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*Am J Physiol Heart Circ Physiol* 284:605-612, 2003. First published Nov 7, 2002;  
doi:10.1152/ajpheart.00697.2002

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# Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries

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**Zhang, David X., Ai-Ping Zou, and Pin-Lan Li.** Ceramide-induced activation of NADPH oxidase and endothelial dysfunction in small coronary arteries. *Am J Physiol Heart Circ Physiol* 284: H605–H612, 2003;10.1152/ajpheart.00697.2002.—We tested the hypothesis that ceramide induces endothelial dysfunction in small coronary arteries via NADPH oxidase-mediated superoxide and resulting peroxynitrite formation. With the use of dihydroethidium as a superoxide indicator, C<sub>2</sub>-ceramide was found to increase superoxide production in the endothelial cells of small coronary arteries, which was inhibited by the NADPH oxidase inhibitors N-vanillylnonanamide, apocynin, and diphenylene iodonium. NADPH oxidase expression was confirmed in endothelial cells, as indicated by the immunoblotting of its subunits gp91<sup>phox</sup> and p47<sup>phox</sup>. C<sub>2</sub>-ceramide increased NADPH oxidase activity by 52%, which was blocked by NADPH oxidase inhibitors but not by inhibitors of NO synthase, xanthine oxidase, and mitochondrial electron transport chain enzymes. By Western blot analysis, ceramide-induced NADPH oxidase activation was found to be associated with the translocation of p47<sup>phox</sup> to the membrane. In isolated and pressurized small coronary arteries, N-vanillylnonanamide, apocynin, or uric acid, a peroxynitrite scavenger, largely restored the inhibitory effects of ceramide on bradykinin- and A-23187-induced vasorelaxation. With the use of nitrotyrosine as a marker, C<sub>2</sub>-ceramide was found to increase peroxynitrite in small coronary arteries, which could be blocked by uric acid. We conclude that NADPH oxidase-mediated superoxide production and subsequent peroxynitrite formation mediate ceramide-induced endothelial dysfunction in small coronary arteries.

lipids; vasorelaxation; endothelium; signal transduction; free radicals; nitric oxide

CERAMIDE, a sphingolipid, has been reported to serve as a signaling molecule in different cell types, including vascular endothelial cells, where it is involved in the regulation of numerous cellular processes, such as ion channel activity, endothelial cell proliferation and apoptosis, and vasomotor responses (7, 15, 16, 19, 27). With respect to vasomotor regulation, previous studies from our laboratory have shown that ceramide attenuates endothelium-dependent vasorelaxation in bovine small coronary arteries, which is associated with an increase in superoxide (O<sub>2</sub><sup>-</sup>) production and a subsequent de-

crease in nitric oxide (NO) concentrations in vascular endothelial cells (31). A ceramide metabolite, sphingosine has also been reported to have a similar inhibitory effect on endothelium-dependent vasorelaxation in pig coronary rings (26). However, the underlying mechanism by which ceramide increases endothelial O<sub>2</sub><sup>-</sup> and subsequently causes endothelial dysfunction has yet to be determined.

In the cardiovascular system, there are several potential enzymatic sources of O<sub>2</sub><sup>-</sup> or other reactive oxygen species (ROS), including NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain, and NO synthase (NOS) (5, 13). Recently, a gp91<sup>phox</sup>-containing phagocyte-type NADPH oxidase has been identified in vascular endothelial cells and is thought to be an important source of O<sub>2</sub><sup>-</sup> in these cells (2, 12, 13, 21). The endothelial NADPH oxidase can be activated by a number of stimuli, such as phorbol esters, tumor necrosis factor- $\alpha$ , angiotensin II, and pulsatile stretch (8, 20, 25). The activation of NADPH oxidase and subsequent increase in O<sub>2</sub><sup>-</sup> production are implicated in the pathophysiology of various cardiovascular diseases including hypertension, atherosclerosis, and diabetes-associated vascular injuries (5, 13). However, it remains unknown how NADPH oxidase is activated under these pathological conditions.

The present study was designed to test the hypothesis that ceramide as an intracellular signaling molecule activates NADPH oxidase to produce O<sub>2</sub><sup>-</sup> and thereby induces endothelial dysfunction by peroxynitrite formation in endothelial cells. We determined NADPH oxidase activity in coronary endothelial cells and its role in ceramide-induced O<sub>2</sub><sup>-</sup> production using dihydroethidium-based fluorescence analysis and isolated small coronary arterial preparations. We then measured peroxynitrite-induced nitrotyrosine formation after incubation of the arteries with ceramide and observed the effects of peroxynitrite blockade on ceramide-induced endothelial dysfunction in these arteries.

