Ceramide Generation in Nitric Oxide-induced Apoptosis

ACTIVATION OF MAGNESIUM-DEPENDENT NEUTRAL SPHINGOMYELINASE VIA CASPASE-3*

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Sodium nitroprusside (SNP), a NO donor, has been recognized as an inducer of apoptosis in various cell lines. Here, we demonstrated the intracellular formation of ceramide, a lipid signal mediator, in SNP-induced apoptosis in human leukemia HL-60 cells and investigated the mechanisms of ceramide generation. The levels of intracellular ceramide increased to, at most, 160% of the control level in a time- and dose-dependent manner when the cells were treated with 1 mM SNP. SNP also decreased the sphingomyelin level to ~70% of the control level and increased magnesium-dependent neutral sphingomyelinase (N-SMase) activity to 160% of the control activity 2 h after treatment. Neither acid SMase nor magnesium-independent N-SMase was affected by SNP. Caspases are thought to be key enzymes in apoptotic cell death. Acetyl-Asp-Glu-Val-Asp-aldehyde, a synthetic tetrapeptide inhibitor of caspases, inhibited magnesium-dependent N-SMase, ceramide generation, and apoptosis. Moreover, recombinant purified caspase-3 increased magnesium-dependent N-SMase in a cell-free system. These results suggest that the findings that SNP increased ceramide generation and magnesium-dependent N-SMase activity via caspase-3 are interesting to future study to determine the relation between caspases and sphingolipid metabolites in NO-mediated signaling.

Nitric oxide is a highly reactive and unstable free radical gas that can cross cell membranes easily by diffusion without depending on any release or uptake mechanisms. NO is involved in several signaling pathways related to a diverse array of cell functions. Low levels of NO constitutively produced by an endothelial nitric-oxide synthase play a physiological role such as in regulation of vasodilatation (1) and platelet aggregation (2). On the other hand, high levels of NO produced by an inducible NO synthase mainly in macrophages and neutrophils mediate cytotoxicity as the first line of self-defense against invading microorganisms (3) or tumor cells (4). Recently, NO-mediated apoptosis was reported in macrophages (5, 6), a pancreatic beta cell line (7), and mouse thymocytes (8). NO-generating compounds such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine, and S-nitroglutathione have been reported to induce apoptosis in human leukemia HL-60 and U937 cells (9–11). Although the mechanisms of NO-mediated cytotoxicity are still controversial, several possible systems described as follows have been proposed; 1) formation of iron-nitrosyl complexes with FeS-containing critical enzymes, which would cause an impairment of mitochondrial function and an energy depletion (12); 2) direct DNA damage by denaturation and cross-linking of DNA, which increase mutagenesis (13); 3) generation of peroxynitrite by reaction of NO with superoxide, which may play a significant role in the cytotoxic process (14, 15); and 4) inactivation of several antioxidant enzymes, including catalase, glutathione peroxidase, and superoxide dismutases (15, 16).

Sphingolipid metabolites including ceramide have been implicated as potential regulatory molecules in signal transductions involving cell growth, differentiation, and death. Many stresses against cell viability such as tumor necrosis factor-α, anti-Fas antibody, ionizing radiation, and serum deprivation (17, 18); anti-cancer drugs (19, 20); heat shock (21); and hydrogen peroxide (22) were reported to be accompanied by an increase in intracellular ceramide. As downstream targets of ceramide, ceramide-activated protein phosphatase (23), protein kinase Cζ (24), a ceramide-activated protein kinase (25), and the interleukin-1β-converting enzyme family of proteases called caspases (26) have been suggested. We have also demonstrated the requirement of the transcription factor component AP-1 and cytosolic translocation of protein kinases Cδ and -ε from the membrane fraction for ceramide-mediated apoptosis (27, 28).

Recently, a family of proteases known as caspases has been implicated as a common executioner of a variety of death signals. Caspase-dependent ceramide generation has been proposed in several apoptosis models (29–31), whereas Mizushima et al. (32) reported that cell-permeable ceramide induced the cleavage and activation of caspase-3. These results may indicate the activation of caspases both upstream and downstream of ceramide production, but the precise relation between the caspase cascade and ceramide generation in apoptosis remains to be clarified. NO was also reported to increase caspase-3 to induce apoptosis (33).

