Ceramide-induced Intracellular Oxidant Formation, Iron Signaling, and Apoptosis in Endothelial Cells

PROTECTIVE ROLE OF ENDOGENOUS NITRIC OXIDE*

Received for publication, January 28, 2004, and in revised form, March 31, 2004
Published, JBC Papers in Press, April 21, 2004, DOI 10.1074/jbc.M400977200

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Sphingolipid ceramide (N-acetylsphingosine), a bioactive second messenger lipid, was shown to activate reactive oxygen species (ROS), mitochondrial oxidative damage, and apoptosis in neuronal and vascular cells. The proapoptotic effects of tumor necrosis factor-α, hypoxia, and chemotherapeutic drugs were attributed to increased ceramide formation. Here we investigated the protective role of nitric oxide (NO) during hydrogen peroxide (H₂O₂)-mediated transferrin receptor (TfR)-dependent iron signaling and apoptosis in C₂-ceramide (C₂-cer)-treated bovine aortic endothelial cells (BAECs). Addition of C₂-cer (5–20 μM) to BAECs enhanced NO generation. However, at higher concentrations of C₂-cer (≥20 μM), NO generation did not increase proportionately. C₂-cer (20–50 μM) also resulted in H₂O₂-mediated dichlorodihydrofluorescein oxidation, reduced glutathione depletion, aconitase inactivation, TfR overexpression, TfR-dependent uptake of ⁵⁵Fe, release of cytchrome c from mitochondria into cytosol, caspase-3 activation, and DNA fragmentation. N⁶-Nitro-L-arginine methyl ester (l-NAME), a nonspecific inhibitor of nitric oxide synthases, augmented these effects in BAECs at much lower (i.e. nonapoptotic) concentrations of C₂-cer. The 26 S proteasomal activity in BAECs was slightly elevated at lower concentrations of C₂-cer (≤10 μM) but was greatly suppressed at higher concentrations (>10 μM). Intracellular scavengers of H₂O₂, cell-permeable iron chelators, anti-TfR receptor antibody, or mitochondria-targeted antioxidant greatly abrogated C₂-cer-and/or l-NAME-induced oxidative damage, iron signaling, and apoptosis. We conclude that C₂-cer-induced H₂O₂ and TfR-dependent iron signaling are responsible for its prooxidant and proapoptotic effects and that NO exerts an antioxidative and cytoprotective role.

Ceramide belongs to a group of naturally occurring sphingolipid second messenger molecules that is formed by sphingomyelin-catalyzed hydrolysis of sphingomyelin (1–3). There is growing interest on the potential role of ceramide-mediated proapoptotic cell signaling in response to treatment with reactive oxygen species (ROS)† (e.g. superoxide and hydrogen peroxide) and other proapoptotic stress factors, including inflammatory cytokines such as tumor necrosis factor-α and lipopolysaccharide, hypoxia, and chemotherapeutic drugs (4–6). Exogenous treatment of endothelial cells and neuronal cells with ceramide also caused oxidative stress and activation of caspase-3 leading to apoptosis (7–12). Ceramide treatment has been shown to trigger both nitric oxide (NO) and superoxide generation in endothelial cells (13–15). The relative ratio between superoxide and NO determine the ultimate cytotoxicity in ceramide-treated cells (14). Exposure of endothelial cells to low concentrations of ceramide (~5 μM) causes an increase in NO formation due to Ca²⁺ activation and translocation of endothelial nitric-oxide synthase (eNOS) (14, 15). At higher concentrations (>20 μM) ceramide treatment induced ROS formation in cells (13, 14).

Previously, we have shown that treatment of endothelial cells with H₂O₂ induced intracellular oxidative stress, iron signaling, and apoptosis through stimulation of the transferrin receptor (TfR) (16, 17). More recently, we showed that NO mitigates peroxide-induced oxidative stress and apoptosis by inhibiting TfR-mediated iron signaling (18). Because ceramide induces both NO and ROS at different concentrations (14), we decided to explore in detail the effect of ceramide on oxidative cell signaling. In this study, we investigated the effects of C₂-ceramide or N-acetylsphingosine, a cell-permeable ceramide analog, and C₂-dihydroceramide, an inactive negative control for C₂-ceramide (Fig. 1), on intracellular oxidant generation and iron signaling. The protective role of endogenously generated NO (19, 20) on ceramide-induced apoptosis was determined. Several fluorescence probes were used to monitor superoxide-, iron-, and peroxide-induced oxidant generation and NO/oxynogen interaction. Results indicate that C₂-ceramide induces ROS- and TfR-mediated iron signaling that are responsible for C₂-cer-induced proapoptotic effects and that NO

