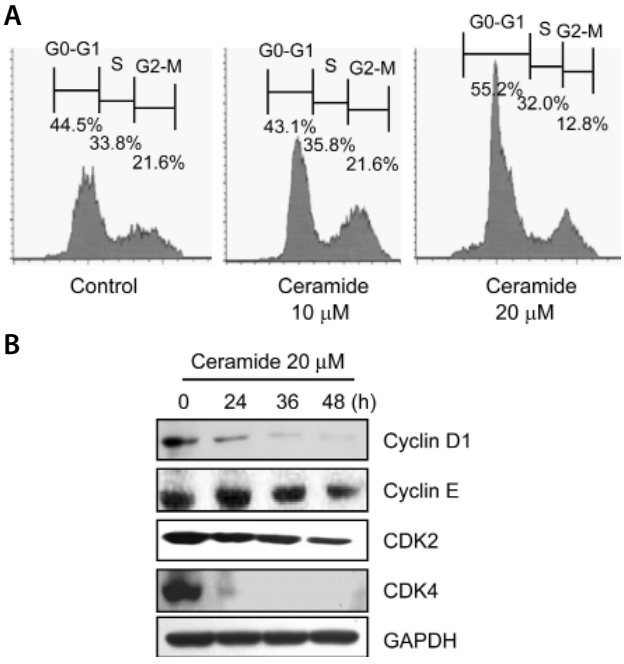

Fig. 3. Ceramide induces cell cycle arrest and regulates cell cycle regulatory proteins. (A) Cells were stimulated with ceramide for 48 h. Nuclei were stained with propidium iodide and analyzed by flow cytometry. (B) The expression levels of cyclin D1, cyclin E, cdk4 and cdk2 were determined by Western blotting.

ern blotting (Fig. 3B).

Ceramide decreased the level of phospho forms of Akt, S6K, and 4E-BP1

Recent studies have shown that ceramide-induced apoptosis is associated with inactivation of Akt kinase pathway (Kim *et al.*, 2008). Therefore, we investigated whether Akt pathway is involved in ceramide-induced apoptosis in U373MG cells. The PI3-kinase/Akt signaling pathway is critical in the inhibition of apoptosis, and molecules that can block Akt activity may have important significance in cancer therapy (Datta *et al.*, 1997; Testa and Bellacosa, 2001). Ceramide treatment resulted in an appreciable down-regulation of phospho-Akt and phospho-Bad without an effect on total Akt and Bad expression in U373MG cells (Fig. 4A). In order to explore the

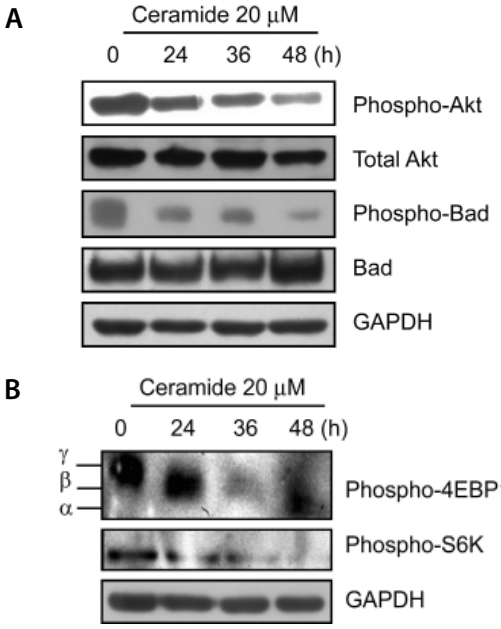

Fig. 4. Ceramide inhibits phosphorylation of Akt, Bad, 4EBP1 and S6K. Cells were treated with 20 μM ceramide for the indicated time periods and the expression levels of phospho-Akt, Akt, phospho-Bad, 4E-BP1 and S6K were determined by Western blotting.

