Original Article

Calcium Antagonist Reduces Oxidative Stress by Upregulating Cu/Zn Superoxide Dismutase in Stroke-Prone Spontaneously Hypertensive Rats

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Recent studies have suggested that the calcium antagonists have an antiatherogenic antioxidant property. The effects of the calcium antagonists on reactive oxygen species (ROS)-related enzymes, however, remain unknown. We hypothesized that the calcium antagonists inhibit oxidative stress in the hearts of stroke-prone spontaneously hypertensive rats (SHRSP) through the ROS-scavenging enzymes known as superoxide dismutases (SODs). Male 12-week-old Wistar-Kyoto rats (WKY) and SHRSP were used for the study. SHRSP were randomized and treated for 6 weeks with a vehicle, amlodipine (5 mg/kg/day), or enalapril (10 mg/kg/day). NAD(P)H oxidase activity was measured by a luminescence assay, and SOD activity was measured spectrophotometrically. Protein expressions were analyzed by immunoblots. Both drugs showed equipotent effects on systolic blood pressure, left ventricular hypertrophy and fibrosis, the wall-to-lumen ratio, the manganese SOD activity, ROS, and the endothelial NO synthase expression in the SHRSP hearts. Furthermore, amlodipine significantly restored copper/zinc-containing SOD (Cu/ZnSOD) expression and its activity in SHRSP hearts to a level equal to that of WKY more effectively than did enalapril ($p < 0.05$), whereas enalapril downregulated NAD(P)H oxidase activity more than did amlodipine ($p < 0.05$) in the SHRSP hearts. Furthermore, amlodipine restored Cu/ZnSOD expression and its activity in SHRSP hearts to a level equal to that in WKY hearts, and this restoration was significantly more effective than that by enalapril ($p < 0.05$); on the other hand, enalapril induced a greater downregulation of NAD(P)H oxidase activity in SHRSP hearts than did amlodipine ($p < 0.05$). Thus, amlodipine may inhibit vascular remodeling and oxidative stress in the SHRSP heart by efficiently upregulating Cu/ZnSOD, suggesting that the calcium antagonist may exhibit an antiatherogenic antioxidative action beyond blood-pressure lowering through the restoration of Cu/ZnSOD activity in the heart in cases of hypertension. (*Hypertens Res* 2004; 27: 877–885)

Key Words: hypertension, calcium antagonist, superoxide dismutase, oxidative stress

Introduction

Increased production of vascular reactive oxygen species (ROS) generated by the activated NAD(P)H oxidase contributes to vascular functional and structural alterations (1),

and the inhibition of the renin-angiotensin system with an angiotensin-converting enzyme (ACE) inhibitor slows the progression of atherosclerosis by reducing ROS and inhibiting vascular remodeling in hypertension (2). Conversely, the enzyme superoxide dismutase (SOD) is a primary cellular defense against ROS (3, 4). Three SOD isozymes have been

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identified: the dimeric, copper/zinc-containing SOD (Cu/ZnSOD) is cytosolic and nuclear; manganese SOD (MnSOD) is mitochondrial; and the tetrameric, proteoglycan-bound Cu/ZnSOD is extracellular (ecSOD). The predominant activity of SOD in peripheral vessels is attributed to Cu/ZnSOD (5), and it may play an important role in the pathogenesis of hypertension (6).

The calcium antagonists are safe and well tolerated (7) and have been widely used in the treatment of patients with hypertension (8), in whom they inhibit cardiovascular events as effectively as ACE inhibitors (9). Interestingly, several studies have shown that the calcium antagonists may have antiatherogenic properties, including an antioxidant property, independent of their effects on vasodilatation (10–12). However, the precise mechanisms by which the calcium antagonists inhibit atherosclerosis *in vivo*, and especially the effects of calcium antagonists on ROS, are largely unknown.

We hypothesized that the calcium antagonists inhibit oxidative stress in the hearts of stroke-prone spontaneously hypertensive rats (SHRSP) through the upregulation of the SOD, ROS-scavenging enzymes, and the purpose of this study was to compare the effects of a dihydropyridine calcium antagonist, amlodipine, with those of the ACE inhibitor, enalapril, on vascular remodeling of intramyocardial arteries and on the antioxidant systems involving NAD(P)H oxidase and SODs in the SHRSP heart.