† The abbreviations used are: ROS, reactive oxygen species; BAEC, bovine aortic endothelial cell; C₂-cer, C₂-ceramide; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; DCFA, 2′,7′-dichlorodihydrofluorescein diacetate; DAF-2-DA, diaminofluorescein diacetate; Fe/TBAP, Fe(III)-tetrais(4-benzoic acid) porphyrin; GSH, reduced glutathione; Tf, transferrin; TfR, transferrin receptor; DHE, dihydroethidium; Mito-Q, mixture of mitoquinone and mitoquinol conjugated to triphenyl phosphonium ion; l-NAME, N⁶-nitro-L-arginine methyl ester; TUNEL, terminal deoxynucleotidyltransferase-mediated nick-end labeling; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; DPBS, Dulbecco’s PBS; eNOS, endothelial nitric-oxide synthase; FITC, fluorescein isothiocyanate; HE, hydroethidium; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[3(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ac-, acetylsalicylic acid; pNA, p-nitroanilide; HPLC, high-performance liquid chromatography; OPA, o-phthalaldehyde.
Ceramide-induced Oxidative Iron Signaling and Apoptosis

Ceramide, and the inactive C2-ceramide analog.

EXPERIMENTAL PROCEDURES

Materials—C2-ceramide and C2-dihydroceramide were purchased from BIOMOL. N2′-nitro-l-arginine-methyl ester (l-NAME) was from Alexis. Glutathione monooethyl ester and desferal (or desferrioxamine) were obtained from Sigma. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) were from Molecular Probes. Fe(III)-tetrakis-(4-benzoic acid) porphyrin (FeTBAP) was synthesized according to a published method (21). Mito-Q was synthesized according to the published procedure (22), and monoclonal antibody 426, against human TfR (lGα class), was obtained from Dr. Ian Trowbridge (Salk Institute, San Diego, CA).

Endothelial Cell Culture—Bovine aortic endothelial cells (BAECs) were obtained from the Clonetics Corp. Cells were obtained at the third passage, transferred to 75-cm2 cell culture flasks (Costar, Cambridge, MA), and grown to confluence (5.2 × 106 cells/75 cm2) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), l-glutamine (4 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml), incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Cells were passaged as described by Balla et al. (23) and used between passages 4 and 12. On the day of the treatment, the medium was replaced with DMEM containing 2% FBS, which contains ~25–30 μg of transferrin/ml. The above experimental conditions were used in all the experiments performed in this study.

Measurement of Oxidative Stress—The level of intracellular oxidative production was estimated by oxidations of DHE and DCFH. Following treatment of BAECs with C2-ceramide, the medium was aspirated, and cells were washed with DPBS and incubated in 2 ml of fresh culture medium without FBS. DHE and DCFH-DA were added at a final concentration (10 μM) and incubated for 20 min, respectively. The cells were then washed twice with DPBS and maintained in 1 ml of culture medium. Fluorescence was monitored using a Nikon fluorescence microscope equipped with rhodamine and FITC filters. The intensity values were calculated using Metamorph software.

Measurement of 55Fe Uptake—55Fe uptake into the cells was measured as described previously (16, 24). Briefly, 0.2 μCi/ml 55Fe (ferric chloride) was added to the medium for 0–8 h, and its levels were measured as a function of time. Cells were washed twice with DPBS and lysed with PBS containing 0.1% Triton X-100, and the cell lysate was counted in a beta counter.

Western Blotting of TfR, PARP, Hsp-70, and Bcl-2—Western blot analysis. Proteins were resolved on SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were washed with TBS (140 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing 0.1% Tween 20 (TBST) and 5% skim milk to block the nonspecific protein binding. Membranes were incubated with 1 μg/ml mouse anti-human transferrin receptor monoclonal antibody (Zymed Laboratories Inc., San Francisco, CA), mouse anti-bovine poly(ADP-ribose) polymerase (PARP) monoclonal antibody (Zymed Laboratories Inc.), mouse anti-human Hsp-70 antibody (Zymed Laboratories Inc.) or hamster anti-human Bcl-2 monoclonal antibody (BD Pharmingen) in TBST 2 h at room temperature, washed 5 times, and then incubated with goat anti-mouse IgG-horseradish peroxidase-conjugated secondary antibody for TfR, PARP, Hsp-70, and mouse anti-hamster (1:5,000) for Bcl-2 for 1.5 h at room temperature. The band was detected using the ECL method (Amersham Biosciences).

Mitochondrial Cytochrome c Release—The release of mitochondrial cytochrome c from the cytosol to the cytosol (26) was measured according to the methods described previously (17, 25). Briefly, BAECs were washed with DPBS and homogenized in PBS supplemented with 40 μg/ml saponin. Lysate was centrifuged at 750 × g for 10 min and followed by 12,000 × g for 20 min. The supernatant was used as the cytosolic fraction to measure the released cytochrome c into the cytosol by Western blot analysis using a mouse anti-cytochrome c antibody (BD Pharmingen). Detection was by horseradish peroxidase-conjugated goat anti-mouse antibody using the ECL method.

