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Ceramide Reduces Endothelium-Dependent Vasodilation by Increasing Superoxide Production in Small Bovine Coronary Arteries

David X. Zhang, Ai-Ping Zou, Pin-Lan Li

Abstract—Ceramide serves as a second messenger in a variety of mammalian cells. Little is known regarding the role of ceramide in the regulation of vascular endothelial function. The present study was designed to determine whether ceramide affects endothelium-dependent vasodilation in coronary arteries and to explore the mechanism of action of ceramide. In isolated and pressurized small bovine coronary arteries, cell-permeable C_2-ceramide (10^{-5} mol/L) markedly attenuated vasodilator responses to bradykinin and A23187 (by 40% and 60%, respectively). In the presence of N^G-nitro-L-arginine methyl ester, ceramide produced no further inhibition on the vasodilation induced by these vasodilators. Ceramide had no effect on DETA NONOate–induced vasodilation. By use of a fluorescence NO indicator (4,5-diaminofluorescein diacetate), intracellular NO was measured in the endothelium of freshly isolated small coronary arteries. It was found that ceramide significantly inhibited bradykinin-induced NO increase within endothelial cells. However, it had no effect on the activity of arterial or endothelial NO synthase. Pretreatment of the arteries with sodium dihydroxybenzene disulfonate (Tiron, 10^{-3} mol/L), a cell-permeable superoxide scavenger, or polyethylene glycol superoxide dismutase (100 U/mL) largely restored the inhibitory effects of ceramide on the vasodilation and NO increase induced by bradykinin or A23187. Moreover, ceramide time-dependently increased intracellular superoxide ($O_2^-\cdot$) in the endothelium, as measured by a fluorescent $O_2^-\cdot$ indicator, dihydroethidium. These results demonstrate that ceramide inhibits endothelium-dependent vasodilation in small coronary arteries by decreasing NO in vascular endothelial cells and that this decrease in NO is associated with increased $O_2^-\cdot$ but not with the inhibition of NO synthase activity within these cells. (***Circ Res***. 2001;88:824-831.)

Key Words: ceramide ∎ vasodilation ∎ coronary vessels ∎ nitric oxide ∎ superoxide

Ceramide, a sphingolipid, has emerged as an intracellular second messenger for various cytokines and hormones, such as tumor necrosis factor (TNF)-α, interleukin-1β, 1,25-dihydroxy-vitamin D_3, and nerve growth factor.[1–4] The ceramide-mediated signaling exists in a variety of mammalian tissues and cells and plays an important role in a number of cellular processes, including cell growth and differentiation, apoptosis, and inflammatory responses.[5,6] Recent studies have indicated that ceramide may also be involved in the regulation of ion channel activity, intracellular Ca^{2+} concentrations, and contractile responses in vascular smooth muscles.[7–12] However, the role of ceramide in the regulation of vascular endothelial function has yet to be clarified.

It is well known that the endothelium-dependent vasodilation (EDVD) in coronary circulation is impaired during myocardial ischemia and reperfusion.[13,14] The mechanism by which ischemia/reperfusion attenuates EDVD is not completely understood but may involve the actions of increased cytokines, such as TNF-α and interleukin-1β, in the myocar-dial tissue during ischemia and reperfusion.[15] TNF-α and other cytokines have been reported to inhibit the release of endothelial NO and EDVD in a variety of vascular beds.[16–19] However, the mechanism mediating these actions remains unclear. Recent studies have indicated that these cytokines can increase intracellular ceramide in vascular endothelial cells, which then mediates a number of cellular responses to these cytokines, such as inflammatory responses and apopto-sis.[20,21] Given the importance of ceramide in the actions of these cytokines, it is possible that increased ceramide in the vascular endothelium also contributes to the impairment of EDVD induced by these cytokines.

Numerous studies have shown that a decrease in the bioavailability of NO plays a central role in the endothelial dysfunction or impairment of EDVD.[22] Although reduced NO bioavailability may be associated with alterations of cellular NO signaling and with a lack of the substrate or cofactors for NO synthase (NOS), there is accumulating evidence suggesting that superoxide ($O_2^-\cdot$) and other reactive oxygen species

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can interact with NO and thereby regulate or modulate the biological actions of NO and endothelial function.[23,24] It has been demonstrated that $O_2^{-\cdot}$ inactivates NO, which reduces arteriolar dilation to acetylcholine or other endothelium-dependent vasodilators. Superoxide dismutase significantly increases the half-life of NO and restores EDVD.[25–28] Because ceramide has been found to increase the $O_2^{-\cdot}$ production in vascular tissues,[29] we hypothesized that the ceramide-stimulated oxidant stress may impair EDVD through NO inactivation, thereby leading to endothelial dysfunction of coronary circulation induced by cytokines during myocardial ischemia/reperfusion. To test this hypothesis, the present study was designed to determine the effect of ceramide on EDVD and to explore the role of NO and $O_2^{-\cdot}$ interaction in mediating the action of ceramide by use of isolated and pressurized small bovine coronary arteries. Then, we directly measured NO and $O_2^{-\cdot}$ concentrations in the endothelium of these arteries and examined the effects of ceramide on NO and $O_2^{-\cdot}$ production with the use of fluorescence microscopy.

