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Role of MAPK in ceramide-induced cell death in primary cultured astrocytes from mouse embryonic brain

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Abstract

Ceramide has been suggested to be involved in a variety of cell signaling pathways including mitogen-activated protein kinases. The present study was undertaken to examine whether mitogen-activated protein kinases are involved in ceramide-induced cell death in primary cultured astrocytes isolated from mouse embryonic brain. Ceramide induced apoptotic death in a dose- and time-dependent manner. Ceramide-induced cell death was dependent on generation of reactive oxygen species. Ceramide caused activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). Pharmacological inhibitors of these kinases prevented ceramide-induced cell death. Ceramide induced an increase in Bax expression, depolarization of mitochondrial membrane potential, and caspase activation. Such effects were inhibited by ERK and JNK inhibitors. These results suggest that activation of ERK and JNK is involved in ceramide-induced apoptosis through a mitochondria-dependent pathway in astrocytes.

Keywords: Ceramide; Apoptosis; MAPK; Bax; Caspase activation; Astrocytes

1. Introduction

Ceramide has been implicated to play an important role in the cell signaling pathway involved in cell growth, proliferation, apoptosis, and other cell responses (Kolesnick and Kronke, 1998; Luberto and Hannun, 1999). Many inducers of apoptosis such as TNFα, Fas ligand, serum deprivation, γ-radiation, chemotherapeutic agents, and ischemia/reperfusion regulate one or more enzymes of ceramide metabolism leading to the accumulation of ceramide (Dbaibo and Hannun, 1998; Pena et al., 1997; Pettus et al., 2002). Ceramide has also been suggested to be involved in a variety of neurological disorders such as epilepsy, Alzheimer's and Parkinson's diseases, and cerebral ischemia (Chen et al., 2001; France-Lanord et al., 1997; Herr et al., 1999; Rose et al., 1999).

Although ceramide induces cell death in various cell types, its underlying mechanism is not clear. Many studies have reported that ceramide induces apoptosis through a mechanism dependent on caspase activation (Dbaibo and Hannun, 1998; Movsesyan et al., 2002; Willaime et al., 2001; Yoshimura et al., 1998a). However, other studies have shown that ceramide induces necrotic cell death (Arora et al., 1997; Gerritsen et al., 1998; Guo et al., 1999) or prevents apoptosis (Irie and Hirabayashi, 1999; Ito and Horigome, 1995; Schwarz and Futerman, 1997). Interestingly, ceramide induces not only apoptosis but also caspase-independent nonapoptotic cell death depending on the cell type. For instance, Mengubas et al. (1999) reported that ceramide triggers cell death by a caspase-dependent apoptosis in Jarkat leukemia cells, but it kills normal human T lymphocytes by a caspase-independent non-apoptotic mechanism. Caspase-independent cell death by ceramide was shown in leukemic U937 cells (Belaud-Rotureau et al., 1999) and glial cells (Mochizuki et al., 2002).

Members of the mitogen-activated protein kinase (MAPK) family constitute important mediators of signal tranduction pathways that serve to coordinate the cellular response to a variety of extracellular stimuli. Based on structural differences, the MAPK family has been classified into three major subfamilies: the extracellular signal-regulated kinase (ERK),

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the c-Jun N-terminal kinase (JNK), and the p38 kinase. The ERK pathway is mainly induced in response to mitogens and growth factors and plays a major role in regulating cell growth, survival, and differentiation (Cobb, 1999; Xia et al., 1995). In contrast, JNK and p38 pathways are activated in response to chemical and environmental stress. Their activations are most frequently associated with induction of apoptosis (Davis, 2000; Xia et al., 1995).

Although involvement of MAPK subfamilies in ceramide-induced cell death have been reported in various cell types including neuronal cells (Verheij et al., 1996; Willaime et al., 2001; Willaime-Morawek et al., 2003), their roles in ceramide-induced astrocyte death are not clear. The present study was therefore undertaken to examine whether ceramide-induced cell death is attributed to apoptosis in astrocytes and, if so, whether MAPK subfamilies are involved in the apoptosis.

