Pranidipine Enhances the Action of Nitric Oxide Released From Endothelial Cells

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Abstract—Nitric oxide (NO) synthesis in vascular endothelium of patients with hypertension is altered. Calcium antagonists have been shown to improve endothelial function in hypertensive patients. Here we report that pranidipine, one of the latest long-acting calcium antagonists in the dihydropyridine group, enhances the actions of NO released from endothelial cells (ECs). Pranidipine significantly enhanced cGMP accumulation in vascular smooth muscle cells cocultured with ECs, whereas amlodipine and nifedipine had no significant effects. In addition, pranidipine also suppressed basal and thrombin-stimulated endothelin-1 production from ECs. Pranidipine also enhanced cGMP accumulation in rat aortic segments with endothelium but not in endothelium-denuded vessels. In contrast, pranidipine had no effect in the presence of N\(^{-}\)-monomethyl-L-arginine, an inhibitor of NO synthesis. Pranidipine did not affect the basal expression of endothelial NO synthase in ECs. However, pranidipine upregulated the activity of superoxide dismutase in ECs. These findings suggest that pranidipine enhances NO action through inhibition of superoxide-induced NO decomposition in the vessel wall. Thus, pranidipine may be useful in the treatment of impaired endothelial function in patients with hypertension. (Hypertension. 2000;35:82-85.)

Key Words: calcium antagonists n nitric oxide n endothelin n superoxide

Calcium antagonists have been widely used for the treatment of patients with angina pectoris and hypertension. It has been shown that these drugs have antiatherosclerotic effects in both experimental and clinical settings.\(^1\) Although the precise mechanisms are not clear, these antiatherosclerotic effects are independent of the reduction of blood pressure and changes in plasma lipid. There is recent evidence that amlodipine, a long-acting calcium antagonist, has been reported in the Prospective Randomized Amlodipine Survival Evaluation Study (PRAISE) to have a substantial beneficial effect in a subgroup of patients with dilated cardiomyopathy.\(^2\) Zhang and Hintze\(^3\) also reported that amlodipine may release nitric oxide (NO) from canine coronary microvessels through modulating the actions or formation of kinins. However, the mechanism by which calcium antagonists release NO is still unclear because there are no known receptors for calcium antagonists in endothelial cells (ECs).

Pranidipine, a new long-acting calcium antagonist in the dihydropyridine group, has potent and long-lasting antihypertensive effects both in vitro and in vivo.\(^4\) It has a unique action on endothelium-dependent relaxation, namely, pranidipine prolongs the duration of endothelium-dependent relaxation, whereas other calcium antagonists including amlodipine had no significant effects. The endothelium-derived relaxing factors include NO, endothelium-derived hyperpolarizing factor, and prostacyclin. We designed the present study to examine whether pranidipine can modulate the function of NO derived from ECs using a coculture system. We found that pranidipine enhanced the actions of NO released from ECs and that the pranidipine-induced effects may be mediated through the inhibition of NO decomposition.

Methods

Materials
Pranidipine, amlodipine, and nifedipine were donated by Otsuka, Sumitomo, and Bayer Pharmaceutical Co, respectively. Anti-human endothelium NO synthase (eNOS) antibody was purchased from Funakoshi Chemical Co. N\(^{-}\)-Monomethyl-L-arginine (L-NMMA), thrombin, and isobutylmethylxanthine (IBMX) were obtained from Dojin Laboratory. For animals used in these experiments, procedures were followed in accordance with our institutional guidelines.

Cell Culture
Human umbilical vein ECs were cultured as described previously.\(^5\) Human vascular smooth muscle cells (VSMCs) were isolated from human aortas as described previously.\(^6\) For the coculture system, ECs were grown to confluence on the collagen-coated microporous membranes of transwells (Coster Corp.). Then the coculture system was prepared as described previously.\(^7\)

Determination of Intracellular cGMP Levels in VSMCs or Isolated Vessels
VSMCs cocultured with ECs were incubated for the indicated times with serum-free medium containing 0.5 mmol/L IBMX and other compounds. For the determination of cGMP levels in the vessels, thoracic aortas were isolated from male Wistar rats (Charles River...
Japan, Tokyo, Japan). In some cases, the endothelium was removed by gently rubbing the intimal surface with a surgical knife. After the incubation, the levels of cGMP were measured with a commercial kit (Amersham International plc).

**Determination of Endothelin-1**
The concentration of endothelin-1 (ET-1) released from ECs was determined as described previously. The protein content was determined with the use of a commercial kit (Bio-Rad Laboratories).