## MATERIALS AND METHODS

*Measurement of intracellular O<sub>2</sub><sup>-</sup> in the endothelium.* Intracellular O<sub>2</sub><sup>-</sup> was measured by fluorescence imaging analysis with dihydroethidium. Dihydroethidium can enter the

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cell and be oxidized by  $O_2^-$  to yield ethidium. Ethidium binds to DNA, which produces strong red fluorescence (6). Assays were performed on the endothelium of isolated small bovine coronary arteries as we described previously (31). Briefly, small intramural arteries (200–400  $\mu\text{m}$  internal diameter) were incubated with 3  $\mu\text{M}$  dihydroethidium (Molecular Probes) with the lumen side upward. The ethidium fluorescence was measured at 490-nm excitation and 610-nm emission. Fluorescent images were captured and analyzed using a personal computer-controlled charge-coupled device camera and MetaMorph software. Previous studies (31) in our laboratory have demonstrated that  $O_2^-$  detected by dihydroethidium in this preparation was located in the endothelium.  $O_2^-$  fluorescence was measured every 5 min in a single area of the endothelial layer for 40 min. Results were expressed as percent changes in averaged fluorescence intensity compared with basal fluorescence within the area imaged.

**Culture of coronary arterial endothelial and smooth muscle cells.** Bovine coronary arterial endothelial cells (BCAECs) and bovine coronary arterial smooth muscle cells (BCASMCs) were cultured as described previously (30). The endothelial and smooth muscle cells were maintained in RPMI 1640 containing 20% FCS, 1% glutamine, and 1% antibiotic solution and in medium 199 containing 10% FCS, 1% glutamine, 1% antibiotic solution, 0.3% gentamycin, 0.3% nystatin, and 0.1% tylosin, respectively. All cells were maintained in an incubator with 5%  $\text{CO}_2$  in air at 37°C and used at passages 3–4.

**Preparation of homogenates and subcellular fractions.** The homogenates, cytosol, or membrane (microsomes) fractions were prepared from cultured BCAECs or BCASMCs as reported previously (30). The protein concentrations were determined by the method of Bradford (Bio-Rad Protein Assays). To evaluate the “purity” of the membrane fraction, the activity of lactate dehydrogenase, a marker enzyme for the cytosol, was assessed using a Sigma diagnostic kit according to the manufacturer’s instructions.

**Western blot analysis.** Western blot analysis was used to determine the relative quantities of cellular or tissue gp91<sup>phox</sup>, p47<sup>phox</sup>, and nitrotyrosine. Briefly, equal amounts (20–40  $\mu\text{g}$ ) of tissue or cell homogenates, cytosol, or microsomes were loaded and then separated by 12% SDS-PAGE (23). The proteins of these samples were then electrophoretically transferred at 100 V for 1 h onto nitrocellulose membranes. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 and probed at room temperature with a monoclonal antibody against gp91<sup>phox</sup> (1:1,000 dilution for 2 h, Transduction Laboratories), a monoclonal antibody against p47<sup>phox</sup> (1:500 dilution for 2 h, Transduction Laboratories), or a polyclonal anti-nitrotyrosine antibody (1:5,000 dilution for 1 h, Upstate Biotechnology). After being washed, the membranes were incubated for 1 h with a horseradish peroxidase-conjugated rabbit anti-mouse IgG (Amersham Pharmacia Biotech) at a dilution of 1:5,000 for monoclonal primary antibodies and with a horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) at a dilution of 1:20,000 for polyclonal primary antibodies. Membranes were washed, incubated for 1 min with SuperSignal West Pico detection reagents (Pierce), wrapped in Saran Wrap, and then exposed to Kodak Omat film. For nitrotyrosine blots, the results were also interpreted and confirmed by another observers without knowledge of the experimental protocol and by scanning and quantitation of different bands using UN-SCAN-IT gel software (Silk Scientific).

**Fluorescence spectrometric assay of  $O_2^-$  production.** A dihydroethidium-based fluorescence spectrometric assay, as recently used in our studies (32), was used to assess  $O_2^-$

production from NADPH oxidase in BCAECs. Briefly, homogenates (20  $\mu\text{g}$ ) freshly prepared from BCAECs were incubated with dihydroethidium (5  $\mu\text{M}$ ) and salmon testes DNA (0.5 mg/ml) in 200  $\mu\text{l}$  PBS. Immediately before fluorescence was recorded, NADPH (final concentration 100  $\mu\text{M}$ ) was added, and ethidium fluorescence was measured using a fluorescence microplate reader (Bio-Tek FL600). To confirm the specificity of the NADPH inhibitors *N*-vanillylnonanamide, apocynin, and diphenylene iodonium, the effects of these inhibitors on mitochondrial respiratory chain-derived  $O_2^-$  was determined using the same assay for NADPH oxidase except that succinate (5 mM) was used as we described previously (32).

**Isolated small coronary artery preparation.** Small intramural coronary arteries (100–200  $\mu\text{m}$  internal diameter) were carefully dissected and cannulated with two glass micropipettes in a water-jacketed perfusion chamber, as we described previously (30). The arteries were pressurized to 60 mmHg and equilibrated in physiological saline solution (PSS) at 37°C. Internal diameter of the arteries was measured with a video recording system.

After a 1-h equilibration period, the vasodilator responses to bradykinin ( $10^{-10}$ – $10^{-6}$  M) and A-23187 ( $10^{-9}$ – $10^{-5}$  M) were determined. All drugs were added into the bath solution unless otherwise indicated. The vasodilator response was expressed as the percent relaxation of U-46619-induced pre-contraction based on changes in the internal diameter. In some experiments, arteries treated with different compounds were collected at the end of protocol, homogenized, and saved for Western blot analysis of nitrotyrosine.

