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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, C2-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 gp91phox and p47phox. C2-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 p47phox 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, C2-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.

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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 described previously (31). Briefly, small intramural arteries (200–400 μm internal diameter) were incubated with 3 μM 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 cells were harvested at ≥95% purity and then used to establish the cell lines. Bovine coronary smooth muscle cells were harvested at ≥95% purity and then used to establish the cell lines. All cells were maintained in an atmosphere with 10% CO2 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 gp91phox, p47phox, and nitrotyrosine. Briefly, equal amounts (20–40 μg) 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 to a PVDF membrane 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 gp91phox (1:1,000 dilution for 2 h, Transduction Laboratories), a monoclonal antibody against p47phox (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 horse-radish 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 other observers without knowledge of the experimental protocol and by scanning and quantitation of different exposures 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 μg) freshly prepared from BCAECs were incubated with dihydroethidium (5 μM) and salmon testes DNA (0.5 mg/ml) in 200 μl PBS. Immediately before fluorescence was recorded, NADPH (final concentration 100 μM) 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 μm 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 (10–8–10–6 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 preconstriction 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 ± 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 μM) (3, 4, 29), apocynin (100 μM) (14, 24), or diphenylene iodonium (DPI; 50 μM). The doses of these inhibitors were based on those reported in previous studies that effectively blocked NADPH oxidase activity. C2-mercaptoethanol (5 μM) was then added, and $O_2^ -$ fluorescence was measured. It was found that C2-mercaptoethanol 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 C2-mercaptoethanol-induced $O_2^ -$ increase was also markedly blocked by apocynin or DPI (data not shown).

Expression of NADPH oxidase subunits. The expression of gp91phox and p47phox proteins was detected in BCAECs by Western blot analysis with monoclonal anti-gp91phox and anti-p47phox antibodies (Fig. 2, A and B). The anti-gp91phox antibody recognized two major bands at ~75 and ~50 kDa in endothelial cells but not in smooth muscle cells from bovine coronary arteries.
gp91phox was mainly expressed in the membrane fraction of endothelial cells with two strong bands at /H1101175 and /H1101150 kDa. This subunit was expressed in the cytosol less abundantly with a weaker /H1101175-kDa band. One specific band was detected at /H1101147 kDa with the anti-p47phox antibody in endothelial cells and smooth muscle cells from bovine coronary arteries. In contrast to gp91phox, p47phox 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 C2-ceramide (5 /H9262 M for 15 min) with or without preincubation with different enzyme inhibitors. The homogenates were then prepared, and NADPH-dependent O2/H11002/H18528 production was measured as described above. It was found that O2/H11002/H18528 production significantly increased in endothelial cells pretreated with C2-ceramide and that this O2/H11002/H18528 production was markedly inhibited by N-vanillylnonanamide (10 /H9262 M), apocynin (100 /H9262 M), or DPI (50 /H9262 M), but not by N\(^\mathrm{G}\)-nitro-L-arginine methyl ester (L-NAME; 100 /H9262 M), a NOS inhibitor, allopurinol (100 /H9262 M), a xanthine oxidase inhibitor, or rotenone (50 /H9262 M), a mitochondrial electron transport chain blocker (Fig. 3).

To exclude potential scavenging of mitochondria-generated O2/H11002/H18528 by NADPH oxidase inhibitors, the effects of these inhibitors on mitochondrial respiratory chain-derived O2/H11002/H18528 production were measured. As shown in Fig. 4, N-vanillylnonanamide (10 /H9262 M), apocynin (100 /H9262 M), and DPI (50 /H9262 M) at the doses that markedly inhibit NADPH oxidase activity had no significant effect on mitochondria-derived O2/H11002/H18528. In con-

**Fig. 1.** Effect of the NADPH oxidase inhibitor N-vanillynonanamide (NVN) on the C2-ceramide (Cer)-induced O2/H11002/H18528 increase in the endothelium of small coronary arteries. Time courses of changes in O2/H11002/H18528 fluorescence in the endothelial cells under control conditions or after treatment with Cer (5 /H9262 M or Cer + NVN (10 /H9262 M) are shown (n = 7–10). *P < 0.05 vs. control; #P < 0.05 vs. Cer.