Materials and Methods

Isolated Small Coronary Artery Preparation

Fresh bovine hearts were obtained from a local abattoir. The left ventricular wall was rapidly dissected and immersed in ice-cold physiological saline solution (PSS) containing the following (in 10^{-3} mol/L): NaCl 119, KCl 4.7, CaCl$_2$ 1.6, MgSO$_4$ 1.17, NaH$_2$PO$_4$ 1.18, NaHCO$_3$ 24, EDTA 0.026, and glucose 5.5, pH 7.4. This myocardial section was transported immediately to the laboratory. Small intramural coronary arteries from the left anterior descending artery were carefully dissected and placed in cold PSS until cannulation (up to 4 hours). Segments of small arteries (100- to 200-μm internal diameter) were transferred to a water-jacketed perfusion chamber and cannulated with 2 glass micropipettes at their in situ length, as we described previously.[10,30] The outflow cannula was clamped, and the arteries were pressurized to 60 mm Hg. The arteries were bathed in the PSS equilibrated with 95% O_2/5% CO_2 and maintained at pH 7.4 and 37°C. The internal diameter of the arteries was measured with a video system composed of a stereomicroscope (Leica MZ8), a charge-coupled device camera (KP-MI AU, Hitachi), a video monitor (VM-1220U, Hitachi), a video measuring apparatus (VIA-170, Boeckeler Instrument), and a video printer (UP890 MD, Sony). The arterial images were recorded continuously with a videocassette recorder (M-674, Toshiba).

After a 1-hour equilibration period, the arteries were precontracted by ≈50% of their resting diameter with the thromboxane A$_2$ analogue U46619. Once steady-state contraction was obtained, cumulative dose-response curves to the endothelium-dependent vasodilators bradykinin (BK, 10^{-10} to 10^{-6} mol/L) and A23187 (10^{-9} to 10^{-5} mol/L) and to the endothelium-independent vasodilator DETA NONOate (10^{-7} to 10^{-4} mol/L) were determined by measuring changes in the internal diameter. To study the effect of ceramide on vasodilator response to BK, A23187, or DETA NONOate, arteries were preincubated with cell-permeable C$_2$-ceramide (10^{-5} mol/L) for 30 minutes, and dose-response curves to the vasodilators were further established. To examine the involvement of NO or $O_2^{-\cdot}$ in ceramide-induced endothelial dysfunction, the arteries were preincubated with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} mol/L; an NO synthase inhibitor), sodium dihydroxybenzene disulfonate (Tiron, 10^{-3} mol/L; a cell-permeable $O_2^{-\cdot}$ scavenger), superoxide dismutase (SOD, 100 U/mL), or polyethylene glycol SOD (PEG-SOD, 100 U/mL) in the presence or absence of C$_2$-ceramide (10^{-5} mol/L) for 30 minutes, and then dose responses to BK and A23187 were determined. All drugs were added into the bath solution. Between pharmacological interventions, the arteries were washed 3 times with PSS and allowed to equilibrate in drug-free PSS for 20 to 30 minutes. The vasodilator response was expressed as

percent relaxation of U46619-induced precontraction on the basis of changes in the internal diameter.

Measurement of Intracellular [NO] in the Endothelium

A novel fluorescent NO indicator, 4,5-diaminofluorescein diacetate (DAF-2 DA), as recently described by Kojima et al,[31] was used to measure intracellular NO concentration ([NO]) within the endothelial cells of freshly isolated small bovine coronary arteries. DAF-2 DA can readily enter the cells and be hydrolyzed by cytosolic esterases to the DAF-2 that is trapped inside the cells. In the presence of NO and oxygen, a relatively nonfluorescent DAF-2 is transformed into the highly green fluorescent triazole form, DAF-2T. Thus, the increases in DAF-2T fluorescence represent the elevation of [NO]. Small intramural arteries (300- to 500-μm internal diameter) were carefully dissected as described above and transferred to a 35-mm dissecting dish coated with Sylgard (World Precision Instruments) and containing ice-cold HEPES-buffered PSS that consists of the following (in 10^{-3} mol/L): NaCl 140, KCl 4.7, CaCl$_2$ 1.6, MgSO$_4$ 1.17, NaH$_2$PO$_4$ 1.18, glucose 5.5, and HEPES 10, pH 7.4. The arterial segment was cut open along its longitudinal axis and pinned onto the dish with lumen side upward. Care was taken not to disrupt the endothelium. After 1 hour of equilibrium at 37°C, the arterial segment was incubated with DAF-2 DA (10^{-5} mol/L, Calbiochem) in 1 mL PSS at room temperature for 30 minutes. The segments were then rinsed 3 times with PSS, and the dish was mounted on the stage of an epifluorescence microscope (Nicon E600) equipped with a ×20 objective and 490-nm excitation and a 510- to 560-nm emission filters. Digital images were captured and analyzed by using a PC-controlled charge-coupled device camera (Roper Scientific RTE/CCD-1300-Y/HS) and MetaMorph imaging and analysis software (Universal Imaging Corp).

