Dual Effect of Ceramide on Human Endothelial Cells
Induction of Oxidative Stress and Transcriptional Upregulation of Endothelial Nitric Oxide Synthase

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Background—Generation of the second-messenger molecule ceramide by stimulated sphingomyelinase activity has been implicated in the inflammatory processes contributing to the pathogenesis of atherosclerosis. However, reports of stimulatory effects of ceramide on endothelial NO production in animal models suggest antiatherosclerotic effects of the molecule. Therefore, we investigated long-term effects of ceramide on NO generation in human endothelial cells.

Methods and Results—In human umbilical vein endothelial cells (HUVECs) and in HUVEC-derived EA.hy 926 endothelial cells, C6-ceramide (N-hexanoyl-d-erythro-sphingosine) reduced the generation of bioactive NO (RFL-6 reporter-cell assay). At the same time, the signaling molecule increased endothelial NO synthase (eNOS) mRNA (RNase protection assay) and protein expression (Western blot). C6-ceramide stimulated eNOS transcription by a signaling mechanism involving protein phosphatase PP2A but did not modify the stability of the eNOS mRNA. Endothelial generation of reactive oxygen species (ROS) was increased by C6-ceramide [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) oxidation-based fluorescence assay], and this effect was partially reversed by the NOS inhibitor $N^\omega$-nitro-L-arginine methyl ester (L-NAME). On the other hand, (6R)-5,6,7,8-tetrahydro-L-biopterin (BH$_4$) normalized in part the ceramide-induced reduction in bioactive NO.

Conclusions—Ceramide produces oxidative stress in human endothelial cells, thereby reducing bioactive NO. The partial reversal of this reduction by BH$_4$ and the diminution of ROS generation by L-NAME suggest that ceramide promotes NADPH oxidase activity of eNOS, leading to ROS formation at the expense of NO synthesis. The ceramide-induced upregulation of eNOS gene transcription can be considered an ineffective compensatory mechanism. The decreased bioavailability of NO is likely to favor a proatherogenic role of ceramide. (Circulation. 2002;106:2250-2256.)

Key Words: atherosclerosis ■ endothelium-derived factors ■ nitric oxide synthase ■ signal transduction

Inflammatory processes play an important role in the pathogenesis of atherosclerosis. Recruitment of mononuclear leukocytes and the migration, growth, and activation of multiple cell types within atherosclerotic lesions are critical features of the chronic inflammatory and fibroproliferative response central to atherosclerosis. The atherosclerotic vascular wall itself contains elevated levels of acute-phase proteins and proinflammatory cytokines, resulting in localized inflammatory effects eventually responsible for intimal thickening and plaque disruption. Predominant cytokines (that are also elevated in plasma) include tumor necrosis factor $\alpha$ (TNF-$\alpha$) and interleukin 6 (IL-6).

Sphingolipids and their metabolic products are known to have second messenger functions in a variety of cellular signaling pathways. Ceramide is found in endothelial cells, smooth muscle cells, monocytes, macrophages, neutrophils, and platelets. Endogenous ceramide is generated from sphingomyelin by the action of sphingomyelinase (SMase) or by de novo synthesis coordinated through the enzyme ceramide synthase. Molecules promoting atherosclerosis such as oxidized low-density lipoprotein (oxLDL), inflammatory cytokines (TNF-$\alpha$ and IL-1$\beta$), or growth factors stimulate sphingomyelin hydrolysis and ceramide generation. Also, the ceramide content of lesional LDL is markedly increased compared with plasma LDL, and ceramide has been found to accumulate in atherosclerotic lesions. Ceramide can stimulate IL-6 gene expression and, subsequently, C-reactive protein, which is likely to have direct proinflammatory effects and to contribute to the atherosclerotic process.