**Statistics.** Data are presented as means  $\pm$  SE. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan’s multiple-range test. Student’s *t*-test was used to evaluate the significant differences between two paired observations.  $P < 0.05$  was considered statistically significant.

## RESULTS

**Role of NADPH oxidase in ceramide-induced  $O_2^-$  production in the endothelium of small coronary arteries.** To determine the role of NADPH oxidase in ceramide-induced  $O_2^-$  production, arterial segments were pretreated with the NADPH oxidase inhibitors *N*-vanillylnonanamide (10  $\mu\text{M}$ ) (3, 4, 29), apocynin (100  $\mu\text{M}$ ) (14, 24), or diphenylene iodonium (DPI; 50  $\mu\text{M}$ ). The doses of these inhibitors were based on those reported in previous studies that effectively blocked NADPH oxidase activity.  $\text{C}_2$ -ceramide (5  $\mu\text{M}$ ) was then added, and  $O_2^-$  fluorescence was measured. It was found that  $\text{C}_2$ -ceramide induced a time-dependent increase in endothelial  $O_2^-$  compared with control, which was significantly attenuated by the NADPH oxidase inhibitor *N*-vanillylnonanamide (Fig. 1). *N*-vanillylnonanamide had no effect on basal  $O_2^-$  fluorescence, because the basal  $O_2^-$  level was very low. The  $\text{C}_2$ -ceramide-induced  $O_2^-$  increase was also markedly blocked by apocynin or DPI (data not shown).

**Expression of NADPH oxidase subunits.** The expression of gp91<sup>phox</sup> and p47<sup>phox</sup> proteins was detected in BCAECs by Western blot analysis with monoclonal anti-gp91<sup>phox</sup> and p47<sup>phox</sup> antibodies (Fig. 2, A and B). The anti-gp91<sup>phox</sup> antibody recognized two major bands at  $\sim 75$  and  $\sim 50$  kDa in endothelial cells but not in smooth muscle cells from bovine coronary arteries.



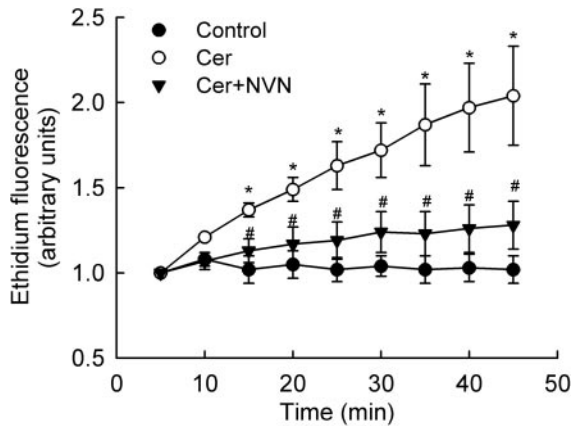


Fig. 1. Effect of the NADPH oxidase inhibitor *N*-vanillylonanamide (NVN) on the C<sub>2</sub>-ceramide (Cer)-induced O<sub>2</sub><sup>-</sup> increase in the endothelium of small coronary arteries. Time courses of changes in O<sub>2</sub><sup>-</sup> fluorescence in the endothelial cells under control conditions or after treatment with Cer (5 μM) or Cer + NVN (10 μM) are shown (n = 7–10). \*P < 0.05 vs. control; #P < 0.05 vs. Cer.

gp91<sup>phox</sup> was mainly expressed in the membrane fraction of endothelial cells with two strong bands at ~75 and ~50 kDa. This subunit was expressed in the cytosol less abundantly with a weaker ~75-kDa band. One specific band was detected at ~47 kDa with the anti-p47<sup>phox</sup> antibody in endothelial cells and smooth muscle cells from bovine coronary arteries. In contrast to gp91<sup>phox</sup>, p47<sup>phox</sup> protein was mainly present in the cytosol.

**Effect of ceramide on NADPH oxidase activity in BCAECs.** To provide further evidence that ceramide activates NADPH oxidase in endothelial cells, BCAECs were treated with C<sub>2</sub>-ceramide (5 μM for 15 min) with or without preincubation with different enzyme inhibitors. The homogenates were then prepared, and NADPH-dependent O<sub>2</sub><sup>-</sup> production was measured as described above. It was found that O<sub>2</sub><sup>-</sup> production significantly increased in endothelial cells pretreated with C<sub>2</sub>-ceramide and that this O<sub>2</sub><sup>-</sup> production was

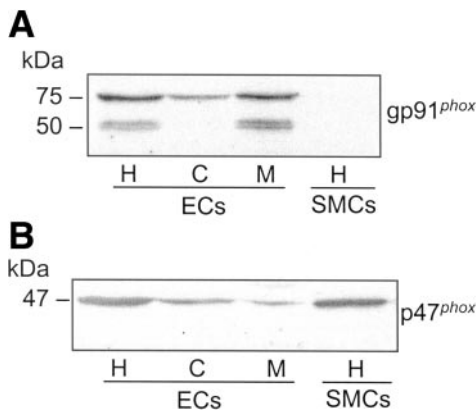


Fig. 2. Protein expression of NADPH oxidase subunits. A and B: Western blots showing expression of gp91<sup>phox</sup> and p47<sup>phox</sup> with a monoclonal antibody. H, homogenate; C, cytosol fraction; M, membrane fraction; ECs, bovine coronary arterial endothelial cells (BCAECs); SMCs, bovine coronary arterial smooth muscle cells (BCASMCs). n = 3.

