Nifedipine Indirectly Upregulates Superoxide Dismutase Expression in Endothelial Cells via Vascular Smooth Muscle Cell–Dependent Pathways

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Background—Calcium antagonists normalize endothelial dysfunction in many cardiovascular diseases. There is no known receptor, however, for calcium antagonists in endothelial cells (ECs). We hypothesized that vascular smooth muscle cells (VSMCs) are involved in the mechanism underlying the normalization of endothelial dysfunction by calcium antagonists.

Methods and Results—Coculture studies with ECs and VSMCs were performed to determine whether VSMCs mediate modulation of endothelial superoxide dismutase (SOD) activity and expression induced by the calcium antagonist nifedipine. Nifedipine induced upregulation of SOD activity in rat aortic segments but had no effect on SOD expression or activity in ECs or VSMCs cultured individually. When ECs were cocultured with VSMCs, however, nifedipine upregulated SOD expression and activity in ECs. Nifedipine stimulated vascular endothelial growth factor (VEGF) production from VSMCs, and this stimulation of VEGF production was abolished by HOE-140, an antagonist of the bradykinin B2 receptor. A neutralizing antibody against VEGF inhibited the upregulation of endothelial SOD by nifedipine. In addition, recombinant VEGF induced an increase in the levels of SOD expression in ECs, and supernatant derived from nifedipine-treated VSMCs enhanced NO production from ECs. This increase in NO production by the supernatant was inhibited by preincubation of ECs with SOD antisense oligodeoxyribonucleotides.

Conclusions—The calcium antagonist nifedipine indirectly upregulates endothelial SOD expression by stimulating VEGF production from adjacent VSMCs. This finding may provide further insight into the mechanism underlying the beneficial effects of calcium antagonists in cardiovascular diseases. (Circulation. 2002;106:356-361.)

Key Words: endothelium ■ nitric oxide ■ muscle, smooth ■ calcium ■ bradykinin

Reactive oxygen species (ROS) arise primarily as byproducts of normal metabolic activities. Increasing evidence suggests, however, that ROS play a critical role in the pathogenesis of endothelial dysfunction in a number of cardiovascular diseases, including atherosclerosis, hypertension, diabetes mellitus, and heart failure.1 The enzyme superoxide dismutase (SOD) is a primary cellular defense against ROS. Three SOD isoforms have been identified. Manganese SOD (Mn-SOD) is mitochondrial; the dimeric, copper/zinc-containing SOD (Cu/Zn-SOD) is cytosolic and nuclear; and the tetrameric, proteoglycan-bound Cu/Zn-SOD is extracellular.2 Distribution of these isoforms varies among species; however, the predominant activity of SOD in peripheral vessels is attributed to the Cu/Zn isoforms.3 SOD deficiency has been shown to result in impairment of endothelium-dependent dilation as a result of increased inactivation of NO in vivo.3 Conversely, SOD has been shown to rescue cerebral endothelial dysfunction in mice overexpressing amyloid precursor protein.4 These findings indicate that upregulation of SOD expression may prevent the development of endothelial dysfunction induced by ROS.

Calcium antagonists have been widely used in the treatment of patients with angina pectoris and hypertension. Interestingly, recent studies have shown that calcium antagonists normalize endothelial dysfunction in many cardiovascular diseases. For example, calcium antagonists reverse the impairment of endothelium-dependent, NO-mediated vasorelaxation in hypercholesterolemic rabbits.5 Nifedipine also improves endothelial function in hypercholesterolemia by enhancing NO function.6 The mechanisms by which calcium antagonists normalize endothelial dysfunction, however, are largely unknown, because there are no known receptors for calcium antagonists in ECs, despite their possible antioxidant properties.7 We hypothesized that the normalization of endothelial dysfunction by calcium antagonists may be mediated through activation of adjacent vascular smooth muscle cells (VSMCs). We have shown that the calcium antagonist nifedipine resulted in an increase in SOD expression and

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activity in endothelial cells (ECs) when ECs were cocultured with VSMCs and that this upregulation by nifedipine was mediated by vascular endothelial growth factor (VEGF) released from VSMCs.