Here, the relation between ceramide generation and caspase-3 in apoptosis induced by NO in HL-60 cells has been investigated. We demonstrated that SNP (a NO donor) increased ceramide generation via activation of magnesium-dependent neutral sphingomyelinase (N-SMase), but not via magnesium-independent and acid SMases. The ceramide generation seemed to be mediated upstream of NO-activated caspase because the inhibition of caspase by acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) prevented NO-induced apoptosis, activation of magnesium-dependent N-SMase, and ceramide formation. Moreover, recombinant purified caspase-3 could increase N-SMase activity in a cell-free system. As far as we...
know, this is the first report suggesting the effect of caspase-3 on magnesium-dependent N-SMase.

MATERIALS AND METHODS

Cells and Reagents—Human leukemia HL-60 cells were kindly provided by Dr. M. Saito (National Cancer Institute, Tokyo, Japan). Ceramide was purchased from Matreya, Inc. [γ-32P]ATP (6000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Diacylglycerol (DAG) kinase was kindly provided by Dr. Y. Hannun (Duke University). Sodium nitroprusside was purchased from E. Merck (Darmstadt, Germany). Acetyl-Asp-Glu-Val-Asp-(4-methylcoumaryl-7-amide) (DEVD-MCA) and DEVD-CHO were purchased from the Peptide Institute (Osaka, Japan), dissolved at 10 mM in Me2SO, and stored at −80 °C. Recombinant purified caspase-3 and -6 were prepared as described (34).

Cell Culture—Human myelogenous leukemia HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in a 5% CO2 incubator. HL-60 cells in exponentially growing phase were washed in RPMI 1640 medium, resuspended in 2% serum-containing medium at a concentration of 2 × 106 cells/ml overnight, and then treated, if not described otherwise below. Viable cell numbers were assessed by the 0.025% trypan blue dye exclusion method under microscopic observation. Cell numbers and survival rates were also measured by the WST-1 assay (cell counting kit, Dojindo, Kumamoto, Japan) using a 96-well microplate reader. Reagents were applied 30 min before adding SNP because the ceramide measurement using DAG kinase was performed as described (37, 38). The solvent system used to separate ceramide phosphate was chloroform/acetone/methanol/acetic acid/H2O (10:4:3:2:1). We confirmed that DAG kinase activity was not increased during the procedure, and the amounts of phospholipid phosphate corresponded to the viable cell numbers (data not shown).

Nitrite Assay—NO undergoes a series of reactions with several molecules present in biological fluids. The final products of NO in vivo are nitrite and nitrate. The sum of nitrite and nitrate can be the index of total NO production. Nitrite, a stable NO oxidation product, was determined using the Griess reaction (nitrite/nitrate assay kit, Cayman Chemical Co., Inc., Ann Arbor, MI). Phenol red-free Dulbecco’s modified Eagle’s medium was harvested after treatment. First, nitrate was converted to nitrite utilizing nitrate reductase, and then Griess reagents were added to convert nitrite into a deep-purple azo compound. The absorbance of the azo chromophore was measured to determine the flow rate.

Flow Cytometry—Flow cytometric DNA analysis was performed for quantification of cell death by apoptosis. Due to DNA degeneration and subsequent leakage from cells (35), apoptotic cells can be detected by diminished staining with DNA-specific fluorochromes. In brief, 2 × 106 cells were harvested, washed with phosphate-buffered saline, and resuspended in phosphate-buffered saline containing 0.5% paraformaldehyde and 0.5% saponin for fixation of cells (36). The cells were then washed and resuspended in fluorochrome solution (50 μg/ml propidium iodide and 1 mg/ml RNase (Bachem California, Torrance, CA)). Red fluorescence was measured with a FACScan (Becton Dickinson Advance Cellular Biology, San Jose, CA). We could assess the number of hypodiploid cells (apoptotic cells) and cells with more than diploid DNA content (non-apoptotic cells).

Ceramide Measurement—Extraction of cellular lipids by the Bligh-Dyer method (34) and ceramide measurement using DAG kinase were performed as described (37, 38). The solvent system used to separate ceramide phosphate was chloroform/acetone/methanol/acetic acid/H2O (10:4:3:2:1). We confirmed that DAG kinase activity was not increased during the procedure, and the amounts of phospholipid phosphate corresponded to the viable cell numbers (data not shown).