**Fig. 2.** Protein expression of NADPH oxidase subunits. A and B: Western blots showing expression of gp91phox and p47phox 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 O2/H11002/H18528 production in BCAECs treated with vehicle or Cer (5 /H9262 M) in the absence or presence of the different enzyme inhibitors N\(^\mathrm{G}\)-nitro-L-arginine methyl ester (L-NAME; 100 /H9262 M), allopurinol (100 /H9262 M), rotenone (50 /H9262 M), NVN (10 /H9262 M), apocynin (100 /H9262 M), or diphenylene iodonium (DPI; 50 /H9262 M) are shown (n = 7–10). *P < 0.05 vs. control; #P < 0.05 vs. Cer.

**Fig. 4.** Mitochondria-derived O2/H11002/H18528 production in the absence or presence of the different enzyme inhibitors rotenone (50 /H9262 M), NVN (10 /H9262 M), apocynin (100 /H9262 M), or DPI (50 /H9262 M) (n = 7–10). *P < 0.05 vs. control.

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contrast, this mitochondrial $O_2^\cdot$ was significantly blocked by rotenone (50 μM).

**Effect of ceramide on p47phox translocation in BCAECs.** We then determined whether ceramide-induced activation of NADPH oxidase is associated with the translocation of p47phox 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 C2-ceramide (5 μM for 15 min), membrane and cytosolic fractions of these cells were then prepared, and immunoblotting for p47phox was performed. As shown in Fig. 5, stimulation of endothelial cells with C2-ceramide induced a quick p47phox translocation to the membrane. N-vanillylnonanamide (10 μM) inhibited C2-ceramide-stimulated p47phox 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 ± 0.07, 2.92 ± 0.41, and 0.47 ± 0.05 nmol·min⁻¹·μg protein⁻¹, respectively. C2-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 C2-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 C2-ceramide (5 μM, 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 μM) 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 C2-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 C2-ceramide-stimulated $O_2^\cdot$ generation, its effect on xanthine/xanthine oxidase-induced endothelial dysfunction was determined. Xanthine (50 μM) and xanthine oxidase (1 mU/ml) were perfused into the arterial lumen for 10 min with or without pretreatment with N-vanillylnonanamide (10 μM). The effectiveness of xanthine/xanthine oxidase at this dose to produce $O_2^\cdot$ was determined before and after C2-ceramide (5 μM, 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 μM) 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 C2-ceramide-induced endothelial dysfunction was also markedly blocked by apocynin.

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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 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 pretreatment of the arteries with uric acid (100 \( \mu \)M; Fig. 9).

**DISCUSSION**

In the present study, we found that gp91\(^{phox}\) and p47\(^{phox}\)-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\(^{phox}\) 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. 7](image1)

**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](image2)

**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.
Recently, a gp91phox-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 gp91phox and p47phox, which are the major membrane and cytosolic subunits, respectively. In agreement with previous reports (13), gp91phox is expressed in endothelial cells but not in smooth muscle cells, while the p47phox found in both types of cells. The anti-gp91phox antibody used in the present study recognized two major protein bands at ~75 and ~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 gp91phox 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 gp91phox (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 gp91phox may colocalize with the endoplasmic reticulum marker in endothelial cells (2, 22). In contrast to the membrane-associated gp91phox, p47phox was primarily detected in the cytosolic fraction of endothelial cells, which is consistent with general notion regarding the cytosolic localization of p47phox in resting endothelial cells (13).

Recently, we (31) reported that ceramide stimulates O₂⁻ production in endothelial cells and consequently induces endothelial dysfunction in small coronary arteries. However, the underlying mechanism of O₂⁻ production remains unknown. In the present study, we provided several lines of evidence indicating that NADPH oxidase may mediate ceramide-induced O₂⁻ production in coronary endothelial cells. First, pretreatment of the arteries with NADPH oxidase inhibitors markedly attenuated the ceramide-induced increase in endothelial O₂⁻ 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 O₂⁻-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 O₂⁻ 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 O₂⁻ production and endothelial dysfunction, had no significant effects on mitochondrial O₂⁻ production. In addition, the inhibitors of mitochondrial electron transport chain enzymes and other potential O₂⁻-generating enzymes including NOS and xanthine oxidase had no effect on ceramide-induced O₂⁻ production in coronary arterial endothelial cells. These results further support the view that ceramide activates NADPH oxidase to produce O₂⁻ 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 p47phox to the cytoplasmic membrane. Given that p47phox 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 p47phox 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 p47phox phosphorylation and subsequent translocation to the membrane are also critical in the activation of endothelial NADPH oxidase induced by tumor necrosis factor-α (10, 20). Because ceramide mediates various actions of cytokines (i.e., tumor necrosis factor-α) 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 O₂⁻ 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.

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