Calcium antagonists normalize endothelial dysfunction and improve the clinical outcome in patients with hypertension. However, the mechanism underlying these beneficial effects remains to be elucidated. Here, we show that the calcium antagonist nifedipine upregulates the expression of manganese superoxide dismutase (Mn SOD), an endogenous antioxidant enzyme, in vascular smooth muscle cells (VSMC) via cellular interactions between VSMC and endothelial cells (EC). Nifedipine induced upregulation of Mn SOD activity and expression in VSMC when cocultured with EC but not when cultured individually. N^G-Monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide (NO) synthesis, inhibited the upregulation of Mn SOD expression induced by nifedipine. Additionally, N-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino) ethanamine, a NO donor, reversed this inhibition by L-NMMA, indicating that NO may be involved in the mechanism underlying the nifedipine-induced upregulation of Mn SOD in VSMC. Preincubation of VSMC with Mn SOD antisense oligodeoxyribonucleotides (ODN) blocked the suppressive effects of nifedipine on DNA synthesis in VSMC cocultured with EC, whereas sense ODN had no effect. We conclude that the calcium antagonist nifedipine induces upregulation of Mn SOD expression in VSMC via NO derived from EC. This finding may provide some insight into the mechanism underlying the beneficial effects of calcium antagonists in patients with hypertension. (Hypertens Res 2003; 26: 503–508)

Key Words: endothelium, nitric oxide, superoxide, smooth muscle, calcium antagonists

Introduction

Nitric oxide (NO), generated by the endothelial isoform of NO synthase (eNOS), has numerous physiological functions such as inhibition of platelet aggregation, induction of vasorelaxation, suppression of adhesion of leukocytes to the endothelium, and inhibition and migration of vascular smooth muscle cells (VSMC). Interestingly, mice lacking eNOS expression develop several pathological conditions such as hypertension, insulin resistance, hyperlipidemia (1), abnormal vascular remodeling (2), heart failure (3), and acceleration of atherosclerosis (4). These data suggest the importance of eNOS expression levels in the determination of NO bioactivity in the cardiovascular system. However, Ozaki et al. (5) recently reported that overexpression of eNOS does not inhibit but accelerates atherosclerosis in apolipoprotein E (apoE)-deficient mice. Similarly, paradoxical reduction of fatty streak formation has been also reported in eNOS-deficient mice (6). Importantly, eNOS is a cytochrome P450 reductase-like enzyme that produces superoxide under pathological conditions. For example, oxidation of tetrahydrobiopterin, a cofactor for eNOS (7), or oxidation of the zinc-thiolate center of eNOS (8) induced by oxidants leads to uncoupling of this enzyme and generation of superoxide in certain cardiovascular diseases including hyperten-
sion in which production of vascular oxidants is upregulated (9). Similarly, it has been reported that hypertension is associated with increased inhibition of NO signaling by superoxide, whereas NO release remains unaffected (10). Taken together, these findings suggest that the scavenging capacity of superoxide may be critical in regulating the bioactivity of NO derived from eNOS. The enzyme superoxide dismutase (SOD) is a primary cellular defense mechanism against superoxide. Three SOD isozymes have been identified. Mang-enese SOD (Mn SOD) is mitochondrial and the dimeric copper/zinc-containing SOD (Cu/Zn SOD) is cytosolic and nuclear, whereas the tetrameric, proteoglycan-bound Cu/Zn SOD is extracellular (11).

Calcium antagonists have been shown to improve endothelial function and clinical outcome in patients with hypertension (12). Although the precise mechanisms underlying these beneficial effects are not clear, a number of possible mechanisms have previously been reported. For example, calcium antagonists normalize endothelial dysfunction through enhancement of NO function (13), antioxidant functions (14, 15), and inhibition of apoptosis (16). We have recently demonstrated that calcium antagonists indirectly up-regulate endothelial SOD expression by stimulating vascular endothelial growth factor (VEGF) production from adjacent vascular smooth muscle cells (VSMC) (17). Upregulation of SOD expression by calcium antagonists results in an increase in NO production from endothelial cells (EC). Interestingly, Fukai et al. (18) recently reported that NO induces upregulation of extracellular SOD expression in VSMC. In the present study, we therefore investigated whether the calcium antagonist nifedipine modulates Mn SOD expression in VSMC via NO-dependent pathways.

Methods

Materials

Nifedipine was donated by Bayer Pharmaceutical Co. (Tokyo, Japan). N-Ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino) ethanamine (NOC-12) and N\textsuperscript{\textalpha}-monomethyl-L-arginine (L-NMMA) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Experimental Design

A coculture experiment with EC and VSMC was performed to determine whether cellular interactions mediate the action of the calcium antagonist nifedipine in these cells. The levels of SOD activity or Mn SOD expression in VSMC cocultured with EC were compared with those in VSMC monocultures.