Analysis of DNA Fragmentation—DNA was isolated using a GENOME kit (Bio 101, Inc., Vista, CA), electrophoresed through a 3% NuSieve agarose minigel (FMC Corp. BioProducts) in 40 mM Tris acetate and 1 mM EDTA at 50 V for 3 h, and visualized under UV light after ethidium bromide staining.
nitrite concentration at 540 nm using the plate reader.

**Sphingomyelin (SM) Quantitation**—The cells were washed with phosphate-buffered saline, seeded at 5 x 10⁵ cells/ml in RPMI 1640 medium containing 2% fetal calf serum, and labeled with [¹⁴C]choline chloride (0.1 μCi/ml) at 37 °C in 5% CO₂ for 36 h. The labeled cells were treated with 1 mM SNP for the indicated times. After harvesting the cells, the lipids were extracted by the Bligh-Dyer method (54) and applied to a Silica Gel 60 TLC plate (Whatman). Inorganic phosphate in the extract was measured to calculate phospholipid content. The TLC plate was developed in solvent containing chloroform/methanol/acetic acid/H₂O (50:30:8:5), and the bands corresponding to SM were detected with a Fuji BAS system.

**Assay Procedure for SMases**—HL-60 cells (1 x 10⁷) were harvested; washed twice with ice-cold phosphate-buffered saline; and homogenized in 0.5 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 1 mM CHAPS, 1 mM dithiothreitol, 100 mM phenylmethylsulfonyl fluoride, 0.15 units/ml aprotinin, and 50 μg/ml leupeptin and centrifuged at 10,000 x g for 10 min to separate the two phases. The clear upper phase (0.4 ml) was removed, placed in a glass scintillation vial, and counted with a scintillation counter (Packard Instrument Co.). Protein concentrations were determined using a Bio-Rad protein assay kit.

**Fluorometric Assay of DEVD-MCA Cleavage Activity**—After each treatment, the cells were homogenized in lysis buffer containing 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM trypsin, 0.15 units/ml aprotinin, and 50 μg/ml leupeptin and centrifuged at 10,000 x g for 10 min.
The supernatant was collected as an enzyme source and added to the reaction mixture (10% sucrose, 10 mM HEPES/KOH (pH 7.4), 5 mM dithiothreitol, 0.1% CHAPS, and 10 mM DEVD-MCA), followed by incubation at 25 °C for 60 min. Fluorescence was measured with a microplate reader (MTP-100F, Corona Electric, Tokyo, Japan) using 360-nm excitation and 450-nm emission filters. Concentrations of 7-amino-4-methylcoumarin liberated as a result of cleavage were calculated comparing with standard 7-amino-4-methylcoumarin solutions.

Preparation of Cell Extracts for Assay of SMase in a Cell-free System—The cells were suspended in lysis buffer containing 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 0.15 units/ml aprotinin, and 50 μg/ml leupeptin; left on ice for 20 min; passed through a 27-gauge needle; and then centrifuged at 10,000 × g for 15 min. Protein concentrations were determined with the Bio-Rad assay.

RESULTS

SNP-induced Apoptosis in HL-60 Cells—SNP showed a time- and dose-dependent induction of apoptosis in HL-60 cells (Fig. 1, A and B). Three h after treatment with 1 mM SNP, HL-60 cells showed morphological changes (blebbing, shrinkage, and chromatin condensation) and DNA fragmentation characteristic of apoptosis (Fig. 1C). The percentage of apoptotic cells measured by flow cytometric analysis increased from 8.6 to 30.5% 4 h after treatment with 1 mM SNP. At higher concentrations, the number of apoptotic cells did not show any more increase, and necrosis was observed judging from trypan blue dye staining. After 24 h, the cell numbers decreased to ~20% of the control level (data not shown). Potassium hexacyanoferrate, which is structurally similar to SNP except for the absence of a nitroso group, did not affect cell growth at the same concentration as SNP (data not shown), suggesting that the effects of SNP on cell growth and apoptosis were due to NO generation, but not to cyanoid effects.