Methods

Materials
Male Wistar rats were from Charles River Japan (Tokyo, Japan). Nifedipine and amlodipine were donated by Bayer and Sumitomo Pharmaceutical Co, respectively. Recombinant human VEGF was from Pepro Tech EC Ltd. d-Arginyl-[Hyp, Thi,’D-Tic, ’Oic”]-bradykinin (HOE-140), a B2 receptor antagonist, and bradykinin were purchased from Peptide Institute, Inc. Anti-human VEGF (16F1) mouse IgG monoclonal antibody was purchased from IBL Co. All animals used in these experiments were treated in accordance with our institutional guidelines.

Experimental Design
A coculture experiment with ECs and VSMCs was performed to determine whether VSMCs mediate the action of calcium antagonists in ECs. The levels of endothelial SOD activity and expression in ECs cocultured with VSMCs were compared with those in EC monocultures.

Cell Culture
Human umbilical vein ECs were purchased from Sanko Junyaku and cultured in EBM-2 (Sanko Junyaku) supplemented with 2% FCS and antibiotics. Human VSMCs were isolated from human aortas and then cultured in DMEM containing 10% FCS. For coculturing, VSMCs were grown to confluence on the collagen-coated microporous membranes of transwells (Costar Corp). The cells were cocultured in EM-2 medium containing 0.5% FCS. Because nifedipine is extremely susceptible to light degradation, all nifedipine solutions were prepared and added to cell cultures under yellow light, and cells were then cultured in the dark to minimize light-induced decomposition.

Determination of SOD Activity
After incubation, the isolated aortas were homogenized in 10 vol 50 mMol/L potassium phosphate (pH 7.4) containing 0.3 mol/L KBr and a cocktail of protease inhibitors (0.5 mmol/L PMSF, 90 mg/L aprotinin, 10 mg/L pepstatin, 10 mg/L leupeptin) followed by sonication (10 seconds) and extraction at 4°C for 30 minutes. Cultured ECs were harvested after incubation for the indicated periods with treatment agents and resuspended in 100 μL of water. Cells were then lysed by freezing/thawing 3 times and centrifuged at 15 000g for 10 minutes at 4°C. After centrifugation, the supernatants were again centrifuged at 20 000g for 30 minutes. The resultant supernatant was then used for the determination of SOD activity by monitoring the inhibition of xanthine oxidase–mediated cytochrome c reduction, with the absorbance measured at 550 nm over 3 minutes.

Western Blot Analysis for SOD
Harvested ECs were treated in 10 μL/106 cells of lysis buffer (1% SDS, 100 mMol/L NaCl, 50 mMol/L Tris-HCl [pH 8.0], 20 mMol/L EDTA) and boiled for 4 minutes. Samples containing 50 μg of protein each were loaded onto a 12.5% SDS-PAGE gel, run, and electroblotted onto nitrocellulose filters. Blots were blocked in 5% skim milk in PBS for 1 hour, treated for 1 hour with antibodies to Cu/Zn-SOD or Mn-SOD, and then incubated with peroxidase-conjugated secondary antibodies for 1 hour. Immunoblots were developed with an ECL Western blotting detection system (Amer sham International plc). The blot was then reprobed with α-tubulin to confirm equal protein loading in each well.

Measurement of VEGF Concentration
The VEGF concentration in the supernatant was measured with a commercial kit (R & D Systems).