Measurement of Caspase Activities—Caspolyptic enzymatic activities of caspase-3, caspase-6, caspase-8, and caspase-9 were measured as described previously (26). Briefly, cells were washed twice with DPBS following treatment with C2-ceramide and then lysed with 50 μl HEPES buffer (pH 7.4) containing 5 mM CHAPS and 5 mM diithiothreitol. After cytosolic fraction was taken by centrifugation at 12,000 × g for 30 min, the activities of caspase-3, caspase-6, caspase-8, and caspase-9 were measured using the substrates of ac-DEV-D-pNA (acetyl-Asp-Glu-Val-Asp-pNA), ac-VEID-pNA (acetyl-Val-Glu-Asp-Glu-pNA), ac-IETD-pNA (acetyl-Ile-Glu-Thr-Asp-pNA), and ac-LEHD-pNA (acetyl-Leu-Glu-His-Asp-pNA) respectively. The absorbance at 405 nm of the released pNA was monitored in a spectrophotometer and quantitated using a pNA standard.

Measurement of Apoptosis by TUNEL Assay—The terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay was performed for microscopic detection of apoptosis (26). This assay is based on labeling of 3′ free hydroxyl ends of the fragmented DNA with fluorescein-dUTP catalyzed by terminal deoxynucleotidyl transferase. Procedures were followed according to a commercially available kit (ApoAlert) from Clontech. Apoptotic cells exhibit a strong nuclear green fluorescence that can be detected using a standard fluorescein filter (520 nm). The areas of apoptotic cells were quantitated using fluorescent microscopy equipped with rhodamine and FITC filters. The quantification of apoptosis was performed using the Metamorph image analysis package.

Measurement of Intracellular NO—Intracellular NO levels were measured using a DAF-2 fluorescent probe (27, 28). The treated cells were washed with DPBS and incubated in 2 ml of fresh culture medium without FBS. DAF-2 was added at a final concentration of 10 μM, and the cells were incubated for 20 min. The cells were washed twice with DPBS and maintained in 1 ml of culture medium. NO levels were determined with a fluorometer using 1 mM hydroxylamine as a standard.

Measurement of Aconitase Activity—The activity of aconitase was measured by HPLC as the peak of the aconitase activity at 240 nm. An extinction coefficient for citraconate of 3.6 mM−1 cm−1 at 240 nm was used (29).

Measurement of Glutathione—The level of glutathione (GSH) was measured by HPLC as the peak of glutathione (GSH) adduct at pH 8.0 containing 100 μM diethylenetriaminepentaacetic acid, and 5 mM citrate in PBS. The activity of aconitase was measured in 100 μM Tris-HCl (pH 8.0) containing 100 μM m-nitrotrisodium isocitrate. An extinction coefficient for citraconate of 3.6 mM−1 cm−1 at 240 nm was used (29).

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Measurement of Aconitase Activity—BAECs were washed twice with cold DPBS and lysed with buffer containing 0.2% Triton X-100, 100 μM diethylenetriaminepentaacetic acid, and 5 mM citrate in PBS. The activity of aconitase was measured in 100 μM Tris-HCl (pH 8.0) containing 100 μM m-nitrotrisodium isocitrate. An extinction coefficient for citraconate of 3.6 mM−1 cm−1 at 240 nm was used (29).

Measurement of Glutathione—The level of glutathione (GSH) was measured by HPLC as the peak of glutathione (GSH) adduct at pH 8.0 (30). BAECs were washed twice with DPBS, suspended in 250 μl of PBS, and lysed by sonication. After centrifugation at 10,000 × g for 2 min, the supernatant was derivatized by incubation for 30 min at room temperature with OA. An aliquot of sample was injected onto a Kromasil C-18 column and eluted isocratically with a mobile phase consisting of 150 mM sodium acetate:methanol (91:8.5, v/v). The OPA-GSH adduct was monitored using a fluoresceine detector operating at excitation and emission wavelengths at 250 and 410 nm, respectively. The levels of intracellular GSH were quantitated using a GSH solution as a standard.

Proteasome Function Assays—For 26 S proteasome, proteasomal activity was measured as previously reported (31, 32). Briefly, cells were washed with buffer 1 (50 mM Tris, pH 7.4/2 mM dithiothreitol/5 mM...
MgCl₂/2 mM ATP) and homogenized with buffer I containing 250 mM sucrose. Twenty micrograms of 10,000 g supernatant was diluted with buffer I to a final volume of 1 ml. The fluorogenic proteasome substrates SucLLVY-AMC (chymotrypsin-like) and Z-Leu-Leu-Lys-7-amido-4-methylcoumarin (trypsin-like) were added in a final concentration of 100 and 80 mM, respectively. Proteolytic activity was measured by monitoring the release of the fluorescent group 7-amido-4-methylcoumarin (excitation, 380 nm; emission, 460 nm).