Increase in Intracellular Ceramide Induced by SNP—To date, our studies (37, 38) and others (17, 18) have demonstrated that ceramide, a lipid second messenger, plays an important role in regulating cell growth, differentiation, and death. To investigate the interrelation between NO and ceramide generation, we measured nitrite concentration in the culture medium after addition of C2-ceramide. We could not detect any change within 6 h, whereas SNP increased nitrite production in a time-dependent manner (Fig. 2). Although ceramide was reported to enhance the expression of inducible NO synthase in rat astrocytes (39), it may be that ceramide does not increase NO generation in HL-60 cells. To examine the possible involvement of the ceramide signaling pathway in NO-induced cell stress, we measured intracellular ceramide levels after addition of SNP. Ceramide generation measured by the DAG kinase assay method began to increase at 90 min after addition of 1 mM SNP and reached a maximum level, which was ~160% of the control level, 4 h after treatment (Fig. 3A). To justify ceramide measurement by the DAG kinase assay, we confirmed that the DAG kinase and phospholipid phosphate activities of the same numbers of cells did not change during treatment with SNP, as described under “Materials and Methods.” Concentrations higher than 1 mM SNP did not increase cera-
Ceramide levels more than 1 mM SNP, probably due to the induction of necrosis (Fig. 3B).

Sphingomyelin Hydrolysis through Magnesium-dependent N-SMase by SNP—Since there are several possible metabolic pathways as a mechanism of ceramide generation, we, first of all, measured changes in labeled SM contents caused by SNP to examine the possible involvement of SMase. SM levels decreased to 70% of the control level 2 h after treatment with 1 mM SNP and then returned to the control level by 6 h (Fig. 3C). We examined the activities of three different types of SMases, which have been reported to be involved in SM hydrolysis (37). As shown in Fig. 3D, magnesium-dependent N-SMase (basal specific activity = 1.08 nmol/mg of protein/h) increased to 156 ± 18% of the control level 2 h after treatment with SNP and returned to the control level by 6 h. The activity of magnesium-independent N-SMase (basal specific activity = 0.65 nmol/mg of protein/h) did not change following treatment with SNP up to 6 h. The activity of acid SMase (basal specific activity = 9.72 nmol/mg of protein/h) slightly decreased, but did not increase after treatment with SNP. The biological meaning of this decrease is unclear at present. These results suggest that ceramide generation induced by SNP results from SM hydrolysis via the increase in magnesium-dependent N-SMase activity.

Increase in DEVD-MCA Cleavage Activity and Its Involvement in SNP-induced Apoptosis—Since many stresses are reported to activate caspase-3 as an executioner of apoptosis, we investigated whether SNP-generated NO activates caspase-3. The activity of caspase-3 was assessed by measuring the proteolytic cleavage of DEVD-MCA, a fluorogenic substrate of caspase-3, and it increased after treatment with SNP (Fig. 4A). The activities 4 h after treatment with 1 mM SNP were 88 and 134 pmol/mg of protein/min, respectively, compared with the control level of 30 pmol/mg of protein/min. SNP increased the caspase-3 activities in a time-dependent manner. DEVD-CHO (200 μM), an inhibitor of caspase-3, completely inhibited the increase in DEVD-MCA cleavage activity induced by SNP (Fig. 4B). Moreover, SNP-induced apoptotic cells markedly decreased from 31 to 11% upon addition of DEVD-CHO (Fig. 4C). These results suggest that the activation of caspase-3 is required to induce HL-60 cell apoptosis by SNP.

Inhibitory Effects of DEVD-CHO on Ceramide Generation and Activation of Magnesium-dependent N-SMase—We investigated whether ceramide generation by magnesium-dependent N-SMase was upstream or downstream of caspase-3 in SNP-induced apoptosis. We examined the effects of DEVD-CHO on ceramide generation and the increase in magnesium-dependent N-SMase activity induced by SNP. The SNP-induced increase in intracellular ceramide, which showed the maximum 4 h after treatment, was completely inhibited by preincubation with 200 μM DEVD-CHO, as shown in Fig. 5A. By the same procedure, the activation of magnesium-dependent N-SMase (146 ± 16% of the control level 4 h after treatment with SNP) was also completely inhibited (Fig. 5B). These results show that the protease including DEVDAse is activated upstream of ceramide generation by magnesium-dependent N-SMase for NO-induced apoptosis.