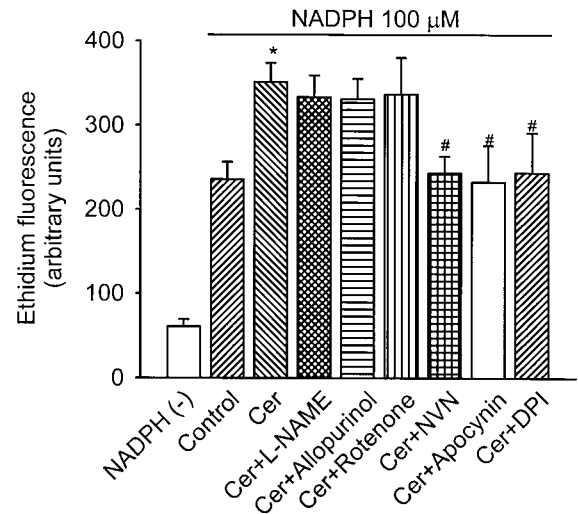


Fig. 3. Effect of Cer on NADPH oxidase activity in BCAECs. The NADPH-dependent O<sub>2</sub><sup>-</sup> production in BCAECs treated with vehicle or Cer (5 μM) in the absence or presence of the different enzyme inhibitors N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 100 μM), allopurinol (100 μM), rotenone (50 μM), NVN (10 μM), apocynin (100 μM), or diphenylene iodonium (DPI; 50 μM) are shown (n = 7–10). \*P < 0.05 vs. control; #P < 0.05 vs. Cer.

markedly inhibited by *N*-vanillylonanamide (10 μM), apocynin (100 μM), or DPI (50 μM), but not by N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME; 100 μM), a NOS inhibitor, allopurinol (100 μM), a xanthine oxidase inhibitor, or rotenone (50 μM), a mitochondrial electron transport chain blocker (Fig. 3).

To exclude potential scavenging of mitochondria-generated O<sub>2</sub><sup>-</sup> by NADPH oxidase inhibitors, the effects of these inhibitors on mitochondrial respiratory chain-derived O<sub>2</sub><sup>-</sup> production were measured. As shown in Fig. 4, *N*-vanillylonanamide (10 μM), apocynin (100 μM), and DPI (50 μM) at the doses that markedly inhibit NADPH oxidase activity had no significant effect on mitochondria-derived O<sub>2</sub><sup>-</sup>. In con-

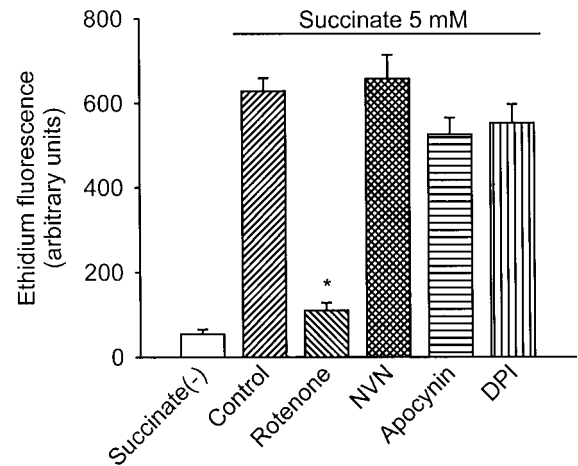


Fig. 4. Mitochondria-derived O<sub>2</sub><sup>-</sup> production in the absence or presence of the different enzyme inhibitors rotenone (50 μM), NVN (10 μM), apocynin (100 μM), or DPI (50 μM) (n = 7–10). \*P < 0.05 vs. control.

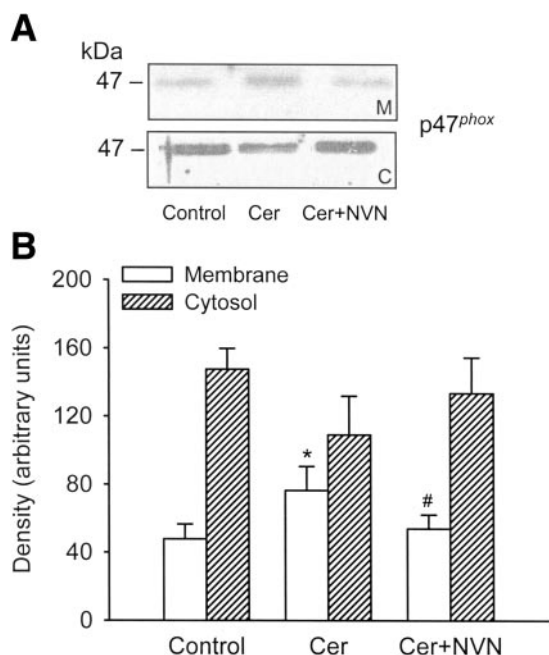


Fig. 5. Effect of Cer on p47<sup>phox</sup> translocation in BCAECs. **A**: immunoblot for p47<sup>phox</sup> in membrane and cytosol fractions of BCAECs treated with vehicle, Cer (5  $\mu$ M), or Cer + NVN (10  $\mu$ M). **B**: summarized data ( $n = 4$ ). \* $P < 0.05$  vs. control; # $P < 0.05$  vs. Cer.

trast, this mitochondrial  $O_2^{\cdot-}$  was significantly blocked by rotenone (50  $\mu$ M).