**RESULTS**

Ceramide Induces Intracellular Superoxide and Hydrogen Peroxide—Intracellular ROS levels were measured in BAECs treated with different concentrations of C₂-ceramide for different time periods. The oxidation of DCFH, a nonfluorescent probe, to a fluorescent dichlorofluorescein (DCF) was used to measure intracellular H₂O₂-derived oxidants. Although H₂O₂ itself does not react with DCFH to form DCF, it was proposed that intracellular peroxidases or redox-active metal ions could catalyze the oxidation of DCFH to DCF in the presence of H₂O₂ (17, 33). Results show that C₂-ceramide induced a dose- and time-dependent increase in DCF staining (Fig. 2, A–C). DCF fluorescence was noticeable in cells treated with 20 μM ceramide and reached a maximum in cells exposed to 50 μM C₂-ceramide for 8 h (Fig. 2, B and C). The oxidation of the probe gradually increased over a 2- to 8-h time period in cells treated with 50 μM C₂-ceramide. Incubation with an inactive form of ceramide, C₂-dihydroceramide, lacking a double bond did not induce any appreciable DCF green fluorescence (Fig. 2, A and B). These results suggest that the active C₂-ceramide induces intracellular oxidant generation as detected by DCF fluorescence.

Next, we determined the effect of C₂-cer in cells treated with dihydroethidium (DHE), a fluorescent probe that reacts with superoxide to form a characteristic red fluorescence. Recent reports indicate that superoxide anion reacts with dihydroethidium to form a product that is distinctly different from ethidium (34). As shown in Fig. 2 (D–F), there was a dose- and time-dependent increase in the intensity of red fluorescence with ceramide treatment, indicative of enhanced superoxide.

Fig. 2. Ceramide-induced superoxide and H₂O₂ generation in endothelial cells using hydroethidine and dichlorodihydrofluorescein probes. A, BAECs were treated with various concentrations of C₂-ceramide (a–d) or 50 μM of C₂-dihydroceramide (e) for 8 h. Also cells were treated with 50 μM C₂-ceramide for different time periods (f–j). After the treatments the medium was aspirated, and the cells were washed twice with DPBS and subsequently incubated with 10 μM DCFH-DA (A) or 10 μM dihydroethidium (D) for 20 min. The cells were then washed with DPBS and maintained in 1 ml of DMEM. The green fluorescence characteristic of DCF (A–C) and red fluorescence caused by ethidium (D–F) were measured using fluorescein isothiocyanate and rhodamine filters, respectively, in a Nikon fluorescence microscope. The data shown are representative of three independent experiments.
Ceramide-induced Oxidative Iron Signaling and Apoptosis

Ceramide-induced Oxidative Stress and Apoptosis—The effects of different antioxidants and iron chelators in C2-ceramide-treated cells were investigated. BAECs were pretreated for 2 h with GSH ester (5 mM), FeTBAP (25 mM), desferal (20 mM), or anti-TfR antibody (12 µg/ml, 42/6, IgA class, which specifically binds to the extracellular domain of the transferrin receptor and inhibits receptor endocytosis) prior to treating cells with 50 µM C2-ceramide for 8 h. These agents inhibited ceramide-induced DCFH oxidation (Fig. 5, A and B) and caspase-3 activation (Fig. 5D). Pretreatment with a mitochondria-targeted antioxidant (e.g., Mito-Q) showed similar inhibitory effects, suggesting that mitochondrial generation of ROS is responsible for ceramide-mediated oxidative stress and apoptosis (Fig. 5C). Under these conditions, these antioxidants and iron chelators also inhibited ceramide-induced DNA fragmentation as shown by TUNEL staining (Fig. 5D).

Effect of C2-ceramide on Intracellular 'NO Generation in Endothelial Cells—At low concentrations (5–20 µM), ceramide enhanced 'NO release in BAECs (15). Thus, intracellular 'NO levels were monitored using DAF-2 fluorescence. It has been shown that DAF-2 forms a fluorescent triazolo-type product in the presence of an oxidant derived from 'NO and molecular oxygen (28). We observed a dose- and time-dependent increase in DAF-2 fluorescence in endothelial cells treated with C2-ceramide, but during longer exposure time there was a disproportionate increase in DAF-2 fluorescence that started to plateau in cells treated with 50 µM C2-cer (Fig. 6, A and B). Ceramide-induced intracellular generation of 'NO was further confirmed using a nitric-oxide synthase inhibitor, L-NAME. Pretreatment of L-NAME for 2 h before the addition of 50 µM C2-ceramide for 8 h inhibited ceramide-induced DAF-2 fluorescence in a dose- and time-dependent manner (Fig. 6, C–F).

Fig. 3. The effect of C2-ceramide on transferrin receptor levels and iron uptake in endothelial cells. BAECs were treated with different concentrations of C2-ceramide (A) and for different time periods either with 50 µM C2-ceramide or 50 µM C2-dihydroceramide (B) and transferrin receptor levels were measured by Western analysis using a monoclonal anti-TfR antibody. C, BAECs were treated with 50 µM C2-ceramide for various time periods along with 0.2 µCi/ml 55Fe, and the uptake of the labeled iron was measured as described under “Experimental Procedures.” Data shown are representative of three separate experiments. *, significantly different (p < 0.05) compared with untreated conditions.