BK (10^{-6} mol/L) was added into the bath solution to stimulate NO production. To study the effect of ceramide on endothelial [NO], the arteries were incubated with C$_2$-ceramide (10^{-5} mol/L) for 30 minutes before the response to BK was determined. To examine whether $O_2^{-\cdot}$ was involved in the effect of ceramide on endothelial [NO], the arteries were incubated with C$_2$-ceramide and Tiron (10^{-3} mol/L) for 30 minutes, and then the response to BK was determined. NO fluorescence was measured every 5 minutes in a single area of endothelial layer. Results were expressed as the integrated fluorescence intensity within the area observed.

Activity of NOS

NOS activity was determined by measuring the conversion of [^{3}H]arginine to [^{3}H]citrulline[32] and by using the isotopic NOS detection kit (Calbiochem) according to manufacturer's protocol. Briefly, the homogenates prepared from cultured bovine coronary endothelial cells (25 μg protein)[33] or small bovine coronary arteries (100 μg protein) were incubated in 50 μL reaction mixture containing the following (in 10^{-3} mol/L): Tris-HCl 25 (pH 7.4), CaCl$_2$ 0.6, β-NADPH 1, tetrahydrobiopterin 0.003, flavin adenine dinucleotide 0.001, flavin mononucleotide 0.001, and cold L-arginine 0.005, along with 1.0 μCi [^{3}H]arginine in the absence or presence of C$_2$-ceramide. After incubation for 15 or 60 minutes (for endothelial cell or arterial homogenates, respectively) at 37°C, the reaction was terminated by the addition of 400 μL of ice-cold stop buffer containing the following (in 10^{-3} mol/L): HEPES 50 (pH 5.5) and EDTA 5. Equilibrated cation exchange resin was added to the samples, and they were then applied to spin columns. After centrifugation, the eluate (containing [^{3}H]citrulline) was collected, and the radioactivity was determined with a liquid scintillation counter. To determine the effect of ceramide on NOS activity in intact endothelial cells, the confluent endothelial cell cultures in 150-mm dishes were treated with C$_2$-ceramide for 30 minutes, followed by harvesting and homogenization. The formation of citrulline was then assayed as described above. In these experiments, the formation rate of citrulline represented NOS activity, which was expressed as picomoles per milligram protein per minute.

Measurement of Intracellular $[O_2^{-\cdot}]$ in the Endothelium

The intracellular $O_2^{-\cdot}$ concentration ($[O_2^{-\cdot}]$) was detected by measuring the fluorescence intensity resulting from oxidation of dihydroethidium (DHE), as reported by Castilho et al.[34] DHE can enter the cell and be oxidized by $O_2^{-\cdot}$ to yield ethidium. Ethidium binds to DNA, which produces strong red fluorescence. Assays were performed on the endothelium of small bovine coronary arteries as prepared for NO measurement. The arterial segment was incubated with 1 mL PSS containing 3×10^{-6} mol/L DHE (Molecular Probes) purified with cationic resin (Dowex-50W-X8), and the ethidium fluorescence was measured every 5 minutes for 30 minutes at 490-nm excitation and 610 nm-emission by using the same imaging system as described above. To study the effect of ceramide on $[O_2^{-\cdot}]$, the arterial segment was incubated with C_2-ceramide (5×10^{-6} mol/L) in addition to DHE.

Statistical Analysis

Data are presented as mean$\pm$SEM. The significance differences in mean values between and within multiple groups were examined by using ANOVA for repeated measures, followed by a Duncan multiple range test (Sigmastat). A value of $P<0.05$ was considered statistically significant.

Results

Effect of Ceramide on EDVD and Endothelial [NO] in Small Coronary Arteries

Endothelium-Dependent Vasodilation

Concentration-response curves of the endothelium-dependent vasodilators BK and A23187 were determined before and after the treatment of arteries with C_2-ceramide (10^{-5} mol/L for 30 minutes). As shown in Figure 1, BK and A23187 produced a concentration-dependent vasodilation in small coronary arteries. Pretreatment of the arteries with ceramide significantly attenuated the vasodilator responses to BK (Figure 1A) and A23187 (Figure 1B), with maximal inhibitions of 40% and 60%, respectively. In contrast, ceramide had no effect on the vasodilation induced by endothelium-independent vasodilator DETA NONOate (Figure 1C).