**Effect of ceramide on p47<sup>phox</sup> translocation in BCAECs.** We then determined whether ceramide-induced activation of NADPH oxidase is associated with the translocation of p47<sup>phox</sup> in endothelial cells, because this translocation mechanism has been reported to play an important role in the activation of NADPH oxidase (1, 10, 18). BCAECs were treated with C<sub>2</sub>-ceramide (5  $\mu$ M for 15 min), membrane and cytosolic fractions of these cells were then prepared, and immunoblotting for p47<sup>phox</sup> was performed. As shown in Fig. 5, stimulation of endothelial cells with C<sub>2</sub>-ceramide induced a quick p47<sup>phox</sup> translocation to the membrane. *N*-vanillylnonanamide (10  $\mu$ M) inhibited C<sub>2</sub>-ceramide-stimulated p47<sup>phox</sup> translocation. The purity of the membrane fraction was confirmed by measuring the activity of lactate dehydrogenase, because this enzyme is only present in the cytosol. We found that lactate dehydrogenase activity was enriched in the cytosolic fraction, with a low activity in the membrane fraction. The activities of this enzyme in the homogenates, cytosol, and membrane fractions were  $1.04 \pm 0.07$ ,  $2.92 \pm 0.41$ , and  $0.47 \pm 0.05$  nmol $\cdot$ min<sup>-1</sup> $\cdot$  $\mu$ g protein<sup>-1</sup>, respectively. C<sub>2</sub>-ceramide treatment did not alter this distribution of lactate dehydrogenase activity. This indicates that despite a possible minor contamination in the membrane fraction by the cytosol, our preparations of subcellular fractions were not different before and after C<sub>2</sub>-ceramide treatment.

**Role of NADPH oxidase in ceramide-induced endothelial dysfunction in small coronary arteries.** Concentration-response curves of the endothelium-dependent

vasodilators bradykinin or A-23187 were determined before and after C<sub>2</sub>-ceramide (5  $\mu$ M, perfused into the lumen of arteries for 30 min) treatment. As shown in Fig. 6, bradykinin and A-23187 produced a concentration-dependent vasorelaxation in small coronary arteries. Pretreatment of the arteries with ceramide significantly attenuated the vasodilator responses to bradykinin (Fig. 6A) and A-23187 (Fig. 6B). *N*-vanillylnonanamide (10  $\mu$ M) had no effect on either basal tone or vasodilator responses to bradykinin and A-23187. However, it largely reversed the inhibitory effect of ceramide on bradykinin- and A-23187-induced vasorelaxation. The C<sub>2</sub>-ceramide-induced endothelial dysfunction was also markedly blocked by apocynin.

To exclude the possibility that *N*-vanillylnonanamide acts by scavenging  $O_2^{\cdot-}$  rather than by blocking C<sub>2</sub>-ceramide-stimulated  $O_2^{\cdot-}$  generation, its effect on xanthine/xanthine oxidase-induced endothelial dysfunction was determined. Xanthine (50  $\mu$ M) and xanthine oxidase (1 mU/ml) were perfused into the arterial lumen for 10 min with or without pretreatment with *N*-vanillylnonanamide (10  $\mu$ M). The effectiveness of xanthine/xanthine oxidase at this dose to produce  $O_2^{\cdot-}$

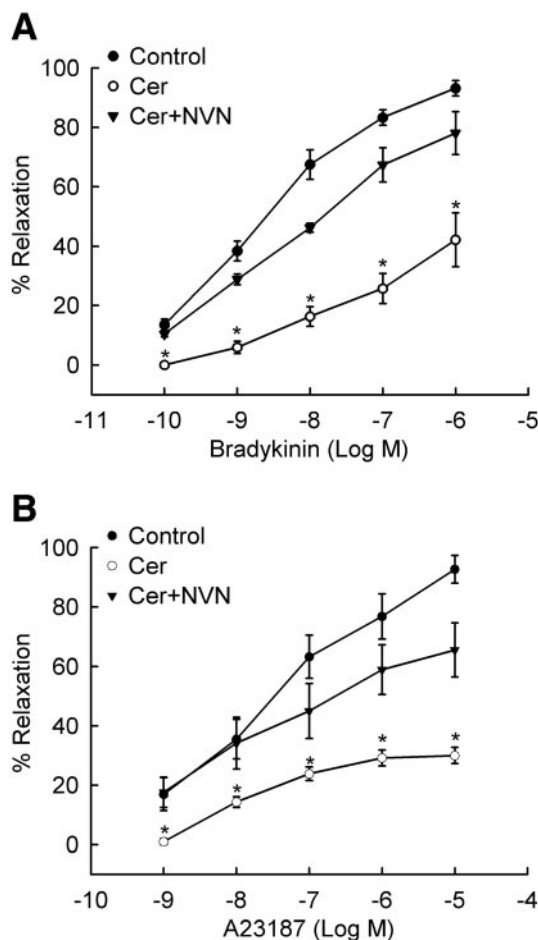


Fig. 6. Effect of NVN on Cer-induced impairment of endothelium-dependent vasorelaxation to bradykinin (**A**) and A-23187 (**B**) in small coronary arteries. The arteries were preincubated with NVN (10  $\mu$ M). Cer (5  $\mu$ M) was perfused into the lumen of arteries and incubated for 30 min ( $n = 6$ ). \* $P < 0.05$  vs. control.