Ceramide-induced oxidative stress and iron signaling in endothelial cells—Fig. 5. Oxidative stress and apoptosis in endothelial cells. C2-ceramide for 8 h caused a dose-dependent increase in the release of cytochrome c from the mitochondria into the cytosol. Incubation of BAECs with C2-ceramide for 8 h caused a dose-dependent increase in the release of cytochrome c into the cytosol (Fig. 4A). BAECs treated with 50 µM of C2-ceramide induced a decrease in the anti-apoptotic protein Bcl-2 located on the outer membrane of the mitochondria (Fig. 4B) as well as in the Hsp-70 protein levels (Fig. 4B). We then identified the active caspase family (caspases 3, 6, 8, and 9) that was activated during ceramide-induced apoptosis. As shown in Fig. 4, only the activity of the effector caspase-3 began to increase as early as 4 h in ceramide-treated cells, reaching a peak value by 8 h. In addition, the increase in caspase-3 activity was detected in cells only when the concentration of C2-ceramide exceeded 20 µM (Fig. 4C). In contrast, the caspase-3 activation was not evident in BAECs treated with an inactive analog of C2-ceramide, namely C2-dihydroceramide (Fig. 1). Although there was a marginal increase in the activation of caspase-6 and caspase-8 (Fig. 4, E and F), their significance in C2-ceramide-induced apoptosis was not apparent, because their activities became noticeable only at 16 h; at this time point, however, the execution of DNA fragmentation had already occurred (Fig. 4H). The marginal increase observed in the caspase-9 activity (Fig. 4G) by 4-h treatment with C2-ceramide would likely activate the caspase-3 activity, initiating a possible feedback loop that further increased their individual activities.

The time-dependent increase in caspase-3 activity was further correlated with its downstream target, namely, poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 4H), which is responsible for DNA fragmentation. The PARP cleavage started to appear at 4 h of treatment with C2-ceramide and significantly increased at 8 and 16 h of incubation with C2-ceramide. The inactive PARP precursor protein (~116 kDa) was cleaved to form a ~85-kDa active fragment (Fig. 4H). This active fragment of PARP translocates into the nucleus and cleaves the DNA. Finally, a dose-dependent increase in DNA fragmentation was shown by TUNEL staining (Fig. 4I) wherein cells were treated with different concentrations of C2-ceramide (0–50 µM) for a period of 8 h. As shown, C2-ceramide treatment increased the TUNEL-positive staining in cells, from 1.5% (0 µM) to 48.4% (50 µM). Treatment with C2-dihydroceramide, the inactive ceramide, did not yield DNA fragmentation as detected by TUNEL staining (Fig. 4C).

Antioxidants and Iron Chelators Mitigate Ceramide-induced Oxidative Stress and Apoptosis—The effects of different antioxidants and iron chelators in C2-ceramide-treated cells were investigated. BAECs were pretreated for 2 h with GSH ester (5 mM), FeTBAP (25 mM), desferal (20 mM), or anti-TfR antibody (12 µg/ml, 42/6, IgA class, which specifically binds to the extracellular domain of the transferrin receptor and inhibits receptor endocytosis) prior to treating cells with 50 µM C2-ceramide for 8 h. These agents inhibited ceramide-induced DCFH oxidation (Fig. 5, A and B) and caspase-3 activation (Fig. 5D). Pretreatment with a mitochondria-targeted antioxidant (e.g., Mito-Q) showed similar inhibitory effects, suggesting that mitochondrial generation of ROS is responsible for ceramide-mediated oxidative stress and apoptosis (Fig. 5C). Under these conditions, these antioxidants and iron chelators also inhibited ceramide-induced DNA fragmentation as shown by TUNEL staining (Fig. 5D).

Fig. 2. Effect of C2-ceramide, amide, did not induce red fluorescence in cells treated with HE
NOS Inhibitor Exacerbates Ceramide-induced Intracellular ROS—Ceramide, at lower concentrations (5–20 μM), induced little or no ROS (Fig. 6A) (15). However, in the presence of L-NAME, ROS generation increased in cells treated with lower concentrations of ceramide, as measured by DCF and hydroethidium fluorescence (Fig. 7, A and B). This suggests that ceramide-induced NO counteracts the effects of ROS or ROS generation induced by ceramide; at higher concentrations of C2-ceramide, ROS generation overwhelms the protective effects of NO. NOS inhibition sensitized these cells to ceramide-induced oxidative stress. This is in agreement with the data showing a disproportionate increase in NO in cells treated with C2-ceramide during prolonged incubation (Fig. 6, A and B), as compared with shorter period of incubation. These results show that L-NAME exacerbates C2-ceramide-induced ROS.