Treatment of human vascular endothelial cells with cell-permeable synthetic ceramide or bacterial SMase elicited E-selectin–dependent adhesion of quiescent leukocytes to endothelial cells, an important onset event in atherogenesis. Development of atherosclerosis is believed to involve prolif-
eration of smooth muscle cells (SMCs). Activation of the sphingomyelin-ceramide pathway has been suggested to play a pivotal role in the oxLDL-induced SMC proliferation. Thus, ceramide seems to be implicated in several ways in the atherogenic process.

Conversely, there is evidence in the literature that ceramide can have positive effects on vascular NO production. NO generated by endothelial NO synthase (eNOS) conveys vasoprotection and antiatherosclerotic effects through several mechanisms. For example, ceramide has been shown to activate eNOS in bovine aortic endothelial cells independently of calcium and to enhance NO production. In rat aortic rings, ceramide induced vasorelaxation that was partially NO mediated. Also, short-term exposure of endothelial cells to TNF-α or binding of high-density lipoprotein to scavenger receptors has been shown to activate eNOS in endothelial cells; intracellular ceramide has been identified as the second messenger for both processes. On the other hand, ceramide has been shown to promote generation of reactive oxygen species (ROS) in bovine coronary arteries, and ROS can rapidly destroy bioactive NO.

Therefore, the present study was designed to investigate long-term effects of ceramide on the biochemical determinants of bioactive NO, namely eNOS expression, eNOS activity, and the concomitant ROS generation.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and HUVEC-derived EA.hy 926 endothelial cells were cultured as previously described. HUVECs of passages 3 to 5 were used.

Analysis of eNOS mRNA and Protein Expression

Confluent cells were incubated with ceramide for the times indicated in the Results section. Kinase and phosphatase inhibitors were given 1 hour before ceramide. The expression of eNOS mRNA was analyzed by RNase protection assay. Protein expression of eNOS was determined by Western blot using a polyclonal rabbit anti-eNOS antibody. The detailed procedure has been described previously.

Analysis of eNOS Promoter Activity

Promoter activity was analyzed by reporter gene assay using the plasmid pGL3-eNOS-Hu-1600 transiently transfected into EA.hy 926 cells. The plasmid contains a 1.6-kb human eNOS promoter fragment (−1600 to +23) cloned before the luciferase gene of pGL3-Basic, as described elsewhere.

Determination of Bioactive NO and Total NO Synthesis (as Nitrite/Nitrate)

Bioactive NO produced by EA.hy 926 cells was bioassayed using RFL-6 rat lung fibroblasts as reporter cells. Confluent EA.hy 926 cells were treated with C6-ceramide for 9 hours. Then EA.hy 926 cells were incubated for 20 minutes with Locke’s solution containing 200 μM superoxide dismutase and 1 μmol/L L-arginine [with or without (6R)-5,6,7,8-tetrahydro-L-biotin (BH4), see Results]. Thereafter, EA.hy 926 cells were stimulated for 2 minutes with 10 μmol/L A23187 (with or without BH4), and the conditioned media from the EA.hy 926 cells were transferred to the RFL-6 cells, as previously described. The cGMP content of the RFL-6 samples was determined by radioimmunoassay. To determine the total synthesis of NO, nitrite and nitrate were measured in the supernatant of EA.hy 926 cells after treatment with C6-ceramide for 9 hours. After reduction of nitrate with nitrate reductase, total nitrate was determined by chemiluminescence using a NOA 280 Nitric Oxide Analyzer (Sievers). Total protein content of the cells was determined (Bradford), and nitrite levels were normalized for protein.

Measurement of Intracellular Reactive Oxygen Species

The determination of intracellular oxidant formation was based on the oxidation of 5-((and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) to yield an intracellular trapped fluorescent compound and the fluorescence measured in a FluoroCount Reader (Packard Bioscience). Confluent EA.hy 926 cells in 96-well plates were treated for 9 hours with the indicated concentrations of C6-ceramide, then washed with Hanks’ buffered saline solution (HBBS, Invitrogen) and incubated for 30 minutes with 10 μmol/L of CM-H2DCFDA in HBBS. Thereafter, cells were washed twice with HBBS and incubated for an additional 2 hours before measuring the fluorescence at excitation and emission wavelengths of 488 nm and 530 nm, respectively.

