Ceramide-Induced Impairment of Endothelial Function Is Prevented by CuZn Superoxide Dismutase Overexpression

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Objective—Ceramide is an important intracellular second messenger that may also increase superoxide. The goal of this study was to determine whether overexpression of CuZn superoxide dismutase (SOD) protects against ceramide-induced increases in vascular superoxide and endothelial dysfunction.

Methods and Results—Carotid arteries from CuZnSOD-transgenic (CuZnSOD-Tg) and nontransgenic littermates were examined in vitro. Immunohistochemistry confirmed that CuZnSOD protein was greater in carotid artery from CuZnSOD-Tg compared with nontransgenic mice. Ceramide (N-acetyl-D-sphingosine; 1 and 10 μmol/L) produced concentration-dependent impairment (P<0.05) of vasorelaxation in response to the endothelium-dependent agonist acetylcholine (ACh) in nontransgenic mice. For example, 100 μmol/L ACh relaxed arteries from nontransgenic mice by 96±4% and 52±5% in the presence of vehicle and 10 μmol/L ceramide, respectively, whereas ceramide (1 or 10 μmol/L) had an effect (P>0.05) on responses of carotid artery to ACh in CuZnSOD-Tg mice. Ceramide had no effect on nitroprusside- or papaverine-induced relaxation in CuZnSOD-Tg or nontransgenic mice. Ceramide increased superoxide in arteries from nontransgenic vessels, and this effect was prevented by polyethyleneglycol-SOD (50 U/mL) or overexpression of CuZnSOD.

Conclusions—These results suggest that ceramide-induced increases in superoxide impair endothelium-dependent relaxation, and that select overexpression of the CuZn isoform of SOD prevents ceramide-induced oxidative stress in vessels. (Arterioscler Thromb Vasc Biol. 2005;25:90-95.)

Key Words: carotid artery ■ genetically altered mice ■ nitric oxide ■ reactive oxygen species ■ SOD1

Ceramide, a sphingolipid second messenger, appears to be involved in the cellular signaling response to inflammatory stimuli or injury.1 For example, lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) have been associated with increases in ceramide and superoxide.2,3 It has been suggested that the increase in superoxide in response to these inflammatory stimuli may be mediated by ceramide.2,3 More recently, ceramide has been identified as having functional effects in cardiovascular physiology and disease, particularly atherosclerosis.4–13

Experimentally, exposure of blood vessels to exogenous ceramide (eg, C2-ceramide, which mimics many of the effects of endogenous ceramide) has been shown to inhibit endothelium-dependent relaxation.14–18 This effect may be mediated by superoxide-mediated inactivation of endothelium-derived nitric oxide (NO).14,17,19 Several potential sources of superoxide exist within the vascular wall of which mitochondria and NAD(P)H-oxidase have been implicated as sources of superoxide in response to ceramide.14,17,20–23 Because increases in superoxide in response to ceramide are thought to be attributable (at least in part) to intracellular sources (ie, mitochondria and NAD(P)H oxidase), we anticipated that overexpression of CuZn superoxide dismutase (SOD; the predominant intracellular SOD)24 would be effective in preventing ceramide-induced increases in vascular superoxide and dysfunction. Thus, the first goal of the present study was to examine the hypothesis that ceramide produces increases in superoxide and endothelial dysfunction in carotid arteries. The second goal was to determine whether overexpression of CuZnSOD prevents ceramide-induced endothelial dysfunction and the accompanying increase in superoxide. To accomplish these goals, we examined vascular responses and superoxide levels in response to ceramide in genetically altered mice that overexpress CuZnSOD.

Methods

Experimental Animals

Mice (male and female) used for this study were derived from breeding male hemizygous CuZnSOD (human)-transgenic (C57BL/6-TgN(SOD1)10Cje) with female C57BL/6J mice obtained from The Jackson Laboratory. Two groups of mice were studied: CuZnSOD-transgenic (CuZnSOD-Tg) and their nontransgenic littermates. Nontransgenic (n=58) and CuZnSOD-Tg (n=43) mice were of similar age (~8 months) and body weight (~32 g; P>0.05). Genotype was ascertained by polymerase chain reaction of DNA.
isolated from tail biopsy samples as described on The Jackson Laboratory web site.23 All experimental protocols were approved by the University of Iowa animal care and use committee.