The next step was to investigate whether Tf-iron could play...
a potential role in mediating ceramide toxicity. To this end, GSH levels were measured in the presence or absence of l-NAME in C2-ceramide treated cells. Results show that l-NAME significantly enhanced the depletion of GSH levels caused by C2-ceramide (≥20 μM) (Fig. 8A). Under these conditions, the total aconitase activity was significantly lower in cells treated with both l-NAME and ceramide (Fig. 8B). The TIR levels were increased at much lower levels of ceramide in cells pretreated with l-NAME (Fig. 8C). This result prompted us to measure the iron uptake under similar conditions. We found that l-NAME treatment synergistically enhanced ceramide-mediated uptake of radiolabeled iron (Fig. 8D). At lower concentrations of C2-ceramide (5 μM), l-NAME treatment increased iron uptake compared with C2-ceramide alone (Fig. 8D). Thus, intracellular depletion of NO augments ceramide-induced iron signaling.

In addition to enhancing oxidant generation, l-NAME treatment exacerbated C2-ceramide-mediated apoptotic effects (Fig. 9A). The release of cytochrome c from the mitochondria was increased in the presence of l-NAME and C2-ceramide, as compared with C2-ceramide or l-NAME alone. Similar results were observed with caspase-3 activity, i.e., lower concentrations of C2-ceramide (10–20 μM) in the presence of l-NAME increased the caspase-3 activity (Fig. 9B). The DNA fragmentation (Fig. 9C) as measured by the TUNEL staining of cells increased in cells treated with 5 μM and 20 μM C2-ceramide for 16 h in the presence of l-NAME as compared with C2-ceramide alone.

In the presence of antioxidants and iron chelator, C2-ceramide/l-NAME-induced ROS generation was inhibited. As shown in Fig. 10, C2-ceramide/l-NAME-mediated DCF fluorescence (Fig. 10, A and B) and caspase-3 activity (Fig. 10C) in cells treated with either GSH ester (5 mM), FeTBAP (25 μM), Mito-Q (1 μM), desferal (20 μM), or anti-TIR antibody (12 μg/ml, IgA class) was considerably decreased. These results indicate that C2-ceramide induces the formation of ROS to a greater extent in l-NAME-treated cells and that TfR-iron plays a predominant role in ceramide-mediated toxicity.

The Biphasic Effect of Ceramide on Proteasomal Activities—Next, we investigated whether C2-cer treatment modulates the proteasomal activity in BAECs. Fig. 11 shows the trypsin-like and chymotrypsin-like activities of the 26 S proteasome in BAECs treated with different concentrations of C2-ceramide and other antioxidants as indicated and DNA fragmentation was measured by TUNEL staining. The data represent mean ± S.D. of three independent experiments.

DISCUSSION

In this study, we demonstrated that a cell-permeable bioactive ceramide analog induces NO at lower concentrations (≥20 μM) and ROS at higher concentrations (>20 μM) in bovine aortic endothelial cells. Results showed that ceramide-induced TIR-dependent iron uptake was responsible for its prooxidant and proapoptotic effects, because pretreatment with TIR antibody or cell-permeable iron chelators greatly mitigated these effects. Depletion of intracellular NO augmented ceramide-induced iron signaling, oxidative stress, and apoptosis. NO suppressed the prooxidant and proapoptotic effects of ceramide by maintaining intracellular iron homeostasis.

DCF Fluorescence as an Indicator of Oxidant-induced Transferrin Iron Signaling—Ceramide-induced oxidative stress was assessed by monitoring the increase in DCF fluorescence (Fig. 2A). Because ceramide-induced DCF fluorescence was inhibited by anti-TIR antibody, we concluded that TIR-transported...
FIG. 6. Effect of ceramide and NOS inhibitor on nitric oxide generation in endothelial cells. A, BAECs were treated with different concentrations of C2-ceramide for either 1 or 8 h and at the end of the experiment, cells were washed twice with DPBS and replaced with 2 ml of culture medium, and 5 μM DAF-2-DA was added and further incubated for 15 min. Cells were washed free of extracellular DAF-2-DA and immediately viewed under the fluorescence microscope equipped with an FITC filter to measure the green fluorescence as an index of nitric oxide levels. B, fluorescence intensity of data shown in A, and C, same as A except that cells were pretreated with different concentrations of L-NAME for 2 h before the addition of 50 μM C2-ceramide for 8 h. D, fluorescence intensity of data shown in C, E, same as A except that cells were treated with 50 μM C2-ceramide for different time points in the absence or presence of 2 mM L-NAME for 8 h. F, fluorescence intensity of data shown in E. Data represent the mean ± S.D. of three separate experiments.

FIG. 7. Effect of NOS inhibitor on ceramide-induced H2O2 and superoxide generation in endothelial cells. BAECs were treated with low concentrations of C2-ceramide (5 and 20 μM) for 8 h either in the presence or absence of 2 mM L-NAME. After the treatments H2O2 (A) and superoxide (B) staining were carried out by DCF and ethidium staining, respectively, as described under "Experimental Procedures." Data shown represent mean ± S.D. of three independent experiments.
Tf-iron was responsible for catalyzing intracellular oxidation of DCFH to DCF. We have recently shown that oxidative inactivation of iron-sensing iron-sulfur proteins (e.g. aconitase) was responsible for cellular iron uptake through increased iron-regulatory protein (IRP) activity (16, 17). Inactivation of aconitase by oxidative disassembly of the [4Fe-4S] cluster is accompanied by enhanced IRP activation through increased mRNA-binding activity associated with the iron-responsive element (35, 36). Increased IRP1/iron-responsive element binding stabilizes the TfR mRNA, leading to enhanced mRNA uptake.