Methods

The Ethics Committee for Animal Experimentation at the Yamaguchi University School of Medicine approved the experimental protocol used in this study. The experiment was performed according to the Guidelines for Animal Experimentation at the Yamaguchi University School of Medicine, and according to the law (No. 105) and notification (No. 6) of the Japanese government.

Chemicals and Antibodies

Amlodipine and enalapril were provided by Pfizer Pharmaceuticals Inc. (Tokyo, Japan), and Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. The following were applied for immunoblots: mouse monoclonal antibodies against human MnSOD (Chemicon International, Temecula, USA), human endothelial NO synthase (eNOS) (BD Transduction Laboratories, San Diego, USA), goat polyclonal antibodies against human p22^{phox} and Cu/ZnSOD (Santa Cruz Biotech, Santa Cruz, USA), and horseradish peroxidase (HRP)-conjugated rabbit anti-goat and anti-mouse IgG (Zymed Laboratories, San Francisco, USA).

Experimental Protocol

Twelve-week-old male Wistar-Kyoto rats (WKY group; $n = 20$) and SHRSP ($n = 60$) were obtained from Charles River

Japan (Yokohama, Japan). The WKY group was treated with a vehicle, and the SHRSP were randomized into 3 groups of 20 rats each and were treated with a vehicle (SHRSP group; $n = 20$), amlodipine (5 mg/kg per day, amlodipine group; $n = 20$), or enalapril (10 mg/kg per day, enalapril group; $n = 20$). The doses used in the experiments were determined by preliminary experiments. Without anesthetizing the rats, systolic blood pressure (SBP) and heart rate were determined by tail-cuff plethysmography. After the 6-week treatment period, rats were weighed and euthanized with a sodium pentobarbital overdose, and hearts were excised and weighed. Some of the hearts were perfused and fixed with heparinized saline followed by Bouin's solution *via* retrograde infusion into the ascending aorta at a pressure of 90 mmHg as previously reported (13, 14), and the left ventricles were separated and cut into three pieces perpendicular to the long axis. A piece of the middle portion of the heart tissue from each heart was paraffin-embedded to obtain 4- μ m-thick sections, which were then stained with Sirius red for histological analysis. The left ventricles of the other hearts were separated, washed with heparinized saline, and weighed and cut into three pieces perpendicular to the long axis. The middle portion and the apex-side heart tissues were frozen in liquid nitrogen and stored at -80°C until use for immunoblotting and for the ROS and ROS-related enzyme assays. To avoid contaminating the epicardial large coronary arteries as much as possible, we did not use the remaining base-side heart tissues for the study.

Histological Analysis

To evaluate the coronary arterial wall thickness and perivascular fractional fibrosis, we scanned short-axis images of intramyocardial arteries at $\times 200$ magnification. In each heart, we evaluated the wall-to-lumen ratio (the medial thickness compared to the internal diameter) and cross-sectional area of at least 10 intramyocardial arteries $<150\ \mu\text{m}$ in diameter, as well as the perivascular collagen volume fraction (the ratio of the collagen deposition area surrounding the vessel to the lumen area) and interstitial collagen area (the ratio of the collagen deposition area in interstitial spaces and the corresponding left ventricular area) in the heart by analyzing Sirius red-stained sections under a microscope fitted with cross-polarization filters. All were evaluated in a blind fashion using a computer-assisted image analysis system with NIH Image software (ver. 1.62), according to the method of Baba *et al.* (15), and the mean value of each heart was used for statistical analysis.

Immunoblotting

Immunoblots were basically performed as previously described (16). The p22^{phox}, Cu/ZnSOD, and MnSOD were separated by sodium dodecyl sulfate (SDS)-15% polyacrylamide gel electrophoresis (PAGE), and eNOS was separated

by SDS-10% PAGE. Primary antibodies against p22^{phox} and Cu/ZnSOD were used at a dilution of 1:500; MnSOD and eNOS were used at a dilution of 1:1,000. Equal amounts of protein of total tissue homogenate from heart tissue were applied in each well (p22^{phox}, 40 μ g; Cu/ZnSOD, 30 μ g; MnSOD, 12 μ g; eNOS, 20 μ g) and then electroblotted and detected with the enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). After immunoblotting, the film was scanned and densitometric analyses were performed using NIH Image software (ver. 1.62).