was confirmed by separate experiments using the fluorescence spectrometric  $O_2^-$  assay. As expected, xanthine/xanthine oxidase significantly inhibited the vasodilator responses to bradykinin and A-23187, which were not affected by *N*-vanillylnonanamide (Fig. 7).

**Role of peroxynitrite in ceramide-induced endothelial dysfunction in small coronary arteries.** To determine whether ceramide-induced endothelial dysfunction is associated with peroxynitrite formation secondary to  $O_2^-$  production, arteries were incubated with uric acid (100  $\mu$ M), a peroxynitrite scavenger, before the addition of  $C_2$ -ceramide (5  $\mu$ M, perfused into the lumen of arteries for 30 min). It was found that uric acid also significantly prevented the ceramide-induced impairment of vasorelaxation to bradykinin and A-23187 (Fig. 8). Perfusion of the arteries with peroxynitrite (100  $\mu$ M) also significantly attenuated vasorelaxation to bradykinin but not to 2-2'-(hydroxynitrosohydrazino)bis-ethanamine, NOC-18, an endothelium-independent vasodilator (data not shown).

**Effect of ceramide on nitrotyrosine in small coronary arteries.** The nitrotyrosine is a biological marker of tissue peroxynitrite. By Western blot analysis, we

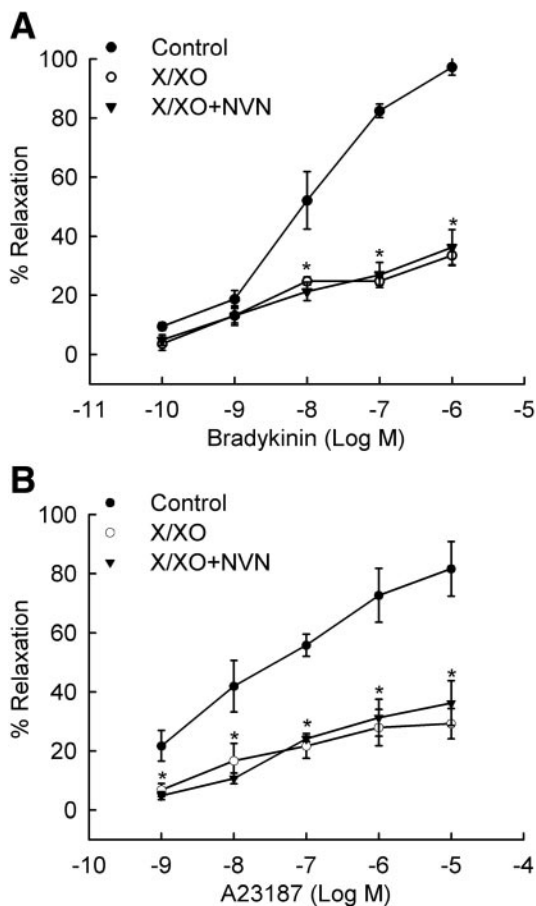


Fig. 7. Effect of NVN on xanthine/xanthine oxidase (X/XO)-induced impairment of endothelium-dependent vasorelaxation to bradykinin (A) and A-23187 (B) in small coronary arteries. Arteries were preincubated with NVN (10  $\mu$ M). Xanthine (50  $\mu$ M) and xanthine oxidase (1 mU) was perfused into the lumen of arteries and incubated for 10 min ( $n = 6$ ). \* $P < 0.05$  vs. control.