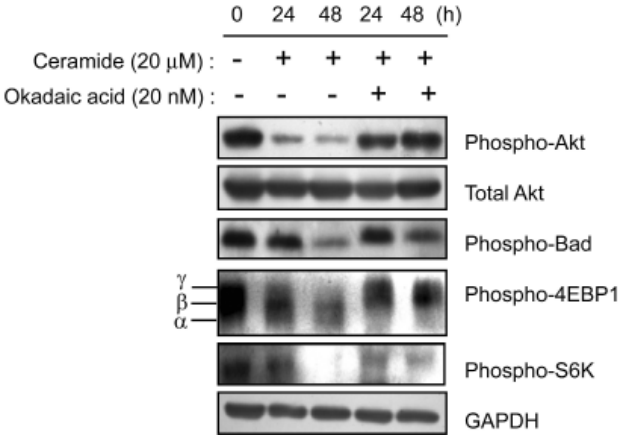

Fig. 5. Inhibition of PP2A prevented ceramide-induced dephosphorylation of Akt, 4E-BP1 and S6K. Cells were treated with 20 μM ceramide for the indicated time periods in the presence or absence of 20 nM okadaic acid.

effect of ceramide in Akt/mTOR kinase cascade, we studied expression of 4E-BP1 and S6K protein, the best characterized downstsream effectors of the mTOR. 4E-BP1 is detected as three distinct bands by Western blot analysis: a slow migrating, hyperphosphorylated γ form, and two faster migrating, hypophosphorylated forms, designated α and β (Guan *et al.*, 2007). The hyperphosphorylated γ form does not interact with eIF4E, thus promotes translation initiation, whereas the α and β forms bind to eIF4E with high and moderated affinity, respectively, and thus inhibit cap-dependent translation inhibition. Ceramide decreased the expression of phospho-S6K and relative level of the γ form of 4E-BP1 (Fig. 4B).

The effects of ceramide on Akt pathway is PP2A-dependent

Ceramide is known to regulate PP2A activity in various cell lines (Law and Rossie, 1995; Ruvolo *et al.*, 1999). PP2A regulates the activities of protein kinase signaling pathways, including the PI3-kinase/Akt pathways (Janssens *et al.*, 2005). We investigated whether PP2A regulates Akt activity and its downstream targets, Bad, 4E-BP1 and S6K after ceramide treatment. To test the role of PP2A in Akt dephosphorylation, we used okadaic acid to block PP2A activity. Cells were treated with okadaic acid, and stimulated with ceramide, and levels of phospho-Akt were analyzed. Down-regulation of PP2A significantly attenuated ceramide-induced Akt dephosphorylation and increased γ form of 4E-BP1 (Fig. 5). We also found that PP2A down-regulates the activation of p70 S6k, suggesting the PP2A phosphatase involves inhibition of Akt/eIF4E survival pathways.

DISCUSSION

Ceramide is a lipid mediator, with proapoptotic activities in cancer cells. The effect of several chemotherapeutic agents are linked to the induction of apoptosis and associated with an increase of cellular ceramide (Bieberich *et al.*, 2000; Macchia *et al.*, 2001). In the present study, we found that ceramide induced apoptotic cell death in U373MG cells, detected by decrease of mitochondrial membrane potential or phosphatidylserine exposure measured by Annexin V binding. In an effort to characterize the molecular mechanisms by which ceramide induces apoptosis in U373MG cells, we evaluated whether inhibition of cell survival signals or activation of cell death signals are involved in inducing apoptosis. Our data showed that ceramide downregulated antiapoptotic Bcl-2 and Bcl-xL whereas upregulated the level of proapoptotic Bax protein. We found that ceramide inhibited antiapoptotic Akt phosphorylation. Akt is known to be an important survival factor in signal transduction pathways involved in cell growth, and is an apoptotic regulator in many cancers (Testa Bellacosa 2001; Cantley, 2002; Osaki *et al.*, 2004). Akt promotes survival by direct phosphorylation of apoptotic regulators, increased cell cycle progression, decreased transcription of pro-apoptotic genes through inhibition of forkhead transcription factors, altered metabolism, or changes in the translation of messenger RNAs that ultimately control cell death (Wendel *et al.*, 2004). The present data showed that ceramide inhibited the activation of Akt detected by decreasing levels of phosphorylation, leading to Bad dephosphorylation. Upon dephosphorylation, Bad promotes cell death by interacting with anti-apoptotic Bcl-2 such as Bcl-xL, which allows the multidomain pro-apoptotic Bcl-2 family members Bax and Bak to aggregate and causes release of apoptogenic molecules (Gross *et al.*, 1999; Zhang *et al.*, 2000; Kuwana and Newmeyer, 2003) Thus, inhibition of Akt pathway may an important mechanism for apoptosis induced by ceramide in U373MG cells.