**Vascular Studies**

Rings of carotid artery (4 rings per mouse) from nontransgenic and CuZnSOD-Tg mice were studied in organ chambers as described previously.26,27 After a 45-minute equilibration period, 2 rings were incubated with vehicle (dimethyl sulfoxide [DMSO]), and 2 rings were incubated with N-acetyl-l-sphingosine (C2-ceramide; 1 or 10 μmol/L) a cell-permeable, biologically active form of ceramide for 30 minutes before and during generation of concentration-response curves. As a control for possible nonspecific effects of ceramide, arteries from nontransgenic mice (in separate experiments) were incubated with vehicle (DMSO) and d-erythro-N-acetylphosphinganine (dihydroceramide; 10 μmol/L), a cell-permeable but biologically inactive form of ceramide.29

After equilibration, vessels were contracted submaximally (50% to 60% of maximum) with the thromboxane analogue 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F2α (U46619). After reaching a stable contraction plateau, concentration-response curves were generated for the endothelium-dependent dilator acetylcholine (ACh; 10 μmol/L) and for the endothelium-independent dilators nitroprusside (0.1 nmol/L to 100 μmol/L) and papaverine (a non-NO–dependent dilator; 0.01 to 10 μmol/L). At the end of each experiment, a full concentration-response curve for U46619 (0.03 to 3.0 μg/mL) was generated to determine the maximal contractile response of each vessel.

Because overexpression of CuZnSOD may increase hydrogen peroxide (a dilator in many vessels), we assessed the role of hydrogen peroxide and NO in mediating responses of carotid arteries in nontransgenic and CuZnSOD-Tg mice under control conditions and in the presence of ceramide. Thus, responses to ACh and nitroprusside were examined in rings of carotid artery incubated with catalase (a scavenger of hydrogen peroxide; 300 U/mL) or N(N)-nitro-L-arginine (L-NNA, an NO synthase inhibitor; 100 μmol/L) in the presence of vehicle or ceramide (10 μmol/L).

**Detection of Superoxide**

Superoxide levels were evaluated in carotid artery using hydroethidine-based confocal microscopy as described previously.26,27 Briefly, sections of carotid artery from nontransgenic (n=22) and CuZnSOD-Tg (n=10) mice were preincubated with either vehicle or ceramide (10 μmol/L) for 30 minutes. In some experiments, sections of carotid artery from nontransgenic (n=6) mice were also incubated with polyethylene glycol (PEG)-SOD (50 U/mL) for 30 minutes. Carotid arteries were frozen in optimal cutting temperature compound (OCT), sectioned (30 μm) onto glass slides, and incubated with hydroethidine (2 μmol/L) for 30 minutes. Positive staining (ethidium; red fluorescence) of carotid artery sections for superoxide was determined with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. Laser settings were identical for acquisition of images, and vessels from nontransgenic and CuZnSOD-Tg mice treated with vehicle or ceramide were processed and imaged in parallel to avoid potential artifacts due to tissue processing. Relative increases in the hydroethidine signal were determined using Scion Image software for the personal computer (version 4.02). Ethidium fluorescence was normalized to cross-sectional area of the vessel wall for each section.

**Immunohistochemistry for CuZnSOD**

Carotid arteries from nontransgenic and CuZnSOD-Tg mice were frozen in OCT and serially sectioned (8 μm) on a cryostat and mounted on microscope slides. Sections were fixed in 2% paraformaldehyde for 15 minutes. Slides were first treated with 3% hydrogen peroxide and rinsed in PBS followed by 8% BSA to quench endogenous peroxidase and to block nonspecific binding of protein, respectively. Slides were then incubated overnight (4°C) with an anti-human CuZnSOD polyclonal antibody (1:250 dilution; provided by Dr Larry Oberley, University of Iowa). Slides were then incubated with a biotinylated anti-rabbit IgG (Kit PK-6101; Vector Laboratories) for 30 minutes. After the slides were rinsed with PBS, avidin-horseradish peroxidase complex (Vector Laboratories) was applied for 30 minutes, followed by incubation with diaminobenzidine. Slides were counterstained with Harrison’s hematoxylin and examined for positive staining of CuZnSOD (brown color) by light microscopy.

**Drugs**

ACh, catalase, ceramide, dihydroceramide, L-NNA, nitroprusside, papaverine, and PEG-SOD were obtained from Sigma, and all were dissolved in saline with the exception of ceramide and dihydroceramide, which were dissolved in DMSO (final concentration <0.01%). U46619 was obtained from Cayman Chemical and dissolved in 100% ethanol, with subsequent dilution being made with saline. Hydroethidine was obtained from Molecular Probes and dissolved in DMSO at a concentration of 0.1 mol/L. All other reagents were of standard laboratory grade.

**Statistical Analysis**

All data are expressed as means±SE. Relaxation to ACh, nitroprusside, and papaverine is expressed as a percent relaxation to U46619-induced contraction. Comparisons of relaxation and contraction were made using ANOVA followed by Bonferroni’s multiple comparison test. Comparison of ethidium fluorescence was made using paired t tests. Statistical significance was accepted at P<0.05.