To determine whether C_2-ceramide acts through an NO-dependent mechanism in the inhibition of EDVD, the arteries were pretreated with the NOS inhibitor L-NAME (10^{-4} mol/L) in the absence or presence of C_2-ceramide (10^{-5} mol/L). As shown in Figure 2, L-NAME markedly attenuated the vasodilator responses to BK and A23187. This confirmed that the response to these vasodilators was NO dependent. In the presence of L-NAME, ceramide produced no further inhibition of BK- or A23187-induced vasodilation. The inhibition of vasodilation by ceramide and L-NAME was similar to that by ceramide or L-NAME alone.

Intracellular NO Concentration

The intracellular [NO] within the endothelial cells was measured in the endothelium of small coronary arteries. Figure 3A presents typical fluorescence microscopic images showing NO-induced DAF-2 green fluorescence within endothelial cells. Incubation of the arteries with BK (10^{-6} mol/L) produced a marked increase in NO fluorescence. In the presence of ceramide (10^{-5} mol/L), this increase was significantly inhibited. Figure 3B summarizes the results of these measurements. BK induced a rapid and time-dependent
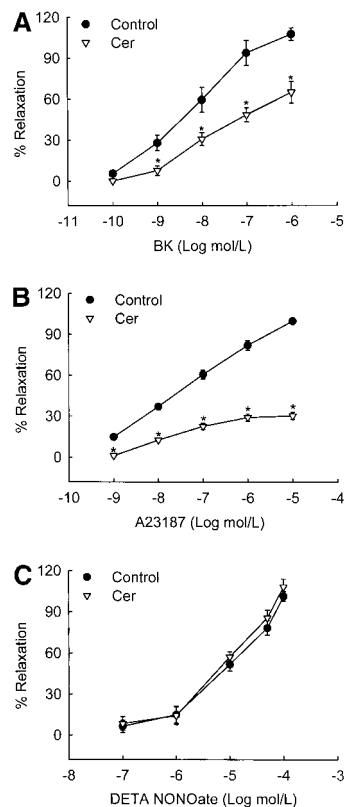

Figure 1. Effect of C_2-ceramide (Cer, 10^{-5} mol/L) on endothelium-dependent and -independent vasodilator responses to BK (n=6) (A), A23187 (n=6) (B), and DETA NONOate (n=6) (C) in small coronary small arteries. *$P<0.05$ vs control.

increase in [NO] within endothelial cells. No significant increase in [NO] was observed in control arteries throughout 30 minutes. Addition of C_2-ceramide into bath solution caused a slight decrease in basal [NO]. In the presence of C_2-ceramide, the BK-induced increase in [NO] was significantly attenuated.
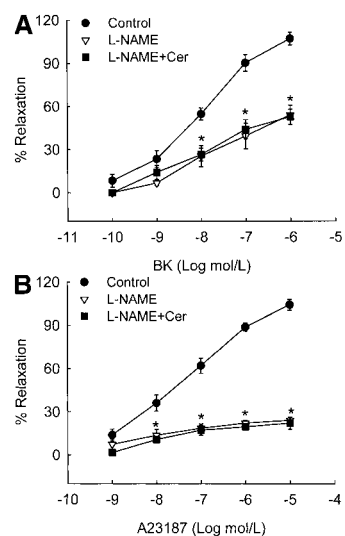

Figure 2. Effect of L-NAME (10^{-4} mol/L) on vasodilator responses to BK (n=6) (A) and A23187 (n=6) (B) in the presence or absence of Cer in small coronary small arteries. *$P<0.05$ vs control.
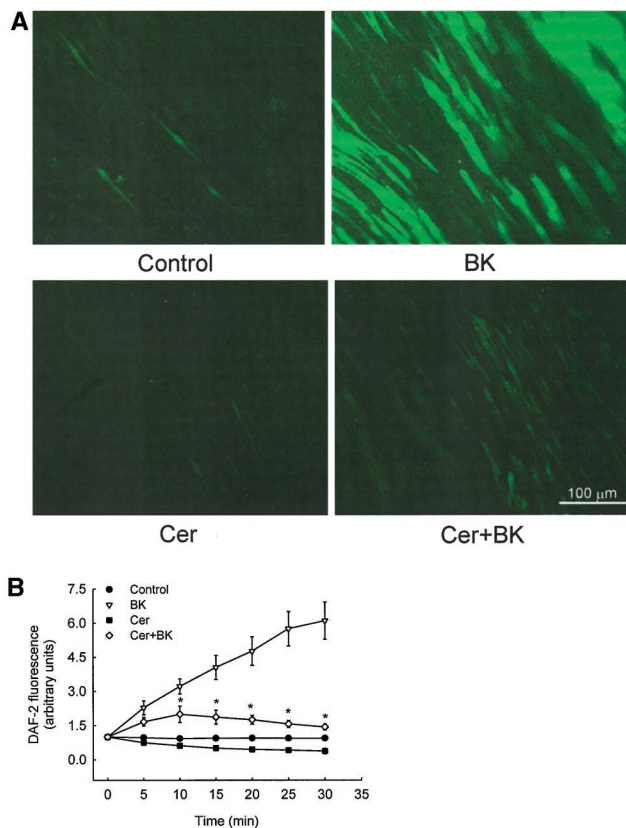
A

B

Figure 3. Effect of Cer on BK-induced increase in [NO] in the endothelium of small coronary arteries. A, Representative fluorescence images of NO in the endothelial cells taken after 30 minutes under control conditions and after treatment with BK (10^{-6} mol/L), Cer (10^{-5} mol/L), or both BK and Cer. B, Time courses for the change in NO fluorescence (n=7 to 10). *$P<0.05$ vs BK.