Measurement of Oxidative Stress

We estimated myocardial oxidative stress by measuring the levels of both 8-*iso*-prostaglandin F_{2 α} (8-*iso*-PGF_{2 α}) and thiobarbituric acid reactive substances (TBARS). The level of 8-*iso*-PGF_{2 α} was measured using an enzyme-linked immunoassay kit (Cayman Chemicals, Ann Arbor, USA) (17). Briefly, heart tissues were homogenized and were protected by the addition of indomethacin (0.001% w/v) to prevent *in vitro* formation of prostanoids due to any leukocyte contamination. Samples were then hydrolyzed with the appropriate excess volume of 2 mol/l KOH 45 °C for 2 h. After hydrolysis, samples were cooled and treated with an equal volume of 2 mol/l HCl, and then the neutralized samples were centrifuged at 3,000 rpm for 20 min. Using 8-*iso*-PGF_{2 α} as the standard, the level of 8-*iso*-PGF_{2 α} was calculated as pg/mg wet tissue.

TBARS levels were determined by a colorimetric method (Wako Pure Chemicals, Tokyo, Japan) (18). Briefly, heart tissue was homogenized in 6.5% trichloroacetic acid (TCA), and a reagent containing 15% TCA, 0.375% thiobarbituric acid, and 0.25% HCl was added to it. The sample was then mixed thoroughly, heated for 15 min in a boiling water bath, cooled, and centrifuged at 2,000 rpm, and the absorbance of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using malondialdehyde as a standard, TBARS levels were calculated as nmol/mg wet tissue.

Measurement of SOD Activity

SOD activities were determined based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm with a spectrophotometric assay (OxisResearch, Portland, USA) (19). Briefly, heart tissues were washed with 0.9% NaCl containing heparin to remove red blood cells, followed by homogenization and centrifugation. Next, 40 μ l of tissue homogenate was added to 900 μ l of 2-amino-2-methyl-1,3-propanediol, containing boric acid and diethylenetriaminepentaacetic acid (DTPA) (pH 8.8), and then 30 μ l of 1,4,6-trimethyl-2,2-vinylpyridinium trifluoromethanesulfonate in HCl was added, briefly vortexed, and incubated at

37 °C for 1 min. We added 30 μ l of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene, in HCl containing DTPA and ethanol, and immediately measured the absorbance at 525 nm spectrophotometrically. We determined the SOD activity based on the ratio of the autoxidation rates in the presence and in the absence of SOD. Absolute ethanol/chloroform, 62.5/37.5 (v/v), was used to inactivate MnSOD and specifically measure Cu/ZnSOD activity according to the manufacturer's recommendations.

Measurement of NAD(P)H Oxidase Activity

NAD(P)H oxidase activities were determined by a luminescence assay (20). Briefly, heart tissues were placed in a chilled, modified Krebs-HEPES buffer (99 mmol/l NaCl, 4.7 mmol/l KCl, 1.9 mmol/l CaCl₂, 1.2 mmol/l MgSO₄, 1.0 mmol/l K₂HPO₄, 25 mmol/l NaHCO₃, 20 mmol/l Na-2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), and 11 mmol/l glucose, pH 7.4). A 10% (w/v) tissue homogenate in a 50-mmol/l phosphate buffer was subjected to centrifugation at 1,000 $\times$ g for 10 min to remove unbroken cells and debris. An aliquot was kept for protein determination, and supernatants (25 μ l) were assayed immediately for superoxide production. A luminescence assay was performed in a 50 mmol/l phosphate buffer, pH 7.0, containing 1 mmol/l O, O'-bis (2-aminoethyl)ethyleneglycol-N, N, N'-tetraacetic acid (EGTA), 150 mmol/l sucrose, 500 μ mol/l lucigenin (bis-N-methylacridinium nitrate) as the electron acceptor, and 100 μ mol/l NAD(P)H as the substrate (final volume 225 μ l). The mixture was poured into a 96-well microplate. This concentration fell well within the linear range of the assay (1 μ mol/l to 10 mmol/l for NAD(P)H), and the NAD(P)H was not rate-limiting over the initial course of the assay. No activity could be measured in the absence of NAD(P)H. After dark adaptation, background counts were recorded and a tissue homogenate was added to the microplate. A lucigenin count was then recorded every 15 s for 10 min, and the respective background counts (without tissue homogenate) were subtracted from the tissue homogenate readings. The lucigenin count was expressed as counts per second per milligram of the tissue homogenate.