**Results**

**CuZnSOD Expression in Carotid Artery**

Consistent with the presence of endogenous mouse CuZnSOD, immunohistochemistry for CuZnSOD revealed light staining (brown color) for CuZnSOD within the wall of carotid arteries from nontransgenic mice (Figure 1). In arteries from CuZnSOD-Tg mice, immunostaining for CuZnSOD (representing endogenous mouse and human CuZnSOD) was markedly increased (Figure 1).

**Ceramide Produces Endothelial Dysfunction in Nontransgenic Mice**

In nontransgenic mice, ACh produced concentration-dependent relaxation of carotid arteries precontracted with...
U46619 (Figures 2 and 3A). This response was markedly attenuated by l-NNa; Figure 3A) but not by catalase (data not shown). These findings suggest that relaxation of the carotid artery to ACh in nontransgenic mice is normally mediated by NO and is consistent with our previous findings in eNOS-deficient mice.28

In vessels treated with ceramide (1 μmol/L), relaxation in response to higher concentrations of ACh was inhibited by ≈25%. For example, relaxation to 100 μmol/L ACh was 92±6% and 71±6% in vehicle-treated and ceramide-treated (1 μmol/L) vessels, respectively (Figure 3A). This effect was selective because vasorelaxation to nitroprusside (Figure 3B) and papaverine (data not shown) was not affected by 1 μmol/L ceramide.

A higher concentration of ceramide (10 μmol/L) produced greater impairment (≈50% inhibition) of ACh-induced relaxation in arteries from nontransgenic mice (Figures 2 and 4A). For example, 100 μmol/L ACh-induced relaxation was 96±4% and 52±5% in the vehicle- and ceramide-treated vessels, respectively (Figure 4A). Relaxation to ACh in the presence of ceramide was markedly reduced (≈90%) by l-NNa but not affected by catalase, suggesting that the residual response to ACh in the presence of ceramide is mediated by NO (Figure 4A). Relaxation to nitroprusside (Figure 4B) was similar in nontransgenic mice in either the absence or presence of 10 μmol/L ceramide (as well as in the absence or presence of catalase or l-NNa; data not shown), demonstrating selectivity.

Incubation of vessels with either vehicle or dihydroceramide (an inactive form of ceramide; 10 μmol/L) had no effect on relaxation to ACh (Figure 5), nitroprusside (Figure 5), or papaverine (data not shown) in nontransgenic mice, providing strong evidence that the endothelial dysfunction observed in this model was attributable to direct effects of ceramide.

**Overexpression of CuZnSOD Protects Against Ceramide-Induced Endothelial Dysfunction**

In CuZnSOD-Tg mice, ACh produced concentration-dependent relaxation, which was similar to that produced in nontransgenic mice (Figures 2 and 3A). This response was markedly attenuated by l-NNa (Figure 3A) but not by catalase (data not shown). These findings suggest that relaxation of the carotid artery to ACh is mediated very predominantly by NO in CuZnSOD-Tg mice.

In contrast to the effects of ceramide (1 and 10 μmol/L) on endothelial function in nontransgenic mice, ceramide-induced endothelial dysfunction was completely prevented by overexpression of CuZnSOD, as evidenced by normal responses to ACh in CuZnSOD-Tg mice (Figures 3A and 4A). This response to ACh in the presence of 10 μmol ceramide was markedly attenuated by l-NNa but not catalase (Figure 4A), indicating the response is mediated by NO. Relaxation in response to nitroprusside (Figures 3B and 4B) was similar in CuZnSOD-Tg mice in either the absence or presence of 10 μmol/L ceramide (as well as in the absence or presence of catalase or l-NNa; data not shown).

**Ceramide-Induced Increases in Superoxide Are Prevented by Overexpression of CuZnSOD**

Basal superoxide levels, as detected by hydroethidine-based confocal microscopy, were similar in carotid arteries from nontransgenic and CuZnSOD-Tg mice (Figure 6). Preincubation of carotid arteries from nontransgenic mice with ceramide (10 μmol/L) increased the hydroethidine signal (11±3 and 18±2×103 relative units in vehicle- and ceramide-treated arteries, respectively; P<0.05; Figure 6). The hydroethidine signal was reduced by PEG-SOD in vehicle- and ceramide-treated nontransgenic vessels (5±1 and 5±1×103 relative units, respectively; P<0.05). Thus, ceramide-induced increases in hydroethidine staining appear to be a result of superoxide and do not appear to be...
attributable to any nonspecific shifts in baseline fluorescence. In addition, increases in the hydroethidine signal in response to ceramide were not observed in carotid arteries from CuZnSOD-Tg mice (Figure 6), suggesting that overexpression of CuZnSOD is sufficient to prevent ceramide-induced increases in superoxide.