To confirm that the NO fluorescence in these arteries is within endothelium, the denuded arteries were used. In these arteries, only weak and nonspecific fluorescence was observed, and there was no significant change in fluorescence after BK treatment. In addition, L-NAME was used to demonstrate the dependence of the NO increase on NOS activity. In the presence of L-NAME (10^{-4} mol/L), the BK-induced increase in fluorescence was markedly blocked, suggesting that the BK-induced increase in [NO] was through the activation of NOS.

Effect of Ceramide on the Activity of Arterial and Endothelial NOS

By determining the formation rate of [³H]citrulline, the NOS activity in small coronary arterial homogenates was found to be 0.67 ± 0.09 pmol/mg protein per minute. The addition of EDTA (10^{-3} mol/L) largely inhibited the formation of [³H]citrulline (by 80%). However, C_2-ceramide at concentrations up to 4×10^{-5} mol/L had no effect on the activity of NOS in these arteries. To further examine the effect of ceramide on NOS activity in endothelial cells, C_2-ceramide was incubated with endothelial cell homogenates (Figure 4A) or intact cells (Figure 4B). Under both conditions, the NOS activity was not altered. However, L-NAME (10^{-4} mol/L) or
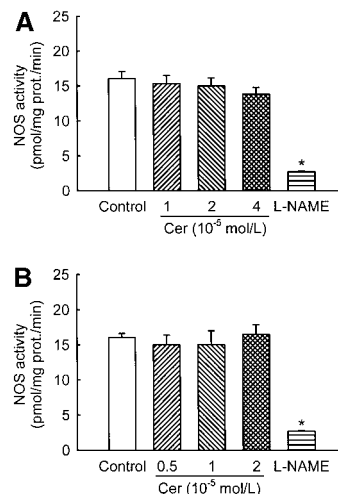

A

B

Figure 4. Effect of Cer on the activity of endothelial NOS. The homogenates of coronary endothelial cells (n=6) (A) and intact endothelial cells (n=6) (B) were incubated with Cer, and the formation of citrulline from [³H]arginine was measured. *$P<0.05$ vs control.

EDTA (10^{-3} mol/L) significantly inhibited the NOS activity of these endothelial cell preparations.

Contribution of $O_2^{-\cdot}$ to Ceramide-Induced Endothelial Dysfunction

Endothelium-Dependent Vasodilation

To explore whether ceramide-induced NO decrease and endothelial dysfunction are associated with $O_2^{-\cdot}$, the arteries were pretreated with Tiron (10^{-3} mol/L). Tiron had no effect on either basal tone or vasodilator responses to BK and A23187. However, it largely reversed the inhibitory effect of ceramide on the vasodilation to BK or A23187 (Figure 5). The inhibitory effect of ceramide was also restored by PEG-SOD (100 U/mL) but not by SOD (100 U/mL) (Figure 6).
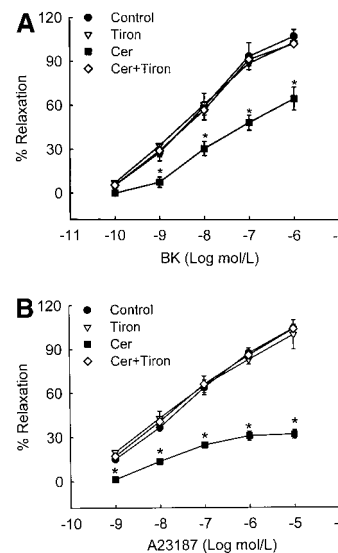

A

B

Figure 5. Effect of Tiron (10^{-3} mol/L) on Cer (10^{-5} mol/L)–induced impairment of vasodilator responses to BK (n=6) (A) and A23187 (n=6) (B). *$P<0.05$ vs control.
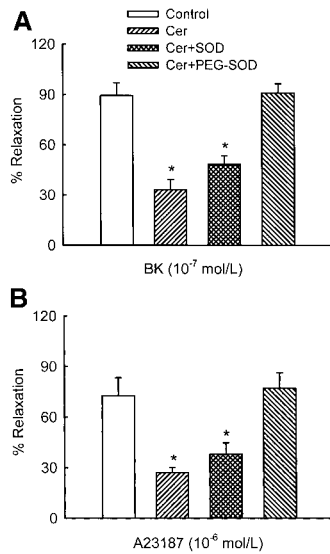
Figure 6. Effect of SOD (100 U/mL) and PEG-SOD (100 U/mL) on Cer (10^{-5} mol/L)–induced impairment of vasodilator responses to BK (n=6) (A) and A23187 (n=6) (B). *$P<0.05$ vs control.