Cell Culture

Human umbilical vein EC were purchased from Sanko Junyaku (Tokyo, Japan) and cultured in EBM-2 (Sanko Junyaku) supplemented with 2% fetal calf serum (FCS) and antibiotics. Human VSMC were isolated from human aortas and then cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS. For coculturing, EC were grown to confluence on the collagen-coated microporous membranes of transwells (Costar Corp., Cambridge, USA). VSMC cultured on 24-well plates were then cocultured with EC in EBM-2 medium containing 2% FCS. In this coculture system, these cells were separately cultured in a 1 mm distance but the culture medium was shared by both cells. Therefore, humoral interchange was allowed between them without direct cellular contact. Since 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 of nifedipine.

Determination of SOD Activity

EC or VSMC were harvested following 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 15,000 g for 10 min at 4°C. After centrifugation, the supernatants were again centrifuged at 20,000 g for 30 min. The resultant supernatant was then used for the determination of SOD activity by monitoring the inhibition of xanthine oxidase-mediated cytochrome c reduction (19), by measuring the absorbance measured at 550 nm over 3 min.

Western Blot Analysis for Mn SOD

Harvested VSMC were treated in 10 µl/10⁶ cells of lysis buffer (1% sodium dodecyl sulfate (SDS); 100 mmol/l NaCl; 50 mmol/l Tris-HCl, pH 8.0; 20 mmol/l ethylenediaminetetraacetic acid (EDTA)) and boiled for 4 min. Samples, each containing 50 µg of protein, were loaded onto a 12.5% SDS polyacrylamide gel electrophoresis (PAGE) gel, run and electroblotted onto nitrocellulose filters. Blots were blocked in 5% skimmed milk in phosphate-buffered saline (PBS) for 1 h, treated for 1 h with an antibody to Mn SOD, and then incubated with peroxidase-conjugated secondary antibodies for 1 h. Immunoblots were developed using an ECL Western blotting detection system (Amersham International Plc, Buckinghamshire, UK). The blot was then reprobed with α-tubulin to confirm equal protein loading in each well.

Cell Proliferation Assay

VSMC were preincubated for 18 h both in the presence or absence of Mn SOD antisense or sense oligodeoxynucleotides (ODN) (1.0 µmol/l). The Mn SOD antisense and sense ODNs used were 5-CCACGGCGCGAGCGACAAACAT TG-3 and 5-CCATGTTTGTTCCGCGCGTG-3 (respec-
tively. VSMC were then cocultured with EC in EBM-2 medium containing 2% FCS for 24 h in the presence or absence of nifedipine. VSMC were labeled with $^3$H-thymidine (2 $\mu$Ci/ml) during the last 4-h period of the incubation. After labeling, cells were washed three times with cold PBS and treated with 5% trichloroacetic acid and ethanol-ethylether (3:1; vol/vol). The residues in the wells were solubilized in 0.3 eq/l NaOH, and the radioactivity of aliquots of the solution was measured after neutralization of the pH.

**Statistical Analysis**

Within-group comparison was made by analysis of variance and Fisher’s PLSD post hoc test was used to test statistical significance. Results are expressed as mean $\pm$ SEM. A value of $p < 0.05$ was considered significant.

**Results**

**Nifedipine Upregulates SOD Activity in VSMC when Cocultured with EC but Not in VSMC Monocultures**

Figure 1 shows the effects of cellular interactions between EC and VSMC on SOD activity in these cells during treatment with the calcium antagonist nifedipine. Interestingly, coculturing resulted in a slight increase in the levels of SOD activity in the other cells. Nifedipine had no effect on the levels of SOD activity in EC or VSMC when these cells were cultured individually. However, it induced a significant increase in the levels of SOD activity in both EC and VSMC when these cells were cocultured.

**Nitric Oxide (NO) Mediates Nifedipine-Induced Upregulation of Mn SOD Expression.**

To define the molecular mechanism by which nifedipine induces upregulation of SOD activity in cocultured vascular cells, we next examined the effect of nifedipine on the levels of Mn SOD expression using Western blot analysis. As
shown in Fig. 2, coculturing with EC resulted in a slight increase in the levels of Mn SOD expression in VSMC, and nifedipine induced upregulation of Mn SOD expression in VSMC cocultured with EC but not in VSMC cultured individually. These results are consistent with the results in Fig. 1. Importantly, L-NMMA, an inhibitor of NO synthesis, partially suppressed the upregulation of Mn SOD expression induced by nifedipine. In addition, NOC-12, a NO donor, reversed this suppression by L-NMMA, suggesting that NO is involved in the mechanism underlying the upregulation of Mn SOD expression in VSMC by nifedipine.