Activation of Magnesium-dependent N-SMase by Purified Caspase-3 in a Cell-free System—It became clear that the caspase inhibitor DEVD-CHO inhibits ceramide generation and magnesium-dependent N-SMase activity in the process of NO-induced apoptosis. Although it is the nature of things that SMase can generate ceramide through hydrolysis of SM, there was no direct evidence that caspase-3 could increase the activity of magnesium-dependent N-SMase. Therefore, we used recombinant purified caspase-3 from Escherichia coli transfected with caspase-3 cDNA, which did not have any types of SMase activity in itself (data not shown), but which could increase magnesium-dependent N-SMase activity with the cell extracts. Addition of recombinant purified caspase-3 (600 ng) to the cell extracts (250 μg) induced a 3-fold increase in magnesium-dependent N-SMase activity (Fig. 6). Since we previously showed that caspase-3 activates caspase-6 in Fas-induced apoptosis (40), we examined whether caspase-6 increased magnesium-dependent N-SMase activity, but this activity did not increase even in the presence of the cell extracts (data not shown). Our results suggest the direct role of caspase-3 in activating magnesium-dependent N-SMase and ceramide generation in NO-induced apoptosis.

DISCUSSION

The sphingomyelin cycle and ceramide generation were first discovered in cell differentiation of HL-60 cells in response to 1α,25-dihydroxyvitamin D3 (37, 38), and recent studies have shown that many cell types respond to diverse stresses with ceramide generation (17, 18). Recently, NO has been reported to be related to induction of apoptosis in various cell lines, including human leukemia HL-60 cells (5–11). In this report,
In this study, N-SMase was activated by caspase-3 because DEVD-CHO, a caspase-3 inhibitor, blocked both SNP-induced SMase activation and ceramide generation (Figs. 4 and 5). To confirm the more direct effect of caspase-3 on SMase, recombinant purified caspase-3 was added to the cell extracts. In this cell-free system, we could detect the increase in magnesium-dependent N-SMase activity in the presence of recombinant caspase-3 (Fig. 6), whereas recombinant caspase-6 could not increase SMase activity (data not shown). Judging from the present data, the NO-generated ceramide signal is the downstream target of caspase-3. However, at present, in terms of the mode of action of caspases on SMase, it is unknown whether caspase-3 cleaved plausible pro-SMase, cleaved and inactivated the SMase inhibitor, or activated the SMase activator. As far as we know, this is the first evidence showing the activation of magnesium-dependent N-SMase via caspase-3, whereas activation of acid SMase activity has been shown to occur in response to caspases (51). In terms of the relation of the caspase cascade and ceramide in induction of apoptosis, many previous reports suggest that ceramide is upstream of the caspase cascade (26). Our data and others related to REAPER in Drosophila (29), which showed that caspase could enhance ceramide generation in vivo, may suggest a new function of ceramide as a modulator of the caspase cascade. At present, unfortunately neither this idea nor the indispensability of caspase-generated ceramide in NO-induced apoptosis can be demonstrated because we do not have any biochemical tool to inhibit directly the activity of N-SMase to generate ceramide.

In contrast to our data, exposure to NO donors such as S-nitroso-N-acetylpenicillamine and SNP or activation of NO synthase was reported to inhibit apoptosis in T lymphocytes and human umbilical vein endothelial cells (52, 53) because NO induced cGMP-dependent or direct inhibition of caspase-3 through protein S-nitrosylation. These data seem inconsistent with our data showing that NO could induce apoptosis by increasing ceramide generation through caspase-3 activation. The discrepancy in the NO effect on apoptosis may be due to differences in intensity and duration of NO exposure and kinds of cells. Indeed, we observed that low concentrations of SNP <100 μM showed a protective effect against serum deprivation-induced apoptosis in HL-60 cells. Induction of apoptosis and an increase in intracellular ceramide were generally observed upon treatment with >250 μM SNP. Caspase-3 activity measured by cleavage of DEVD-MCA was also enhanced at higher (but not lower) concentrations of SNP.

NO could generate peroxynitrite by reacting with superoxide anion. NO could also modulate endogenous antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutases (15, 16). Since intracellular ceramide was reported to be increased by hydrogen peroxide (22), changing of redox status may be another one of the mechanisms regulating apoptotic signals between NO and ceramide generation. Finally, it remains to be elucidated in the future how NO activates caspase-3 and what are the mechanisms of activation of magnesium-dependent N-SMase to understand the biochemical and physiological implications of the ceramide signal in NO-induced apoptosis.

REFERENCES

6. Y. Takeda and T. Okazaki, unpublished data.
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