Statistics

Statistical differences between mean values were determined by ANOVA followed by Fisher’s protected least-significant difference test for comparison of different means.

Results

C6-Ceramide Incubation Reduced Bioactive NO

When HUVEC-derived EA.hy 926 endothelial cells were treated with C6-ceramide for 9 hours, bioactive NO was decreased in a concentration-dependent manner (Figure 1).

C6-Ceramide Upregulated eNOS mRNA Expression in Human Vascular Endothelial Cells in a Concentration- and Time-Dependent Manner

To test whether the reduced NO production in response to ceramide correlated with a reduced eNOS expression, we analyzed eNOS mRNA expression with RNase protection assay. Surprisingly, C6-ceramide enhanced eNOS mRNA expression in a concentration- and time-dependent manner in EA.hy 926 cells (Figure 2A through 2C). Also, in HUVECs treated with 10 μmol/L C6-ceramide for 9 hours, a significant upregulation of eNOS mRNA was observed (Figure 2D). In human endothelial cells, C6-ceramide did not induce the mRNA expression of inducible-type NOS (3 experiments, data not shown).
C6-Ceramide Upregulated eNOS Protein Expression in Human EA.hy 926 Endothelial Cells
EA.hy 926 cells were incubated with 10 μmol/L C6-ceramide for 10 or 16 hours. A significant enhancement of eNOS protein expression by C6-ceramide was seen at both time points (Figure 3A and 3B).

C8-Ceramide and Ceramide-Generating SMase Increased eNOS mRNA Expression in Human EA.hy 926 Endothelial Cells
Human EA.hy 926 endothelial cells were incubated with 10 μmol/L dihydro-C6-ceramide, 10 μmol/L C8-ceramide, or 0.01 or 0.1 U/mL SMase for 9 hours. C8-ceramide increased eNOS mRNA expression to a similar extent (200.0±13.0% of control, P<0.001) as C6-ceramide. Dihydro-C6-ceramide is an inactive analog of C6-ceramide, which has almost the same chemical structure as C6-ceramide except for lacking the C4-C5 double bond of sphingoid backbone. Dihydro-C6-ceramide did not affect eNOS expression (124.5±7.0% of control, no significance), indicating the biological specificity of the ceramide effect on eNOS expression. Bacterial SMase, which has been shown to increase intracellular ceramide levels in HUVECs, increased eNOS mRNA expression as well (to 140.0±9.0% [P<0.01] and 176.8±7.8% [P<0.001] of control at 0.01 or 0.1 U/mL, respectively). Data represent mean±SEM of 3 experiments (*P<0.05, **P<0.01, ***P<0.001 vs untreated cells, ie Co or time zero).

C6-Ceramide Enhanced eNOS Promoter Activity but Did Not Change the Stability of the eNOS mRNA
In EA.hy 926 cells transfected with pGl1-eNOS-Hu-1600, the eNOS promoter fragment showed a significant basal activity (control) compared with the vector pGl1-Basic (Figure 4A). Incubation with 10 μmol/L C6-ceramide for 12 hours increased the activity of the human eNOS promoter (Figure 4A).

In other experiments, EA.hy 926 cells were treated with actinomycin D to analyze the decay of human eNOS mRNA. C6-ceramide (10 μmol/L) did not change the stability of the eNOS mRNA (Figure 4B).
C6-Ceramide–Induced eNOS Upregulation Was Reduced by Inhibitors of Protein Phosphatase 2A but Not by the Inhibitors of Protein Phosphatase 1, Protein Kinase C, or Mitogen-Activated Protein Kinase

Several downstream targets of ceramide signaling have been identified in recent years. These include protein kinase C (PKC), the mitogen-activated protein kinase (MAPK) cascades, and protein phosphatase (PP) 2A or PP1. We used specific inhibitors to characterize the protein kinase or phosphatase possibly involved in ceramide-induced eNOS expression.