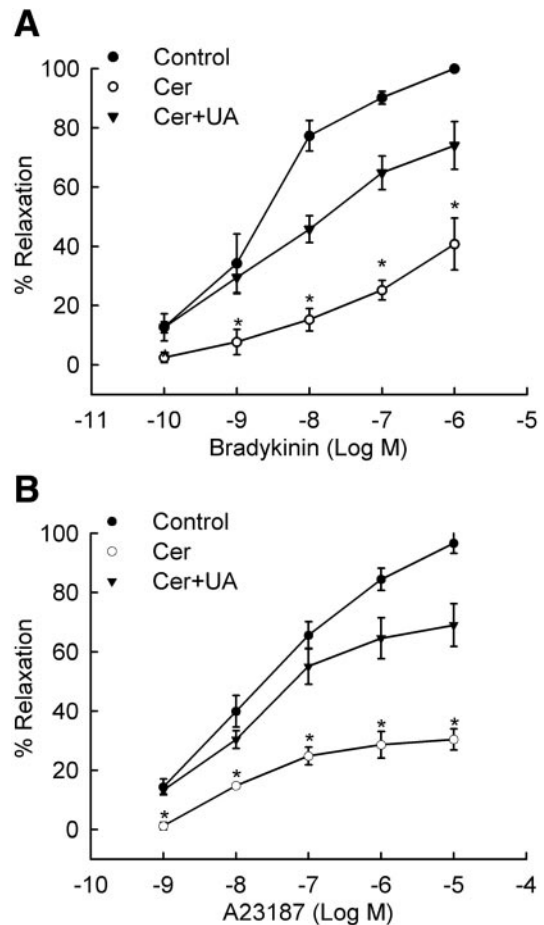


Fig. 8. Effect of the peroxynitrite scavenger uric acid (UA) on Cer-induced impairment of endothelium-dependent vasorelaxation to bradykinin (A) and A-23187 (B) in small coronary arteries. Arteries were preincubated with UA (100  $\mu$ M). Cer (5  $\mu$ M) was perfused into the lumen of arteries and incubated for 30 min ( $n = 6$ ). \* $P < 0.05$  vs. control.

found that treatment of the arteries with  $C_2$ -ceramide (5  $\mu$ M, perfused into the lumen of arteries for 30 min) markedly increased the number and amount of proteins with nitrotyrosine epitopes. This  $C_2$ -ceramide effect, however, was significantly blocked by the pre-treatment of the arteries with uric acid (100  $\mu$ M; Fig. 9).

**DISCUSSION**

In the present study, we found that gp91<sup>phox</sup>- and p47<sup>phox</sup>-containing NADPH oxidase is present in the endothelial cells of small coronary arteries and contributes importantly to ceramide-induced vascular  $O_2^-$  production and endothelial dysfunction in these arteries. The activation of endothelial NADPH oxidase by ceramide is associated with a rapid translocation of the cytosolic subunit p47<sup>phox</sup> to the cytoplasmic membrane of endothelial cells. In addition, ceramide-induced endothelial dysfunction is largely mediated by the formation of peroxynitrite resulting from increased  $O_2^-$  production in endothelial cells.



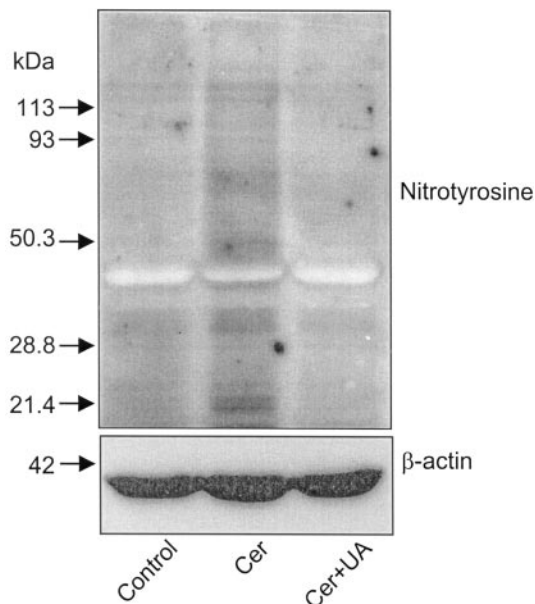


Fig. 9. Effect of Cer on peroxynitrite-induced nitrotyrosine formation in small coronary arteries. Arteries were preincubated with UA (100  $\mu$ M). Cer (5  $\mu$ M) was perfused into the lumen of arteries and incubated for 30 min using the same protocol as vasorelaxation studies. The homogenates of these arteries were prepared, followed by nitrotyrosine detection using Western blotting ( $n = 4$ ).

Recently, a gp91<sup>phox</sup>-containing phagocyte-type NADPH oxidase has been reported in endothelial cells of various vascular beds (2, 12, 13, 21). In the present study, we found that the NADPH oxidase is also expressed in endothelial cells of small coronary arteries, as indicated by the immunoblotting of gp91<sup>phox</sup> and p47<sup>phox</sup>, which are the major membrane and cytosolic subunits, respectively. In agreement with previous reports (13), gp91<sup>phox</sup> is expressed in endothelial cells but not in smooth muscle cells, while the p47<sup>phox</sup> found in both types of cells. The anti-gp91<sup>phox</sup> antibody used in the present study recognized two major protein bands at  $\sim$ 75 and  $\sim$ 50 kDa, a pattern similar to endothelial cells of other vascular beds and thought to represent the presence of variably glycosylated forms of NADPH oxidase (21). The gp91<sup>phox</sup> subunit was primarily present in the 100,000-g membrane fraction, suggesting its membrane localization. This is generally consistent with previous studies indicating that the activity of NADPH oxidase is mainly detected in the membrane fraction of the vasculature (13, 28). However, the possibility of other subcellular distribution of gp91<sup>phox</sup> (i.e., in the endoplasmic reticulum, which is also present in the membrane fraction) cannot be excluded. With the use of fluorescent confocal microscopy, it has been shown recently that gp91<sup>phox</sup> may colocalize with the endoplasmic reticulum marker in endothelial cells (2, 22). In contrast to the membrane-associated gp91<sup>phox</sup>, p47<sup>phox</sup> was primarily detected in the cytosolic fraction of endothelial cells, which is consistent with general notion regarding the cytosolic localization of p47<sup>phox</sup> in resting endothelial cells (13).