Intracellular O_2^- Concentration

To provide direct evidence for the role of O_2^- in the action of ceramide, the intracellular $[O_2^-]$ was measured in the endothelium of small coronary arteries. Figure 7A presents the typical fluorescence microscopic images showing O_2^--
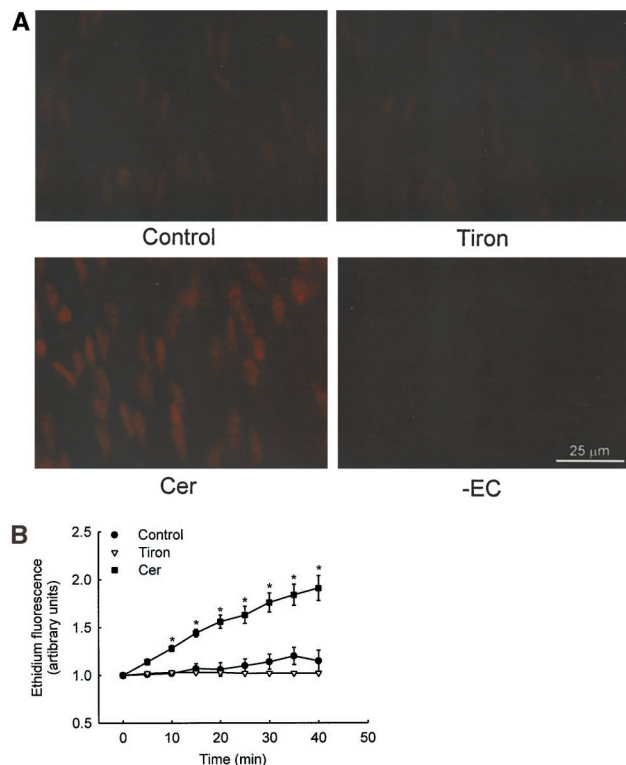

Figure 7. Effect of Cer on intracellular $[O_2^-]$ in the endothelium of coronary small arteries. A, Representative fluorescence images of O_2^- in the endothelial cells taken after 40 minutes under control conditions, after treatment with Tiron (10^{-3} mol/L) or Cer (5×10^{-6} mol/L), or after removal of endothelial cells (−EC). B, Time courses for the change in O_2^- fluorescence (n=7 to 10). *$P<0.05$ vs control.
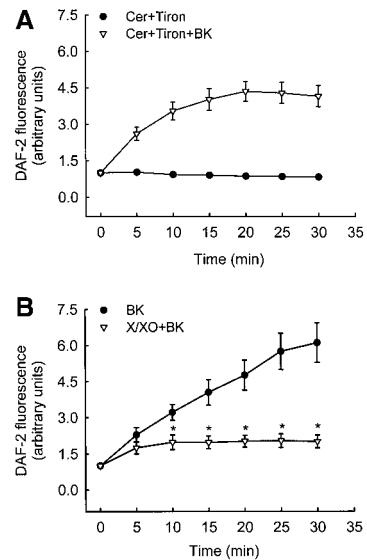

Figure 8. Effect of endogenous and exogenous O_2^- on [NO] in the endothelium of small coronary arteries. A, Effect of Tiron (10^{-3} mol/L) on the inhibitory action of Cer (10^{-5} mol/L) on endothelial [NO] under basal conditions and after treatment with BK (10^{-6} mol/L) (n=7). B, Effect of xanthine (X, 2.5×10^{-5} mol/L)/xanthine oxidase (XO, 0.1 U/mL) on BK (10^{-6} mol/L)–induced [NO] increase (n=7). *$P<0.05$ vs BK.

induced red fluorescence within endothelial cells. The nuclei were the primary fluorescent structures labeled. Incubation of the arteries with C_2-ceramide (5×10^{-6} mol/L) produced a significant increase in O_2^- fluorescence compared with control conditions. Under resting conditions, Tiron (10^{-3} mol/L) was without effect on O_2^- fluorescence, and removal of the endothelium resulted in the loss of cell-specific fluorescence, confirming that the O_2^- detected was within the endothelium. Figure 7B summarizes the results of these measurements. C_2-ceramide induced a time-dependent increase in endothelial $[O_2^-]$ compared with control conditions. Tiron was without effect on endothelial $[O_2^-]$.