Statistical Analysis

All values were expressed as the means $\pm$ SEM. The experimental groups were compared with ANOVA followed by Scheffe's multiple comparisons; values of $p < 0.05$ were considered statistically significant.

Results

Throughout the experiments, SBP in the vehicle SHRSP group was significantly higher than that in the WKY group. Both enalapril and amlodipine significantly and equally reduced SBP compared to the levels in the vehicle SHRSP

Fig. 1. Systolic blood pressure and heart rate in the vehicle SHRSP, amlodipine, and enalapril groups. * $p < 0.01$ vs. the WKY group, † $p < 0.01$ vs. the vehicle SHRSP group. Experiments, $n = 5-7$.

Fig. 2. Representative micrograph of the effects of amlodipine and enalapril on vascular remodeling and perivascular collagen deposition in an intramyocardial artery. The sections were stained with Sirius red F3BA (a through d) and viewed through a polarized-light microscope (e through h). Shown are WKY (a and e) and SHRSP treated with vehicle (b and f), amlodipine (c and g), or enalapril (d and h). Bar, 50 μ m.

group. However, the two drug-treated SHRSP groups showed significantly higher SBP than did the WKY group (Fig. 1A). The heart rates were unaltered throughout the experiments among the four groups (Fig. 1B).

Figure 2 shows representative micrographs of the effects of amlodipine and enalapril on vascular remodeling and

perivascular collagen deposition in an intramyocardial artery. Table 1 summarizes the body weight, left ventricular (LV) weight, wall-to-lumen ratio of intramyocardial arteries, and cardiac fibrosis in 18-week-old WKY and SHRSP. The body weight was greater in the WKY group than in the three SHRSP groups, but there was no difference in body weight

Table 1. Body Weight, Left Ventricular Weight, Wall-to-Lumen Ratio of the Intramyocardial Artery, and Cardiac Fibrosis in 18-Week-Old Rats

Parameter	WKY	SHRSP	Amlodipine	Enalapril
BW (g)	413 ± 10	296 ± 7*	299 ± 2*	296 ± 6*
LVW (mg)	818 ± 22	863 ± 24	757 ± 20†	754 ± 14†
LVW/BW (mg/g)	1.90 ± 0.05	2.77 ± 0.11*	2.40 ± 0.05*,†	2.42 ± 0.04*,†
Wall-to-lumen ratio	0.30 ± 0.06	0.58 ± 0.06*	0.37 ± 0.03†	0.28 ± 0.03†
Perivascular collagen volume fraction	0.83 ± 0.10	2.23 ± 0.22*	1.23 ± 0.18†	0.94 ± 0.18†
Interstitial collagen area (%)	1.00 ± 0.18	2.91 ± 0.34*	1.61 ± 0.14†	0.99 ± 0.12†

Values are the mean ± SEM. WKY and SHRSP were treated with vehicle, enalapril (10 mg/kg per day), or amlodipine (5 mg/kg per day) for 6 weeks. * $p < 0.01$ vs. the WKY groups, † $p < 0.01$, ‡ $p < 0.05$ vs. the SHRSP group. BW, body weight; LVW, left ventricular weight; WKY, Wistar Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats. Experiments, $n = 8$.

Fig. 3. Levels of 8-iso-PGF_{2α} and TBARS in the rat heart. Bars indicate SEM. * $p < 0.01$, # $p < 0.05$ vs. the WKY group, † $p < 0.01$, ‡ $p < 0.05$ vs. the vehicle SHRSP group. Experiments, $n = 8$. 8-iso-PGF_{2α}, 8-iso-prostaglandin F_{2α}; TBARS, thiobarbituric acid reactive substances.

between the vehicle and the two drug-treated SHRSP groups.

The LV weight/body weight ratio was significantly higher in the vehicle SHRSP group than in the WKY group. Both drugs significantly and similarly reduced the LV weight/body weight ratio in the SHRSP hearts. However, both drug-treated SHRSP groups showed a significantly higher LV weight/body weight ratio than that of the WKY group. The wall-to-lumen ratio of the intramyocardial arteries in the vehicle SHRSP group was significantly greater than that in the WKY group. Animals in the two drug-treated group showed a significant and approximately equivalent reduction in the wall-to-lumen ratio compared to those in the vehicle SHRSP group. In addition, there were no differences in the wall-to-lumen ratio values among the WKY group and the two drug-treated SHRSP groups. The perivascular collagen volume fraction and interstitial collagen area in the left ventricle were significantly higher in the vehicle SHRSP group than in the WKY group. Both drugs significantly reduced these values compared with those in the vehicle SHRSP group, and there were no significant differences in these values between the WKY group and the two drug-

treated SHRSP groups.