**FIG. 8.** Effect of NOS inhibitor on ceramide-induced GSH depletion, aconitase activity, TfR expression, and iron uptake in endothelial cells. A, BAECs were treated with different concentrations of C2-ceramide in the presence or absence of 2 mM L-NAME (pretreatment for 2 h), and the GSH levels were determined by measuring the GSH-o-phthalaldehyde adduct using HPLC. B, same as A except that the total aconitase activity was measured spectrophotometrically at 240 nm. C and D, same as A except that the transferrin receptor levels and 55Fe uptake were measured with and without L-NAME. Cells were treated with different concentrations of C2-ceramide in the presence or absence of 2 mM L-NAME for 8 h, and the uptake of labeled iron was measured as described under “Experimental Procedures.” Data represent mean ± S.D. of at least three separate experiments. *, significantly different (p < 0.05) compared with untreated conditions.

**FIG. 9.** NOS inhibitor exacerbates ceramide-induced apoptosis in endothelial cells. A, BAECs were pretreated with 2 mM L-NAME for 2 h and subsequently treated with 20 μM C2-ceramide for 8 h, and the release of cytochrome c from the mitochondria was measured by Western analysis using an anti-cytochrome c antibody. B, cells were treated with low concentrations of C2-ceramide (5–20 μM) for 8 h in the presence or absence of 2 mM L-NAME (2-h pretreatment), and caspase-3 activity was measured spectrophotometrically. C, same as in B except that cells were treated for 16 h with 5 and 20 μM of C2-ceramide either in the presence or absence of L-NAME, and DNA fragmentation was measured by TUNEL-positive staining as described under “Experimental Procedures.”
translation, TfR synthesis, and Tf-iron uptake (37). The exogenous addition of bolus or continuously generated H$_2$O$_2$ to endothelial cells caused enhanced oxidation of DCFH to DCF that was regulated by TfR-mediated uptake of Tf iron (17). Pretreatment of cells with anti-TfR antibody that specifically binds to the extracellular domain of TfR inhibited H$_2$O$_2$-induced iron signaling and ROS-mediated DCF fluorescence. The present data indicate that intracellular oxidation of DCFH is catalyzed by ceramide-induced H$_2$O$_2$ and Tf-iron transported through TfR.

Superoxide/Hydroethidine-induced Intracellular Red Fluorescence—Hydroethidine or dihydroethidium has been widely used to detect intracellular superoxide (16, 34). This assay is based on the fact that the product formed from the reaction between superoxide and dihydroethidium exhibits a red fluorescence. This product long been thought to be ethidium (38). We recently reported that the fluorescence characteristics of the superoxide/hydroethidine product are distinctly different from those of ethidium (34). In addition, the HPLC retention time of ethidium is different from that of the product formed from the reaction between superoxide and hydroethidine (34). Although the exact structure of this product is not known, HE is still a viable fluorescent probe for detecting intracellular superoxide by monitoring the “red fluorescence” formed from HE. Preliminary experiments show that cells treated with hydroethidine and C$_2$-ceramide exhibit a product whose fluorescence

Fig. 10. Effect of co-incubation of NOS inhibitor and antioxidants and iron chelators on ceramide-induced H$_2$O$_2$ generation and caspase-3 activation in endothelial cells. A, BAECs were treated with 20 μM C$_2$-ceramide alone or ceramide plus l-NAME or ceramide plus l-NAME plus antioxidants or iron chelators for 8 h, and H$_2$O$_2$ generation was measured as an index of DCF fluorescence. Note that l-NAME or other compounds were pretreated for 2 h before the addition of C$_2$-ceramide. In the case of antioxidant or iron chelator plus l-NAME plus ceramide groups, antioxidant or iron chelator was added 2 h prior to the addition of l-NAME, which was added 2 h prior to the addition of ceramide. B, fluorescence intensity of data shown in A; C, same as A except that caspase-3 activity was measured spectrophotometrically at 405 nm by following the release of 5-nitroanilide. +++, cells were pretreated with both l-NAME (2 mM) and different antioxidants before they were treated with C$_2$-ceramide (20 μM). Data represent the mean ± S.D. of three separate experiments.