**Western Blot Analysis**
After the incubation, cells were harvested and lysed in 10 mL/10^6 cells of lysing solution (1% SDS; 100 mmol/L NaCl; 50 mmol/L Tris-HCl, pH 8.0; 20 mmol/L EDTA) and boiled for 4 minutes. Samples of 50 μg protein each were electrophoresed and electroblotted on nitrocellulose filters. Blots were blocked in 3% skimmed milk in PBS for 1 hour, treated for 1 hour with a monoclonal antibody to eNOS, and then incubated with peroxidase-conjugated secondary antibodies for 1 hour. Immunoblots were developed by ECL detection system (Amersham International plc).

**Determination of Superoxide Dismutase Activity**
The total superoxide dismutase (SOD) activity was measured by a spectrophotometric assay as described previously. Briefly, ECs cultured on 10-cm dishes were pelleted after the incubation for indicated times with pranidipine, other calcium antagonists, or tumor necrosis factor-α (TNF-α) (R&D Systems Inc) and resuspended in 100 mL of water. Cells were then lysed 3 times by freezing/thawing and centrifuged at 15,000g at 4°C for 10 minutes. The SOD activity in the supernatant was measured with a commercial kit (Calbiochem-Novabiochem Corporation). The precipitate was used for protein assay.

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

**Results**

**Effects of Pranidipine on cGMP Accumulation in VSMCs Cocultured With ECs**
Pranidipine at a concentration of 1 μmol/L significantly enhanced the cGMP accumulation in VSMCs from 30 minutes and up to 150 minutes after coculturing with ECs (Figure 1A). In addition, pranidipine induced a dose-dependent enhancement of cGMP accumulation in VSMCs cocultured with ECs (Figure 1B).

**Effects of Amlodipine and Nifedipine on cGMP Accumulation**
In contrast, neither amlodipine nor nifedipine at a concentration of 1 μmol/L enhanced the cGMP accumulation in VSMCs at 30 and 60 minutes after coculturing with ECs (Figure 2).

**Effects of Pranidipine on ET-1 Production From ECs**
As shown in Figure 3A, pranidipine also suppressed basal and thrombin-stimulated ET-1 production from ECs. However, pranidipine had no suppressive effect in the presence of L-NMMA, an inhibitor of NO synthesis (Figure 3B).

**Effects of Pranidipine on cGMP Accumulation in Rat Aortic Segments**
To examine whether pranidipine can enhance NO action in the vessel wall, we next measured cGMP accumulation after incubation with pranidipine in rat aortic segments with or without endothelium. Incubation for 60 minutes with pranidipine (1 μmol/L) significantly increased the levels of cGMP accumulation in rat aortic segments with endothelium (Figure 4). However, L-NMMA inhibited these effects. In addition, these pranidipine-induced effects were not observed in endothelium-denuded vessels (Figure 4).

**Effects of Pranidipine on eNOS Protein**
Pranidipine might change the expression of eNOS. This possibility was tested by incubating ECs with or without pranidipine for 6 or 12 hours before quantifying eNOS.
The degree of an increase induced by pranidipine was almost time-dependent increase in the levels of SOD activity in ECs. In contrast, nifedipine at a concentration of 1 \( \mu \)mol/L showed no significant effects.

Figure 3. Effects of pranidipine on ET-1 production from ECs. A, Dose-responsive effects of pranidipine on the basal and thrombin-stimulated ET-1 production from ECs. Confluent ECs were incubated for 24 hours with serum-free medium containing various concentrations of pranidipine in the presence or absence of 10 U/mL thrombin. After the incubation, ET-1 concentration in the medium was measured as described in the text. B, Confluent ECs were incubated for 24 hours with serum-free medium containing 1 \( \mu \)mol/L pranidipine or 10 U/mL thrombin in the presence or absence of 3\( \times 10^{-5} \) \( \mu \)mol/L L-NMMA. Values are mean\( \pm \)SD of 4 individual experiments, each containing 2 replicates. *P<0.01 vs control; **P<0.01 vs thrombin; +++P<0.01 vs pranidipine alone; +++P<0.01 vs thrombin and pranidipine.

Protein. Figure 5 shows a representative Western blot analysis of eNOS protein levels of ECs either exposed to pranidipine (1 \( \mu \)mol/L) or not exposed to pranidipine. The amounts of eNOS proteins were similar under these conditions.