Human EA.hy 926 endothelial cells were incubated for 9 hours with 10 μmol/L C6-ceramide, alone or in combination with PP2A inhibitors (okadaic acid or calyculin A, both 10 nmol/L), a PP1 inhibitor (tautomycin, 100 nmol/L), PKC inhibitors (GF 109203X or Gö 6983, both 1 μmol/L), or the inhibitor of the MAPK kinase, MEK-1 (PD 98059, 10 μmol/L). As shown in Figure 5, none of the inhibitors affected the eNOS expression when used alone. The PP2A inhibitors, but not the inhibitors of PP1, PKC, or MEK-1, reduced the upregulation of eNOS expression by C6-ceramide.

Effect of Ceramide on ROS Production and eNOS Uncoupling

Upregulation of eNOS (Figures 2 and 3) was associated with a decreased production of bioactive NO (Figure 1). However, the total synthesis of NO (measured as nitrite and nitrate) increased in parallel with eNOS expression after exposure of EA.hy 926 cells to ceramide for 9 hours: 119.5±5.8% of control after 3 μmol/L C6-ceramide and 142.4±12.9% of control after 10 μmol/L C6-ceramide (mean±SEM, P<0.05, 4 experiments). This suggests that NO synthesis is indeed increased but there is an accelerated oxidation of NO after ceramide.

Because ROS can rapidly oxidize and inactivate NO, we measured ROS in EA.hy 926 cells after a 9-hour incubation with C6-ceramide. As shown in Figure 6, ROS generation was increased by C6-ceramide. ROS production can result from several enzymes, including the NADPH oxidase activity of uncoupled eNOS itself. Indeed, ceramide-induced ROS...
production was partially prevented by a NOS inhibitor, N\textsuperscript{-}G-nitro-L-arginine methyl ester (L-NAME, Figure 6). A major cause of eNOS uncoupling in vivo can be a BH\textsubscript{4} deficiency.\textsuperscript{25} Therefore, we supplemented ceramide-treated cells (and controls) with BH\textsubscript{4} in the incubation media. The ceramide-induced reduction in bioactive NO was normalized by BH\textsubscript{4} (Figure 7).

**C6-Ceramide–Induced eNOS Upregulation Was Not Mediated by ROS**

ROS, especially hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), has been shown to upregulate eNOS expression in endothelial cells.\textsuperscript{26,27} Thus, ceramide-induced eNOS expression could have been the consequence of an increased H\textsubscript{2}O\textsubscript{2} production. However, the eNOS mRNA expression induced by ceramide was not prevented by catalase (1000 U/mL) or by the antioxidant N-acetylcysteine (5 mmol/L, 3 experiments each, data not shown). Moreover, H\textsubscript{2}O\textsubscript{2}-induced eNOS upregulation has been reported to be mediated by Ca\textsuperscript{2+}/calmodulin-dependent kinase II and janus kinase 2.\textsuperscript{27} Inhibitors of these kinases (KN-93 and AG 490, both 10 \textmu mol/L) did not affect the ceramide-induced eNOS mRNA expression in EA.hy 926 cells (3 experiments each, data not shown).

**Discussion**

Ceramide is a second messenger molecule that seems to play an important role in the process of atherogenesis.\textsuperscript{3,10,28} However, potential antiatherosclerotic effects also have been reported for this signaling molecule. Particularly, the reports demonstrating a stimulation of endothelial NO synthesis would suggest antiatherosclerotic properties.