Fig. 11. Effect of ceramide on the proteasomal activity in BAECs. BAECs were treated with different concentrations of C$_2$-ceramide in the presence and absence of l-NAME (2 mM), Mito-Q (1 μM), or desferal (20 μM) for 6 h. The chymotrypsin-like (A) and trypsin-like (B) activities of 26 S proteasome were measured in cell lysates as described under “Experimental Procedures.” Data represent the mean ± S.D. of three separate experiments.
yield was enhanced in the presence of DNA and whose HPLC profile was distinctly different from that of ethidium (not shown). Clearly, elucidating the reaction mechanism between hydroethidine and superoxide is pivotal to our understanding of the oxidative reactions induced by ceramides and other bioactive lipid mediators.

Mitochondria as a Source of Superoxide Generation in Ceramide-treated Cells—Based on the published data (4, 39–41), we propose that ceramide-induced ROS generation inhibits mitochondrial enzymes (aconitase and complex activity) associated with the electron transport chain, which in turn leads to more superoxide and H2O2. Inhibition of complex-1 activity stimulated superoxide formation through increased auto-oxidation of ubisemiquinone (43, 44). Ceramide-induced oxidative stress was thought to result from inhibition of mitochondrial complex III activity (45). The molecular signaling events induced by the lipid second messenger, C2-ceramide, and their role in inhibiting the respiratory enzymes are not fully known. The activation of rac 1, the protooncogene family member and a regulatory component of NADPH oxidase, in ceramide-treated endothelial cells was reported to induce mitochondrial oxidative stress (39). The role of mitochondria in ceramide-induced ROS generation is implicated, because pretreatment with mitochondria-targeted antioxidants (Mito-quione or Mito-vitamin E) (22) greatly inhibited C2-cer-induced DCF and HE fluorescence (data not shown).

Nitrile Oxide, Iron Homeostasis, and Proteosomal Activation—It has been previously shown that addition of low concentrations of C2-ceramide (5 μM) to BAECs significantly increases the eNOS activity due to translocation of eNOS from the endothelial cell membrane to intracellular sites (15). However, at higher concentrations of ceramide, even though there was an increased eNOS mRNA and protein expression, there was a decrease in the levels of bioactive ‘NO (13). These findings are in accordance with the present data in that at low concentrations of C2-ceramide (< 20 μM), there was an increase in the generation of ‘NO and a decrease in ROS generation, whereas, at higher concentrations of ceramide (> 20 μM), ROS generation becomes dominant. Although the reasons for the shift in mechanism are not completely understood, it is likely that ROS can rapidly react with ‘NO, thereby reducing its bioactivity. The antioxidative and cytoprotective effects of ‘NO were recently attributed to ‘NO-induced proteosomal activation (18). Proteosomal inhibitors abrogate ‘NO-mediated cytoprotection and antioxidative effects (18). ‘NO as a stimulator of proteosomal function is still a nascent concept (18). It was suggested that ‘NO mitigated peroxide-induced transferrin iron uptake, DCFH oxidation, and apoptosis by enhancing the proteolytic activity in endothelial cells (17, 18). Depletion of endogenous ‘NO with l-NAME decreased the trypsin-like activity of 26 S proteasome in endothelial cells (18). Previous results also indicated that ‘NO controls peroxide-induced iron signaling, intracellular iron homeostasis, and oxidative stress through increased proteosomal activation (18). In the present study, C2-cer-induced Ti-iron uptake, DCFH oxidation, and caspase-3 activation were greatly enhanced in the presence of l-NAME. Treatment with C2-cer activated the proteosomal function at lower concentrations (which induced ‘NO), and higher concentrations of C2-cer (which induced more ROS and less ‘NO) inhibited the intracellular proteosomal function (Fig. 11). C2-cer-induced proteolytic activation was inhibited by l-NAME, suggesting a role for ‘NO. In contrast, l-NAME did not inhibit the proteosomal inactivation observed at higher concentrations of C2-cer (Fig. 11). If any, l-NAME enhanced the proteosomal inactivation induced by C2-cer at higher concentrations (Fig. 11). This is consistent with the enhanced iron uptake and oxidant generation observed in BAECs treated with C2-cer (5–20 μM) and l-NAME (Fig. 3C).

**Ceramides: Prooxidant Lipid Mediators in Cardiovascular and Neurodegenerative Diseases?—**Ceramide-induced oxidative stress was proposed to play a key role in the pathogenesis of atherosclerosis, amyotrophic lateral sclerosis, and Alzheimer’s disease (46, 49, 50). The ceramide-induced ROS-mediated apoptotic signaling pathway has been suggested to play a key role in the degeneration of dopaminergic neurons in patients with Parkinson’s disease (7, 12). In these pathologies, perturbed iron metabolism or elevated iron levels were shown to be prominent. Thus, ceramide-induced oxidative stress and iron signaling reported in the present work explain in part the mechanism of initiation and propagation of lipid peroxidative processes in the pathogenesis of these diseases. ‘NO levels are impaired in age-related cardiovascular and neurodegenerative diseases (42, 51). The present data suggest that there exists an inverse relationship between endogenous ‘NO and cellular iron levels. Clearly, future studies should be directed at investigating more thoroughly the intriguing connection between ceramide, ‘NO, and iron in neurovascular and cardiovascular pathologies.

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