Effects of Pranidipine, Amlodipine, and Nifedipine on SOD Activity

As shown in Figure 6A, pranidipine (1 \( \mu \)mol/L) induced a time-dependent increase in the levels of SOD activity in ECs. The degree of an increase induced by pranidipine was almost the same as that induced by TNF-\( \alpha \) at a concentration of 1 ng/mL. Amlodipine (1 \( \mu \)mol/L) also induced an increase in the levels of SOD activity at 4 and 12 hours after the stimulation (Figure 6B). However, the degrees were less than those induced by pranidipine. In contrast, nifedipine at a concentration of 1 \( \mu \)mol/L showed no significant effects.

Discussion

The endothelium plays numerous physiological roles in the maintenance of vascular homeostasis by releasing vasoconstrictor and vasodilator substances. One of these factors is NO, which induces vasodilation by increasing cGMP through activation of soluble guanylate cyclase within VSMCs. NO also inhibits the production of ET-1, an endothelium-derived vasoconstricting factor, from ECs.\(^9\) Balance between these relaxing and contracting factors is important in the regulation of vascular tone. On the other hand, endothelium is also a source of superoxide, which can mediate endothelium-dependent contractions by the breakdown of NO.\(^10\) The present study demonstrated that pranidipine, a long-acting calcium antagonist, may enhance the actions of NO released from ECs through inhibition of NO decomposition. This conclusion was based on our findings that pranidipine enhanced cGMP accumulation in VSMCs cocultured with ECs and in the vascular segments with endothelium. Pranidipine also enhanced the NO-induced suppression of ET-1 production from ECs. However, pranidipine had no effect in the presence of the NO synthesis inhibitor L-NMMA. In addition, pranidipine did not affect the basal expression of eNOS protein. On the other hand, pranidipine directly upregulated the activity of SOD, a superoxide scavenger.

Hypertension is associated with structural and functional alterations of the vessel wall. Recent studies have suggested that the endothelium-derived NO system is impaired in patients with hypertension, which may be important in the
pathogenesis of hypertension and its cardiovascular complications.\textsuperscript{11} PRAISE reported that there was a trend toward a reduction in the relative risk of mortality of patients with heart failure due to nonischemic dilated cardiomyopathy in the amiodipine group.\textsuperscript{2} These findings suggest that long-acting calcium antagonists may have unexpected beneficial actions during treatment of hypertensive patients. Recently, Zhang and Hintze\textsuperscript{1} reported that unlike nifedipine and diltiazem, amiodipine stimulates NO release from canine coronary microvessels. Thus, NO could be a key molecule that mediates beneficial actions of long-acting calcium antagonists. Although the mechanism by which patients with hypertension have a defect in the endothelium-derived NO system is not clear, it should be noted that Tschudi et al\textsuperscript{12} reported that in rat mesenteric resistant arteries hypertension is associated with increased NO decomposition by superoxide and not an altered release of NO. ECs contain various superoxide anion–generating systems such as xanthine oxidase\textsuperscript{13,17} and NADH oxidase.\textsuperscript{14} Hishikawa and Luscher\textsuperscript{15} also reported that pulsatile stretch stimulates superoxide production in human aortic ECs via NADPH oxidase and endothelial NO synthase. These findings suggest that the prevention of NO decomposition is a primary therapeutic target for the impaired endothelium-derived NO system in patients with hypertension.

Recently, Chen et al\textsuperscript{16} reported that amiodipine may preserve plasma SOD activity in an atherosclerotic rabbit model. In this study, amiodipine slightly upregulated the SOD activity in ECs. However, it showed a weak but not significant enhancing effect on cGMP accumulation induced by coculturing with ECs. Nakayama et al\textsuperscript{4} also reported similar findings that pranidipine induced a prolongation of endothelium-dependent relaxation in the rat aortic ring preparation, whereas amiodipine was without this effect. Since pranidipine induced a stronger enhancement of SOD activity than other calcium antagonists, it could more effectively improve the endothelium-derived NO system in patients with hypertension. Although it has been shown that nifedipine may stimulate cellular cGMP production through modulation of phosphodiesterase activity,\textsuperscript{17} pranidipine-induced enhancement of cGMP accumulation was observed in the presence of the phosphodiesterase inhibitor IBMX. Thus, it is unlikely that modulation of phosphodiesterase activity is responsible for the mechanism of enhancement by pranidipine.

In conclusion, pranidipine, a new long-acting calcium antagonist, enhances the actions of NO derived from ECs, which may be mediated through the inhibition of NO decomposition. Thus, pranidipine may be useful for the improvement of the endothelium-derived NO system in patients with hypertension.

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References
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