2. Materials and methods

2.1. Chemicals

C_2-ceramide, propidium iodide, fibroblast growth factor (FGF), epidermal growth factor (EGF), Hoechst 33258, superoxide dismutase (SOD), catalase, *N*-benzyloxycarbonyl-Val-Ala-Asp (*O*-Me) fluoromethyl ketone (z-DEVD-FMK), poly-D-lysine, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). B-27 supplement was purchased from Invitrogen Corporation (Grand island, NY, USA). Tween 20, PD98059, U0126, and SP600125 were purchased from Calbiochem (California, USA). 3,3′-Dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR, USA). Antibodies of MAPK subfamilies and Bax were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals were of the highest commercial grade available.

2.2. Primary culture of mouse embryonic brain

Astrocytes were isolated from 13-day-mouse embryos and cultured by the method of Renau-Piqueras et al. (1989) with some modification. Briefly, the cerebral cortex obtained under sterile conditions were dissected free of meninges and dissociated mechanically by pipetting 10 times with a 10-ml pipette in Neurobasal™ Media (NB media, Invitrogen Corporation, NY, USA) containing trypsin (50 μg/ml). The cell suspension was vortexed for 1 min in NB media and plated on 100-mm culture dishes (2.0×10^6 cells per dish) coated with poly-D-lysine in NB media containing essential factors (50 ng/ml FGF, 50 ng/ml EGF, 2 mM L-glutamine, and B-27). The culture medium was changed every 3 days of culture. Cultures were grown in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The purity of primary cultured astrocytes was assessed by immunocytochemistry using a primary antibody of glial fibrillary acidic protein (Dako A/S, Denmark). Contamination by neurons was assessed by immuncytoochemistry using a monoclonal antibody of β-tubulin (Sigma–Aldrich Chemical, MO, USA). Approximately 95% of cells were astrocytes and contamination by neurons was not observed. Cells were used for experiments after 10 days of culture in which cells were grown to confluence.

Glial intermediate filaments (IF) have been known to be most characteristic morphologic feature of normal astrocytes both in vivo and in vitro, and in reactive astrocytic gliosis (Lazarides, 1982; Trimmer et al., 1982). Glial fibrillary acidic protein is the major component in glial IF and has been used as a specific marker for glial cells, particularly in development of astrocytes in vivo, as well as in primary cultures (Bock et al., 1977; Trimmer et al., 1982). Glial fibrillary acidic protein-positive cells have been reported to have normal astrocyte functions including modulation of neuronal proliferation (Gomes et al., 1999; Yoshimura et al., 1998b).

2.3. Cell viability assay

Cell viability was evaluated by a MTT assay. After washing cells, culture medium containing 0.5 mg/ml of MTT was added to each well. Cells were incubated for 2 h at 37° C, the supernatant was removed and the formazan crystals formed in viable cells were solubilized with 110 μl of dimethyl sulfoxide. A 110 μl aliquot of each sample was then transferred to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader (Bio-Tek instrument, Winooski, VT, USA).

2.4. Measurement of apoptosis

2.4.1. Cytochemical staining

Cells were grown on 22-mm glass coverslips in 12-well plates. After treatment with ceramide, cells were washed twice and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at 4 °C. The fixed cells were stained with 10 μg/ml Hoechst 33258 for 15 min at 37 °C. Morphological evaluation of nuclear condensation and fragmentation was performed immediately after staining using a visualized fluorescent microscope (Leica, Wetzlar, Germany).

2.4.2. Flow cytometric analysis

Cells were grown in six-well plates and were treated as indicated. Cells were washed in phosphate-buffered saline and fixed with cold 70% ethanol containing 0.5% Tween 20 at 4 °C. Cells were washed and resuspended in 1.0 ml of propidium iodide solution containing RNase A (10 mg/ml) and propidium iodide (50 μg/ml) and incubated for 30 min at 37 °C. Apoptosis cells were analyzed by Flow Cytometer using FL2 detector (FACsort Becton Dickinson, CA) and data were analyzed with CELLQuest Software. Cells with sub-G1 propodium iodide incorporation were considered as apoptotic. The percentage of apoptotic cells was calculated as the ratio of events on sub-G1 to events from the whole population.