Measurement of NO Production
Both the NO-reactive dyes 4,5-diaminofluorescein diacetate (DAF-2 DA) and DAF-2 were used to determine NO production in our experiments. After treatment with the supernatant, cells were loaded at 37°C for 20 minutes with DAF-2 DA (10 μmol/L) in HEPES buffer containing 0.1 mmol/L L-arginine. Cells were then rinsed 3 times in HEPES buffer and analyzed immediately under a Nikon EF2 fluorescence microscope equipped with an excitation 489 nm, emission 515 nm, BP green filter (×400). DAF-2 was used to determine the levels of NO released from ECs into the medium. ECs were incubated for 2 hours with DAF-2 in HEPES buffer containing 0.1 mmol/L L-arginine at 37°C after treatment with the supernatant.

Statistical Analysis
Statistical analysis was performed by 1-way ANOVA after a post hoc test. Results are expressed as mean±SEM. A value of P<0.05 was considered significant.

Results
Nifedipine Upregulates SOD Activity in Rat Aortic Segments but Not in ECs or VSMCs When Cultured Individually
Incubation for 24 hours with nifedipine significantly increased the levels of SOD activity in rat aortic segments (Figure 1). Nifedipine had no direct effect, however, on SOD activity in ECs (Figure 2) or VSMCs (data not shown) cultured individually.

Nifedipine Induces Upregulation of SOD in ECs Cocultured With VSMCs
As shown in Figure 2, coculturing with VSMCs resulted in a slight upregulation of the basal SOD activity in ECs, whereas incubation with nifedipine induced a dose-dependent increase in the levels of SOD activity in ECs when these cells were cocultured with VSMCs. SOD
activity in VSMCs was also upregulated by nifedipine when VSMCs were cocultured with ECs (data not shown). In addition, nifedipine induced a dose-dependent increase in the expression levels of both Mn-SOD and Cu/Zn-SOD in ECs cocultured with VSMCs but not in ECs cultured individually (Figure 3). Furthermore, amlodipine, another calcium antagonist, also induced upregulation of SOD activity and expression in ECs cocultured with VSMCs (see Figure 5).

VEGF Derived From VSMCs Mediates Upregulation of SOD by Nifedipine

Importantly, the supernatant obtained from VSMCs treated with nifedipine significantly upregulated SOD expression and activity in ECs (Figure 4, A and B), suggesting that VSMC-derived factors may be involved in the mechanism underlying the upregulation of endothelial SOD by nifedipine. We next examined the possibility that VEGF, an EC-specific growth factor derived from VSMCs, may mediate this upregulation of endothelial SOD by nifedipine. As shown in Figure 4B, a neutralizing antibody against VEGF inhibited upregulation of SOD activity induced by the supernatant from nifedipine-treated VSMCs, whereas a control antibody had no effect. This neutralizing antibody also suppressed the upregulation of SOD expression by nifedipine or amlodipine in ECs...
cocultured with VSMCs (Figure 5, A and B). In addition, VEGF induced a time-dependent increase in the levels of both Mn-SOD and Cu/Zn-SOD expression in ECs cultured individually (Figure 6).

Nifedipine Enhances VEGF Production From VSMCs Through Activation of the Bradykinin Receptor

Next, we determined whether nifedipine affects the levels of VEGF released from VSMCs. As shown in Figure 7A, nifedipine induced a significant increase in the release of VEGF from VSMCs after incubation for 24 hours. This increase was shown to be dose-dependent (Figure 7B). Interestingly, HOE-140, an antagonist of the bradykinin B2 receptor, abolished this increase by nifedipine (Figure 7C). In addition, bradykinin itself induced a significant increase in the levels of VEGF release from VSMCs, suggesting that nifedipine stimulates VEGF production through activation of the B2 receptor.

Nifedipine Enhances NO Production From ECs via VSMC-Dependent Pathways

Next, we investigated whether upregulation of endothelial SOD by nifedipine resulted in an increase in NO production by ECs. As reported previously, incubation for 24 hours with VEGF enhanced NO production from ECs. When ECs were incubated for 24 hours with medium from an incubation of VSMCs treated with nifedipine, NO production was significantly enhanced (Figure 8A). As shown in Figure 8B, incubation for 24 hours with the medium from nifedipine-treated VSMCs increased the levels of NO released from ECs, as did VEGF. The medium containing nifedipine incubated without VSMCs, however, had no effect. In addition, a neutralizing antibody against VEGF suppressed the enhancement of NO production by the supernatant from nifedipine-treated VSMCs.