Recently, we (31) reported that ceramide stimulates O<sub>2</sub><sup>-</sup> production in endothelial cells and consequently

induces endothelial dysfunction in small coronary arteries. However, the underlying mechanism of O<sub>2</sub><sup>-</sup> production remains unknown. In the present study, we provided several lines of evidence indicating that NADPH oxidase may mediate ceramide-induced O<sub>2</sub><sup>-</sup> production in coronary endothelial cells. First, pre-treatment of the arteries with NADPH oxidase inhibitors markedly attenuated the ceramide-induced increase in endothelial O<sub>2</sub><sup>-</sup> in the endothelium of isolated small coronary arteries by using fluorescence imaging analysis. Second, ceramide significantly stimulated the activity of NADPH oxidase in endothelial cells. Finally, inhibition of NADPH oxidase by different NADPH oxidase inhibitors largely prevented ceramide-induced and O<sub>2</sub><sup>-</sup>-mediated endothelial dysfunction in small bovine coronary arteries.

In previous studies, ceramide has been shown to interact with the mitochondrial electron transport chain, leading to generation of O<sub>2</sub><sup>-</sup> or other ROS in isolated rat mitochondria (9, 11). Therefore, there was a concern about the specificity of NADPH oxidase inhibitors used in the present study. However, it is confirmed that those NADPH oxidase inhibitors with different mechanisms of action, at the doses that consistently inhibit ceramide-induced O<sub>2</sub><sup>-</sup> production and endothelial dysfunction, had no significant effects on mitochondrial O<sub>2</sub><sup>-</sup> production. In addition, the inhibitors of mitochondrial electron transport chain enzymes and other potential O<sub>2</sub><sup>-</sup>-generating enzymes including NOS and xanthine oxidase had no effect on ceramide-induced O<sub>2</sub><sup>-</sup> production in coronary arterial endothelial cells. These results further support the view that ceramide activates NADPH oxidase to produce O<sub>2</sub><sup>-</sup> and consequently results in endothelial dysfunction.

Although NADPH oxidase has been implicated in the signaling of ceramide effect in endothelial cells, the mechanism by which ceramide activates this enzyme has yet to be determined. In the present study, ceramide-induced activation of NADPH oxidase was found to be associated with a rapid translocation of p47<sup>phox</sup> to the cytoplasmic membrane. Given that p47<sup>phox</sup> translocation leads to the recruitment of other cytosolic subunits of NADPH oxidase to the membrane and then the activation of this enzyme in phagocytes (1, 18), these data suggest that p47<sup>phox</sup> translocation may be also involved in ceramide-induced activation of NADPH oxidase in endothelial cells of small coronary arteries. In line with these findings, it has been reported recently that p47<sup>phox</sup> phosphorylation and subsequent translocation to the membrane are also critical in the activation of endothelial NADPH oxidase induced by tumor necrosis factor- $\alpha$  (10, 20). Because ceramide mediates various actions of cytokines (i.e., tumor necrosis factor- $\alpha$ ) in a number of cells, including endothelial cells (7, 15, 16, 19, 27), it is possible that ceramide serves as a signaling molecule in NADPH oxidase activation induced by cytokines in endothelial cells.

With respect to the mechanism for the action of O<sub>2</sub><sup>-</sup> to induce endothelial dysfunction, it has been proposed

previously that  $O_2^-$  and other ROS may interact with NO, thereby modulating bioavailability of NO in the vasculature (5). Recently, an important mechanism by which ROS can affect endothelial production of NO has been proposed, which involves the uncoupling of endothelial NOS induced by peroxynitrite, an end product of the reaction of  $O_2^-$  and NO (17, 33). Interestingly, the present study found that pretreatment of the arteries with uric acid, a peroxynitrite scavenger (17), substantially restored the responses to bradykinin and A-23187 in ceramide-treated small coronary arteries. With the use of Western blot analysis of nitrotyrosine, a well-accepted biological marker for the formation of peroxynitrite, we found that ceramide significantly increased nitrotyrosine levels in small coronary arteries and uric acid blocked this increase in nitrotyrosine. These results further support the role of peroxynitrite in ceramide-induced and  $O_2^-$ -mediated endothelial dysfunction in small coronary arteries.

In summary, our data demonstrate that NADPH oxidase-mediated  $O_2^-$  production and subsequent peroxynitrite formation contribute to ceramide-induced endothelial dysfunction in small coronary arteries. Given the increasing evidence on the role of ceramide in cytokine signaling, this ceramide-induced NADPH oxidase activation may be importantly implicated in vascular endothelial dysfunction associated with cytokines as occurred in various cardiovascular diseases such as myocardial ischemia and reperfusion.

The authors thank Gretchen Barg for secretarial assistance.

This study was supported by National Institutes of Health Grants HL-57244 (to P.-L. Li) and DK-54927 (to A.-P. Zou) and American Heart Association Established Investigator Grant 9940167N (to P.-L. Li) and Predoctoral Fellowship 0010185Z (to D. X. Zhang).

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