Effect of Endogenous and Exogenous O_2^- on Endothelial [NO]

To determine whether O_2^- interacts with NO and thereby reduces DAF-2 fluorescence and whether ceramide reduces [NO] through O_2^-, the endothelial [NO] was measured in the presence of Tiron or an O_2^--producing system. Consistent with the results in arterial preparations, the inhibitory effect of ceramide on the BK-induced increase in [NO] was largely restored by pretreatment with 10^{-3} mol/L Tiron (Figure 8A), suggesting that O_2^- can decrease [NO] within endothelial cells.

To further confirm this interaction of O_2^- and NO, the arteries were treated with xanthine (2.5×10^{-5} mol/L)/purified xanthine oxidase (0.1 U/mL), a well-known O_2^--producing system, 15 minutes before BK (10^{-6} mol/L). It was found that xanthine/xanthine oxidase significantly attenuates the BK-induced increase in endothelial [NO] (Figure 8B).

Discussion

The present study demonstrated that ceramide inhibited BK- and A23187-induced vasodilation in small coronary arteries.

In the presence of L-NAME, ceramide had no further inhibition on the responses to these vasodilators. However, ceramide was without effect on the vasodilation induced by the endothelium-independent vasodilator DETA NONOate. These results suggest that ceramide has a detrimental effect on NO-mediated EDVD in coronary microcirculation and thereby may lead to endothelial dysfunction.

Recently, there has been accumulating evidence suggesting that ceramide and other sphingolipids play an important role in regulating vascular function. These sphingolipids may affect cell proliferation and differentiation, cell apoptosis, and vasomotion of different arteries.[7,8] With respect to vasomotor regulation, cell-permeable ceramide and/or sphingomyelinase treatment has been shown to induce the relaxation of phenylephrine-contracted rat thoracic aorta[9,11] but to produce contraction in canine cerebral arteries, rat mesenteric resistance and capacitance vessels, and bovine coronary resistance arteries.[10,12] It seems that the effects of ceramide on vasomotor response are dependent on the species, vascular beds, or arterial size used in different studies. The present studies have demonstrated that ceramide attenuates L-NAME–sensitive EDVD, providing the first direct evidence that ceramide impairs NO-mediated EDVD in coronary resistant arteries. This effect of ceramide on EDVD is similar to that of another sphingolipid, sphingosine. It has been reported that sphingosine attenuates thrombin- and A23187-induced relaxation without affecting sodium nitroprusside–induced relaxation in pig coronary rings. The impairment of EDVD induced by sphingosine was shown to contribute to vascular dysfunction in atherosclerotic arteries.[35] In those studies, sphingosine was proposed to inhibit endothelial NOS activity by a calmodulin-dependent mechanism, inasmuch as it may directly bind to calmodulin and thereby inhibit the activity of calmodulin-dependent enzymes. In addition, sphingosine may modify receptor–G-protein coupling by altering membrane fluidity, thus leading to impaired agonist-induced release of NO.[35] However, there is no direct evidence supporting those mechanisms for the action of sphingosine.

In the present study, it was also found that ceramide exhibits a greater inhibitory effect on A23187-induced EDVD than on BK-induced EDVD. Treatment of the arteries with ceramide almost completely abolished the vasodilator response to A23187, whereas it inhibited BK-induced vasodilation by $\approx$40%. This difference in the inhibitory effects of ceramide on vasodilator responses to A23187 and BK was comparable to that obtained in L-NAME–pretreated arteries. Previous studies have shown that BK stimulates endothelial cells to release endothelium-derived hyperpolarizing factor (EDHF) in addition to NO,[36] and it is likely that A23187-induced EDVD is primarily mediated by NO, whereas BK-induced EDVD is associated with both NO and EDHF in the present study. These results provide further evidence that a deficiency of NO is mainly responsible for ceramide-induced impairment of EDVD. This view is also supported by the findings that ceramide did not significantly affect the vasodilator response of coronary arteries to arachidonic acid (authors' unpublished data, 2000), which primarily stimulates the release of EDHFs, such as epoxyeicosatrienoic acids from endothelial cells of the coronary arteries.[37] Although these studies are preliminary, they suggest selective impairment of NO-mediated vasodilation by ceramide in coronary arteries.

To explore the mechanisms mediating the action of ceramide, we examined the effect of ceramide on intracellular [NO] and NOS activity in the endothelium. As expected, BK was found to induce a marked and time-dependent increase in [NO] in the endothelium of these freshly dissected arteries. In the presence of ceramide, the BK-induced [NO] increase was significantly attenuated. These results provide a direct evidence that the inhibitory effect of ceramide on EDVD is mediated by decreasing [NO] in the endothelium. However, a previous study has found that ceramide increases [NO] in cultured bovine aortic endothelial cells.[38] The reason for this discrepancy is unclear. It is possible that compared with cultured aortic endothelial cells, the endothelium of freshly isolated small coronary arteries may exhibit different behavior in NO regulation. Moreover, the ceramide-induced activation of endothelial NOS in cultured cells[38] did not occur in our freshly isolated coronary arteries. By measuring the conversion rate of L-arginine to citrulline, we found that ceramide had no effect on NOS activity. Our findings indicate that the inhibitory effect of ceramide on endothelial [NO] is not associated with the changes in NOS activity.