Figure 3 shows that both the 8-iso-PGF_{2α} and TBARS levels were significantly higher in the vehicle SHRSP hearts than in the WKY group. Both amlodipine and enalapril significantly inhibited the rise in both the 8-iso-PGF_{2α} and TBARS levels in the SHRSP hearts compared with the vehicle SHRSP group, and there were no significant differences in these values among the WKY group and the two drug-treated groups.

Figure 4 shows the results of quantitative analysis of p22^{phox}, Cu/Zn-, and MnSOD expression in the rat heart. Expression of p22^{phox} in the heart was significantly higher in the vehicle SHRSP group than in the WKY group. Both drugs significantly downregulated p22^{phox} expression compared to that in the vehicle SHRSP group. The enalapril group showed a significant decrease in p22^{phox} expression to nearly the level seen in the WKY group, whereas the level of p22^{phox} expression in the amlodipine group was significantly higher than that of the WKY group, and significant differences were seen between the amlodipine and enalapril groups in p22^{phox} expression in SHRSP hearts. Conversely,

Fig. 4. Quantitative analysis of p22^{phox}, Cu/Zn-, and MnSOD expression in the rat heart. Bars indicate SEM. * $p < 0.01$, # $p < 0.05$ vs. the WKY group, † $p < 0.01$, ‡ $p < 0.05$ vs. the vehicle SHRSP group, § $p < 0.01$, ¶ $p < 0.05$ vs. the amlodipine group. Experiments, $n = 8$.

Fig. 5. Quantitative analysis of NAD(P)H oxidase activity, and Cu/Zn- and MnSOD activity in the rat heart. Bars indicate SEM. * $p < 0.01$, # $p < 0.05$ vs. the WKY group, † $p < 0.01$ vs. the vehicle SHRSP group, ‡ $p < 0.05$, § $p < 0.01$ vs. the amlodipine group. Experiments, $n = 4-5$.

Cu/ZnSOD expression was significantly downregulated in the vehicle SHRSP group compared with that in the WKY group. Compared to that in the vehicle SHRSP group, Cu/ZnSOD expression in SHRSP hearts was significantly upregulated in both the amlodipine and enalapril groups. However, only the amlodipine group showed the restoration of Cu/ZnSOD expression to nearly the same level as seen in the WKY group, and a significant difference was seen between the amlodipine and enalapril groups in Cu/ZnSOD expression in SHRSP hearts. MnSOD expression in the heart was unaltered among the four groups.

Figure 5 shows the results of the quantitative analysis of

NAD(P)H oxidase and SOD activities in rat hearts. NAD(P)H oxidase activity was significantly upregulated in the vehicle SHRSP group compared with that in the WKY group. There was a significant reduction in NAD(P)H oxidase activity in the enalapril group to nearly the same level as seen in the WKY group. In contrast, amlodipine significantly reduced NAD(P)H oxidase activity in the SHRSP heart compared with that in the vehicle SHRSP group; however, the levels of NAD(P)H oxidase activity in the amlodipine group were still significantly higher than those of the WKY group. Moreover, significant differences were seen between the enalapril and amlodipine groups in NAD(P)H oxi-

Fig. 6. Quantitative analysis of eNOS expression in the rat heart. Bars indicate SEM. * $p < 0.05$ vs. the WKY, amlodipine, and enalapril groups. Experiments, $n = 8$.

dase activity in SHRSP hearts. Conversely, Cu/ZnSOD activity was significantly lower in the vehicle SHRSP group than in the WKY group. Compared to that in the vehicle SHRSP group, Cu/ZnSOD activity was significantly higher in the hearts of the vehicle SHRSP group than in those of the amlodipine and enalapril groups, and only the amlodipine group restored Cu/ZnSOD activity to a level nearly equal to that of the WKY group. Furthermore, significant differences were seen between the amlodipine and enalapril groups in Cu/ZnSOD activity in SHRSP hearts. MnSOD activity in the heart was unaltered among the four groups.

The level of eNOS expression in the rat heart was significantly lower in the vehicle SHRSP group than in the WKY group (Fig. 6). Both amlodipine and enalapril restored eNOS expression in SHRSP hearts to the same degree as in WKY hearts, while there were no differences in the degree of up-regulation of eNOS expression in the heart between the two drug-treated SHRSP groups.