Nifedipine Enhances Inhibition of VSMC Growth by EC through Upregulation of Mn SOD Expression

In order to define the physiological importance of the observed upregulation of Mn SOD expression in VSMC, we used Mn SOD antisense ODN to determine whether the increase in the levels of Mn SOD is related to the inhibitory effect of EC on VSMC growth. Preincubation of VSMC with Mn SOD antisense ODN abolished the nifedipine-induced upregulation of Mn SOD expression, whereas sense ODN had no effect (Fig. 3A, B). As shown in Fig. 3C, coculturing with EC resulted in a slight inhibition of VSMC growth, and nifedipine significantly enhanced this inhibitory effect. Preincubation with Mn SOD antisense ODN significantly reversed this nifedipine-induced enhancement, whereas sense ODN had no effect. These results indicate that nifedipine-induced enhancement of the inhibitory effect of EC on VSMC growth is primarily due to upregulation of Mn SOD expression.

Discussion

In the present study we showed that cellular interactions between EC and VSMC are important in the regulation of antioxidant enzyme Mn SOD expression in VSMC. Upregulation of vascular Mn SOD expression by the calcium antagonist nifedipine is mediated through these cellular interactions. Finally, upregulation of Mn SOD expression by nifedipine results in the enhancement of VSMC growth inhibition by EC.

Hypertension is associated with increased vascular superoxide production (9). Increased levels of vascular superoxide induce endothelial dysfunction by promoting inactivation of NO. Superoxide also induces down-regulation of contractile type smooth muscle myosin heavy chain isofrom SM2 expression in medial smooth muscle cells, which leads to an increase in aortic stiffness and reduction of vascular responsiveness in the rabbit aorta (20). In addition, the reaction between NO and superoxide results in the formation of peroxynitrite, highly toxic metabolite that induces vascular damage. Importantly, eNOS exposure to peroxynitrite results in increased enzymatic uncoupling and generation of superoxide, further contributing to vascular injury (7, 8). These ob-
servations indicate that scavenging of superoxide is very important in preventing endothelial dysfunction and vascular injury in patients with hypertension. In keeping with these observations, the present data that the calcium antagonist nifedipine upregulated Mn SOD expression in VSMC may provide some insight into the mechanism underlying the beneficial effects of calcium antagonists in patients with hypertension.

Calcium antagonists have previously been shown to normalize endothelial dysfunction in many cardiovascular diseases including hypertension (13, 21). Although the underlying mechanism by which calcium antagonists normalize endothelial dysfunction remains to be elucidated, several possible mechanisms have been previously reported. For example, Zhang and Hintze (22) reported that calcium antagonists enhance NO production in canine coronary microvessels. Their possible antioxidant properties have also been suggested (23). We have recently demonstrated that calcium antagonists indirectly upregulate the SOD expression in EC by stimulating VEGF production from adjacent VSMC (17). We also demonstrated that upregulation of endothelial SOD expression by calcium antagonists resulted in an enhancement of NO production from EC. In the present study we showed that the calcium antagonist nifedipine induced upregulation of Mn SOD expression in VSMC as well. Although the precise mechanism underlying the upregulation of Mn SOD in VSMC by nifedipine is not clear at present, NO derived from adjacent EC appears to be involved in this mechanism, because L-NMMA, an inhibitor of NO synthesis, inhibited this upregulation by nifedipine. However, whether or not a similar increase in Mn SOD expression could be obtained by treatment with organic nitrates or NO donors remains to be determined. This is in agreement with the previous report by Fukai et al. (18) who showed that endothelial NO stimulates extracellular SOD expression in adjacent VSMC via cGMP-mediated pathways, thus preventing superoxide-induced degradation of NO. In the present study, we also demonstrated that upregulation of Mn SOD resulted in the enhancement of inhibitory effects of EC on VSMC growth, suggesting that upregulation of Mn SOD by nifedi-
pine leads to the enhancement of NO bioactivity. Taken together, these findings suggest that the ability of calcium antagonists to upregulate both endothelial NO production and vascular Mn SOD expression may strengthen their efficiency and physiological usefulness in patients with hypertension.

In conclusion, the results of this study demonstrate that the calcium antagonist nifedipine induces upregulation of Mn SOD expression in VSMC via cellular interactions between EC and VSMC. Thus, cellular interactions between these two cell types is considered to be involved in the molecular mechanism underlying the normalization of endothelial dysfunction by calcium antagonists.

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