In precontracted rat aortic rings, bacterial SMase and cell-permeable synthetic ceramide caused vasorelaxation in a concentration-dependent manner; removal of the endothelium reduced these relaxations.\textsuperscript{13} Ceramide has been shown to produce translocation and activation of eNOS in bovine aorta endothelial cells through a Ca\textsuperscript{2+}-independent mechanism.\textsuperscript{12} Moreover, short-term TNF-\textalpha exposure\textsuperscript{14} or binding of high-density lipoprotein to scavenger receptors\textsuperscript{15} leads to a ceramide-dependent activation of eNOS in endothelial cells. On the other hand, in isolated bovine coronary arteries,
ceramide has been shown to increase $O_2^-$ generation, thereby producing endothelial dysfunction and reducing NO-mediated vasodilation.16

Against this background of NO-increasing and NO-reducing effects of ceramide reported in different animal models, we decided to determine the release of biologically active NO (measured by the stimulation of soluble guanylyl cyclase) from human endothelial cells. C6-ceramide clearly reduced bioactive NO (Figure 1). At the same time, the producing enzyme, eNOS, was upregulated at the mRNA and protein levels (Figures 2 and 3). This was a transcriptional effect on the eNOS promoter that involved PP2A (Figures 4 and 5), one of the known target enzymes of ceramide.22,23

The discrepancy between increased expression and decreased activity of eNOS is resolved in Figure 6, which demonstrates that ceramide also stimulated ROS production in human endothelial cells. ROS can rapidly oxidize NO, thereby reducing its bioactivity. Thus, ceramide produced oxidative stress in endothelial cells, a finding that is in accordance with a recent study in small bovine coronary arteries.16 The enzyme systems in EA.hy 926 cells responsible for ROS production in response to ceramide remain to be determined. Potential sources of $O_2^-$/ROS include NAD(P)H oxidase, xanthine oxidase, and the mitochondrial respiratory chain. In human aortic smooth muscle cells, ceramide activates NAD(P)H oxidase and increases $O_2^-$ production.29 In HUVECs, ceramide has also been shown to interact with the mitochondrial electron transport chain, leading to the generation of ROS.30

An increased oxidation of BH$_4$ by peroxynitrite/O$_2^-$ may cause BH$_4$ deficiency, which favors NADPH oxidase activity of eNOS leading to the formation of ROS ($O_2^-$ and H$_2$O$_2$) in lieu of NO.24 This phenomenon is often referred to as eNOS uncoupling.24 Interestingly, in EA.hy 926 cells, the ceramide-induced reduction in bioactive NO could be largely normalized when the cells were treated with BH$_4$ (Figure 7). This suggests that ceramide led to an uncoupling of eNOS and that eNOS itself contributed to ROS production (Figure 6). This assumption is substantiated by the finding that the NOS inhibitor L-NAME partially prevented endothelial ROS generation (Figure 6). NO production by eNOS correlates closely with the intracellular concentration of BH$_4$, and supplementation with BH$_4$ has been shown to correct eNOS dysfunction in several types of pathophysiology, such as hypertension, experimental diabetes, and hypercholesterolemia, and in smokers.24 However, BH$_4$ supplementation does not affect ROS generation from sources other than eNOS, which may explain why NO bioavailability was not increased parallel to eNOS expression, even when eNOS uncoupling was corrected by BH$_4$ supplementation (Figures 2 and 7).

Several pathophysiological conditions of oxidative stress, such as angiotensin II–induced hypertension,31 streptozotocin-induced diabetes,32 or nitroglycerin tolerance,33 have been shown to go along with an upregulation of eNOS expression, which may be considered an unsuccessful compensatory mechanism. The same seems to be true for ceramide signaling. Future research may determine whether ceramide signaling is involved in some of those pathophysiological situations.

In conclusion, we identified the lipid second-messenger ceramide as a regulator of eNOS expression in human endothelial cells. Incubation of human endothelial cells with exogenous cell-permeable C6- or C8-ceramide or with bacterial SMase (which increases intracellular ceramide contents by hydrolysis of sphingomyelin) increased eNOS transcription. However, despite the upregulation of eNOS expression, ceramide produces oxidative stress in endothelial cells and reduces bioactive NO. The decreased NO bioavailability is likely to favor a proatherogenic role of ceramide.

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