SOD antisense ODNs were used to determine whether the increase in the levels of SOD by nifedipine is related to NO production. Preincubation with Mn-SOD antisense ODNs abolished upregulation of SOD expressions induced by the supernatant from nifedipine-treated VSMCs, whereas sense ODNs had no effect (data not shown). As shown in Figure 8B, preincubation with Mn-SOD antisense ODNs significantly suppressed the enhancement of NO production induced by the supernatant from nifedipine-treated VSMCs, indicating that the enhancement of NO production by nifedipine is primarily a result of upregulation of SOD expression.

Discussion

The present study highlights the following novel findings on the mechanisms responsible for the normalization of endothelial dysfunction by calcium antagonists. (1) The calcium antagonist nifedipine indirectly upregulates SOD activity and expression in ECs through activation of adjacent VSMCs. (2) VEGF released from VSMCs is involved in the mechanism underlying the upregulation of endothelial SOD activity by nifedipine. (3) Nifedipine stimulates the release of VEGF from VSMCs through activation of the bradykinin B2 receptor. (4) Upregulation of endothelial SOD by nifedipine results in the enhancement of NO production from ECs.
Calcium antagonists are widely used in the treatment of hypertension and angina pectoris. Recent evidence suggests that these drugs improve clinical outcome in patients with certain cardiovascular diseases.\textsuperscript{12,13} It is noteworthy that calcium antagonists have been shown to normalize endothelial dysfunction in many cardiovascular diseases,\textsuperscript{14} because endothelial dysfunction is critical for the development of cardiovascular events.\textsuperscript{15} The underlying mechanism by which calcium antagonists normalize EC dysfunction was unknown, however, because these cells have no receptors for calcium antagonists. In the present study, we have demonstrated for the first time that calcium antagonists indirectly upregulate SOD expression and activity in ECs via VSMC-dependent pathways. This finding may be important, because it is speculated that upregulation of endothelial SOD by calcium antagonists might be less efficient in VSMC-poor vessels, such as a vessel with vulnerable plaques.\textsuperscript{16} Our findings may also provide evidence that calcium antagonists could modulate the function of L-type calcium channel–negative cells through activation of L-type calcium channel–positive cells.

Increased superoxide concentrations play a critical role in the pathogenesis of endothelial dysfunction in many cardiovascular diseases. For example, hypertension is associated with increased inhibition of NO signaling by superoxide, whereas NO release remains unaffected.\textsuperscript{17} Bauersachs et al\textsuperscript{18} also reported that endothelial dysfunction in ischemic heart failure was induced by an increase in vascular superoxide production despite enhanced vascular endothelial nitric oxide synthase (eNOS) and soluble guanylate cyclase expression. Importantly, eNOS is a cytochrome P450 reductase–like enzyme that produces superoxide under certain pathological conditions.\textsuperscript{19} Vergnani et al\textsuperscript{20} reported that oxidized LDL, an atherogenic factor, may uncouple eNOS activity, allowing the enzyme to become a source of superoxide. The interaction between NO and superoxide results in the formation of peroxynitrite, a highly toxic metabolite that can induce tissue damage, suggesting that the scavenging capacity of superoxide by endothelial SOD plays a key role in regulating the production of 2 interrelated but distinct free radicals from eNOS. In the present study, we have shown that SOD antisense ODNs suppressed endothelial NO production induced by the conditioned medium from nifedipine-treated VSMCs. In addition, NO released by an NO donor did not result in any increase in the levels of SOD expression in ECs (data not shown). Thus, the enhancement of NO production from ECs by nifedipine appears to be primarily a result of the upregulation of endothelial SOD expression. Taken together, these findings suggest that upregulation of