Ceramide-induced apoptosis is associated with mTOR downstream protein expression. Phosphorylation and activation of S6K by mTOR phosphorylates ribosomal protein S6, leading to the enhancement of translation of mRNAs with 5' terminal oilgopyrimidine (Park *et al.*, 2002). Phosphorylation of 4E-BP1 decreases the affinity of the protein for eIF4E, allowing eIF4F to assemble with other translation initiation factors to initiate cap-dependent translation. Conversely, cap-dependent mRNA translation is blocked after hypophosphorylation of S6K and 4E-BP. The hypophosphorylated 4E-BP1 binds eIF-4E and inhibits cap-dependent protein translation (Fingar *et al.*, 2004). Ceramide decreased levels of phospho-S6K and reduced the translation permissive γ form of 4E-BP1, indicating that ceramide-induced apoptosis in U373MG cells is associated with inhibition of cap-dependent mRNA translation. Regulation of the G1 to S transition of the cell cycle is considered to be important for control of cell proliferation. Cell cycle progression is regulated by sequential activation of cdks complexed with cyclins. Cdk-bound cyclins activate cdk, promoting cell cycle progression, while cdk inhibitors negatively regulate the kinases and cause cell cycle arrest (Ekholm and Reed, 2000). mTOR has been shown to control cell cycle progression through S6K and 4E-BP1 (Fingar *et al.*, 2004). Previous study has shown that ceramide-induced apoptosis is linked to cell cycle arrest (Kim *et al.*, 2000). Present study showed that ceramide induced G1 phase arrest with simultaneous decrease in Cdk2, Cdk4, cyclins D1 and E. We also found that ceramide inhibits phosphorylation of S6K and 4E-BP1, suggesting involvement of mTOR pathway in ceramide-regulation of cell cycle.

PP2A controls the activities of several major protein kinase families in the cell, and functions as a positive regulator of apoptosis by dephosphorylating and inactivating antiapoptotic proteins such as Bcl-2 and Akt kinase (Millward *et al.*, 1999; Janssens *et al.*, 2005; Yin *et al.*, 2006). Ceramide is known to stimulate PP2A activity through binding of its catalytic subunit (Law and Rossie, 1995). Therefore, we studied the possible involvement of PP2A in the regulation of Akt signaling in ceramide-treated U373MG cells. Inhibition of PP2A with a PP2A inhibitor, okadaic acid significantly inhibited ceramide-induced dephosphorylation of Akt and S6K, and reversed ceramide-suppressed expression of γ form of 4E-BP1 protein level. Thus, these results demonstrate that PP2A is involved in the induction of apoptosis via inhibition of Akt/mTOR pathway.

Overall, our results indicate the presence of multiple pathways for the ceramide-induced apoptosis in U373MG cells. Ceramide-induced apoptosis in glioblastoma cancer cells is associated with mitochondrial signaling which involves decrease in mitochondrial membrane potential, and reduction of antiapoptotic Bcl-2 and phospho-Akt levels. In addition, PP2A inhibits Akt activity and promotes the translational and growth repressor functions of 4E-BP1.

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