Discussion

There are several major new findings of the present study. First, although immunohistochemistry revealed that CuZnSOD protein is markedly increased in CuZnSOD-Tg mice, relaxation of the carotid artery to ACh was unaltered by overexpression of CuZnSOD. This finding suggests that overexpression of CuZnSOD does not alter endothelial function under normal conditions. Second, ceramide impairs endothelial function in nontransgenic mice. Overexpression of CuZnSOD protected against ceramide-induced endothelial dysfunction. Third, ceramide increased superoxide levels in carotid artery of nontransgenic but not CuZnSOD transgenic mice. These findings combined with the functional responses seen in the CuZnSOD-Tg mice provide direct evidence that ceramide-induced superoxide formation produces endothelial dysfunction, possibly resulting from superoxide-mediated inactivation of NO bioavailability.

Ceramide-Induced Endothelial Dysfunction

Ceramide has been shown to have direct effects on vascular tone that appear to vary depending on the species and vascular bed. In addition, acute incubation with ceramide has been shown to impair endothelium-dependent relaxation. Consistent with these findings, we found that incubation of carotid arteries from nontransgenic mice with ceramide impaired relaxation to ACh in a concentration-dependent manner. The concentrations of ceramide used in the present study are physiologically relevant. In addition, we found that dihydroceramide, a cell-permeable but biologically inactive form of ceramide, had no effect on vascular responses to either endothelium-dependent or -independent stimuli, suggesting that the effects of ceramide are selective.
Mechanism of Ceramide-Induced Vascular Dysfunction

It has been shown previously that selective overexpression of CuZnSOD increases CuZnSOD protein and activity levels in nonvascular tissue and aorta from CuZnSOD-Tg mice. Consistent with these findings, we found using immunohistochemistry that CuZnSOD protein is markedly increased in carotid arteries from CuZnSOD-Tg mice compared with nontransgenic mice. In relation to vascular function, ACh produced concentration-dependent relaxation in carotid arteries from CuZnSOD-Tg that was similar to that observed in nontransgenic mice under basal conditions. In carotid arteries from nontransgenic and CuZnSOD-Tg mice, relaxation in response to ACh was attenuated by ~90% in the presence of l-NAME. In contrast, catalase had no effect on responses to ACh in either nontransgenic or CuZnSOD-Tg mice. These findings provide evidence that relaxation to ACh is mediated by NO in nontransgenic and CuZnSOD-Tg mice, and that overexpression of CuZnSOD does not alter this response.

On the basis of studies using pharmacological approaches only, it has been suggested that ceramide-induced endothelial dysfunction is attributable to increases in superoxide, with concomitant reductions in endothelial-derived NO. To test this concept with a genetic and perhaps more definitive approach, we examined the effect of ceramide in CuZnSOD-Tg mice. Overexpression of CuZnSOD completely prevented the ceramide-induced endothelial dysfunction that was observed in nontransgenic mice (ie, relaxation to ACh in carotid arteries from CuZnSOD-Tg mice was similar to that observed in vessels treated with either ceramide or vehicle). Furthermore, in CuZnSOD-Tg mice, relaxation in response to ACh in the presence of ceramide was markedly attenuated by l-NAME but not catalase, suggesting that overexpression of CuZnSOD prevents ceramide-induced impairment of an NO-mediated response. The use of CuZnSOD-Tg mice also extends pharmacological studies in that it demonstrates that select overexpression of the major intracellular isoform of SOD per se is sufficient to protect endothelial function in response to ceramide.

In cells in culture, ceramide produces increases in superoxide. In the present study, ceramide increased superoxide in carotid arteries from nontransgenic mice as detected using hydroethidine. Thus, our findings are in agreement with the observation that prevention of ceramide-induced oxidative stress in vessels.

Functional Implications

The present study suggests that selective overexpression of CuZnSOD prevents ceramide-induced increases in superoxide and endothelial dysfunction. Increases in ceramide have been described in diseases associated with inflammation. For example, angiotensin II, endothelin, LPS, and TNF-α all increase ceramide levels and are known to impair endothelium-dependent relaxation via a mechanism that may involve increased superoxide. In addition, ceramide has also been shown to accumulate within atherosclerotic lesions, which are clearly associated with increases in vascular oxidative stress and impairment of vascular function. Thus, increases in ceramide may promote endothelial dysfunction. In summary, our results indicate that ceramide impairs endothelium-dependent relaxation via increases in superoxide, and that selective overexpression of the CuZn isoform of SOD is very effective in preventing ceramide-induced oxidative stress in vessels.

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