Numerous studies have been performed to examine the regulation of NO production in the arterial endothelium and to explore the mechanism of NO-mediated endothelial dysfunction in various pathological states, such as myocardial ischemia and reperfusion, atherosclerosis, and hypertension.[23,24] However, most of those studies used pharmacological interventions to block or enhance NOS activity and then examined the alteration of endothelial function, such as EDVD. There were few studies to address these issues by directly measuring endothelial NO. By using a novel fluorescent NO indicator and freshly isolated coronary small arteries, we were able to detect intracellular [NO], whereby changes in [NO] within endothelial cells in response to endothelium-dependent vasodilators, such as BK and A23187, could be dynamically examined. This preparation provides a valuable model for studying NO regulation and NO-mediated endothelial dysfunction in the vasculature.

To further ascertain how ceramide reduces endothelial [NO] and consequently inhibits EDVD in coronary arteries, the role of $O_2^{-\cdot}$ was examined. Recent studies have indicated that ceramide and/or other sphingolipids may stimulate the production of $O_2^{-\cdot}$ in vascular cells.[29,39,40] Because $O_2^{-\cdot}$ can interact with NO and thereby decrease [NO] within endothelial cells,[25–28] we hypothesized that ceramide may stimulate the $O_2^{-\cdot}$ production in the vascular endothelium, which then interacts with NO and results in the impairment of EDVD in coronary circulation. The present study provided several lines of evidence to support this hypothesis. First, pretreatment of the arteries with Tiron, an antioxidant that is capable of scavenging $O_2^{-\cdot}$ from both the intracellular and extracellular environment,[41] prevented the ceramide-induced inhibition of NO production and endothelial dysfunction. Second, ceramide significantly increased $[O_2^{-\cdot}]$ in the endothelium, as measured by DHE fluorescence microscopy. Finally, exogenous $O_2^{-\cdot}$ produced by xanthine/xanthine oxidase inhibited the NO increase in the endothelium.

The cellular site at which O_2^- is produced by ceramide and O_2^- interacts with NO in the coronary arteries is still undetermined. Although pretreatment of the arteries with SOD, a cell-impermeable O_2^- scavenger, did not prevent the inhibitory effect of ceramide on EDVD, PEG-SOD significantly blocked the action of ceramide. It seems that O_2^- from either the intracellular or extracellular space contributes to the ceramide-induced impairment of EDVD. With respect to extracellular O_2^-, it is possible that O_2^- stimulated by ceramide is released or transferred into the vascular interstitium,[42–44] where it interacts with NO and consequently blocks the action of NO. This interstitial O_2^- can be scavenged by PEG-SOD, a chemically modified SOD that may exhibit enhanced adherence to the cell surface compared with native SOD adherence. It is also possible that the coupling of PEG may increase the cell permeability of SOD into endothelial cells, thereby increasing its efficiency in scavenging the O_2^- induced by ceramide. Indeed, a previous study has shown that catalase and SOD can be effectively delivered into endothelial cells from the liver by chemical modification (ie, succinylation).[45]

In the cardiovascular system, there are several potential enzymatic sources of O_2^- or other reactive oxygen species, including NAD(P)H oxidase, xanthine oxidase, NO synthase, and the mitochondrial respiratory chain.[23,24] Previous studies have shown that ceramide activates NAD(P)H oxidase and increases the O_2^- production in human aortic smooth muscle cells.[29] Ceramide has also been shown to interact with the mitochondrial electron transport chain, leading to the generation of reactive oxygen species.[46,47] Further studies are required to determine whether ceramide increases the production of O_2^- in the coronary arterial endothelium through these or other mechanisms.

The present study did not attempt to determine the pathogenic significance of ceramide-induced endothelial dysfunction in the coronary circulation. Previous studies have shown that ceramide or other sphingolipids are increased in cardiac tissue during myocardial ischemia and reperfusion, which may contribute to the detrimental effects of TNF-α or other cytokines under these conditions.[15,48] Given the important role of ceramide in mediating the effects of cytokines, it is plausible that increased cytokines during myocardial ischemia/reperfusion increase ceramide levels in the coronary vascular endothelium or other tissues and that ceramide, in turn, causes the impairment of EDVD. In this respect, ceramide may represent an important signaling molecule mediating endothelial dysfunction in the coronary circulation during myocardial ischemia/reperfusion.

In summary, the present study has demonstrated that ceramide inhibits NO-mediated EDVD in small coronary arteries. It has been suggested that the inhibitory effect of ceramide is associated with increased [O_2^-] and the subsequent decrease in [NO] in endothelial cells of the coronary arteries. These results led to a hypothesis that ceramide-induced inhibition of EDVD may contribute to vascular endothelial dysfunction of the coronary circulation associated with cytokines, such as TNF-α, during myocardial ischemia and reperfusion.

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