Figure 7. Nifedipine stimulates production of VEGF from VSMCs. Time-course (A) and dose-dependent effect (B) of nifedipine and effect of HOE-140, an antagonist of bradykinin B\textsubscript{2} receptor, (C) on changes in VEGF levels in supernatant released from VSMCs. VSMCs were treated for 24 hours with bradykinin (1 \(\mu\)mol/L) or nifedipine in presence or absence of HOE-140 (1 \(\mu\)mol/L). After incubation, medium was collected and VEGF concentrations in supernatant were measured as described in text. Values are mean±SEM of 4 individual experiments, each containing 3 replicates. \(*P<0.05, \text{significantly different from control.} \) **\(P<0.05, \text{significantly different from ECs treated with nifedipine.} \)

Figure 8. Supernatant derived from nifedipine-treated VSMCs enhances NO production in ECs. ECs were treated for 24 hours with supernatant from VSMCs treated with nifedipine (1 \(\mu\)mol/L) or EM-2 medium containing 0.5% FCS with or without VEGF (10 ng/mL). A, To determine NO production in cells, ECs were loaded at 37°C for 20 minutes with NO-reactive dye DAF-2 DA (10 \(\mu\)mol/L) as described in text. Production of NO in ECs was visualized by emission of green light (515 nm) on excitation at 489 nm. B, To determine levels of NO released from ECs into medium, ECs were incubated for 2 hours with DAF-2 (10 \(\mu\)mol/L) after treatment with supernatant as described in text. Mn-SOD antisense or sense ODNs were preincubated at 18 hours before treatment with supernatant. Values are mean±SEM of 3 individual experiments, each containing 2 replicates. \(*P<0.05, \text{significantly different from control.} \) **\(P<0.05, \text{significantly different from ECs treated with supernatant from nifedipine-treated VSMCs.} \)
endothelial SOD by calcium antagonists may serve to reduce the pro-oxidant potential of eNOS in pathological conditions.

The concentrations of nifedipine used in our experiments were 0.1, 1, and 5 μmol/L, whereas the clinical plasma concentration of nifedipine is reported to be ≈0.2 μmol/L.6 In addition, it has been shown that nifedipine may accumulate in membranous structures, resulting in higher localized concentrations of the compound.21 Thus, the concentrations of nifedipine used in our experiments are comparable to the clinical plasma concentrations achieved in the treatment of patients with cardiovascular disease.

In the present study, we have demonstrated that nifedipine-induced stimulation of VEGF production from VSMCs was abolished by HOE-140, an antagonist of the B2 receptor. Zhang and Hintze22 reported that NO production by amlodipine was dependent on stimulation of the B2 receptor in canine coronary microvessels. In addition, Knox et al.23 recently reported that bradykinin can increase VEGF secretion in human airway smooth muscle cells. These observations and our findings suggest that activation of the B2 receptor may be involved in the mechanism by which nifedipine stimulates VEGF production from VSMCs. We have also demonstrated that VEGF derived from VSMCs induced upregulation of endothelial SOD expression. Because scavenging superoxide by SOD prolongs the half-life of NO, the ability of VEGF to upregulate both eNOS and SOD expression may increase the physiological importance of VEGF derived from VSMCs with regard to the regulation of NO function in ECs. Fukui et al.24 recently reported that endothelial NO stimulates extracellular SOD expression in adjacent VSMCs through cGMP-dependent pathways, thus preventing superoxide-mediated degradation of NO. This and our present findings suggest that cellular interactions between ECs and VSMCs are critical in the regulation of the vascular redox state and that VEGF is an important messenger molecule between the 2 cell types.

In conclusion, the results of this study demonstrate for the first time that the calcium antagonist nifedipine stimulates the release of VEGF from VSMCs, which then induces upregulation of SOD activity in adjacent ECs. These findings may provide further insight into the mechanism underlying the normalization of endothelial dysfunction by calcium antagonists.

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