Discussion

Amlodipine and enalapril contributed equally to an overall reduction in oxidative status and to the inhibition of vascular remodeling of intramyocardial arteries and cardiac fibrosis in SHRSP to the same levels as those in the WKY group with the doses used in the study. Even though both drugs demonstrated equipotent blood pressure-lowering actions, they still induced significantly higher SBPs in the drug-treated SHRSP groups than in the WKY group. These results suggest that both drugs may have additional benefits for the reduction of these indices in the SHRSP hearts beyond blood-pressure lowering, indicating that these processes are redox-sensitive

in SHRSP (21). The different mechanisms by which amlodipine and enalapril influence these processes could be related to the direct action of the ROS-related enzyme systems, since they belong to different classes of drugs. In this study, we clearly showed that enalapril reduced ROS in the SHRSP hearts by decreasing NAD(P)H oxidase more selectively and efficiently than did amlodipine, whereas amlodipine reduced ROS by selectively restoring the Cu/ZnSOD more effectively than did enalapril.

Our results for the inhibitory effects of enalapril on NAD(P)H oxidase in the SHRSP heart indicate that angiotensin II (Ang II) may play a critical role in the regulation of NAD(P)H oxidase, a major source of superoxide anion in vascular cells (1, 2). Enalapril might prevent the activation of Ang II type 1 receptors and, thus, inhibit the phosphorylation of p47^{phox}, which promotes its binding to the membrane NAD(P)H oxidase components through c-Src (22). The decreased activity of this enzyme by the administration of enalapril resulted in reduced generation of superoxide anion and inhibited vascular remodeling of intramyocardial arteries and cardiac fibrosis in the SHRSP heart in our study, independent of blood-pressure lowering (23).

Clinically, amlodipine inhibits cardiovascular events as effectively as ACE inhibitors (9), and it has also been shown to significantly slow the progression of atherosclerosis (10). Several potential antiatherosclerotic mechanisms of action independent of calcium channel modulation have been reported for amlodipine (10–12), including inhibition of lipid peroxide formation, increase in NO production, modification of smooth muscle cell atherosclerotic membrane defects *via* reduction of the swelling of the smooth muscle cell membrane, a proteoglycan-mediated mechanism by vascular smooth muscle cells and low-density lipoprotein-proteoglycan interaction (24), and inhibition of smooth muscle cell proliferation, migration, and phenotypic change (25). These cellular actions by amlodipine would inhibit vascular remodeling. As shown in this study, the reduced activation of Cu/ZnSOD in the SHRSP heart suggests that exposure to oxidative stress induced by the activation of NAD(P)H oxidase may exhaust the antioxidative capacity in the SHRSP heart. In contrast, the restoration of Cu/ZnSOD activity by amlodipine in the SHRSP heart would lead to decreased superoxide anion concentration and the inhibition of vascular remodeling of intramyocardial arteries and cardiac fibrosis to the same extent as that of the WKY and enalapril groups at the doses used.

Our results for the effects of amlodipine on Cu/ZnSOD in SHRSP hearts are similar to the results described in a previous report in hypercholesterolemic rabbits (26). In addition, there have also been several reports (6, 11, 27) showing the involvement of SOD or Cu/ZnSOD in blood-pressure lowering, attenuated vascular remodeling, decreased vascular superoxide concentration, and increased antioxidant status in hypertension. Furthermore, Cu/ZnSOD limits the increases in superoxide anion under basal conditions and also results

in altered responsiveness in both large arteries and microvessels in Cu/ZnSOD-deficient mice (5). These results suggest that Cu/ZnSOD plays a crucial role in the pathogenesis of vascular dysfunction in hypertension, and that amlodipine may exert antiatherosclerotic effects by preserving Cu/ZnSOD. In our study, we did not examine which cells are important for upregulating Cu/ZnSOD in the SHRSP heart, and the precise mechanisms by which amlodipine upregulates Cu/ZnSOD remain unknown. However, Fukuo *et al.* have recently demonstrated that the calcium antagonist nifedipine indirectly upregulates endothelial SOD expression and enhances endothelial NO production in the vasculature, and induces upregulation of MnSOD expression in vascular smooth muscle cells *via* NO derived from endothelial cells (28, 29).

Hypertension is a central pathogenic factor of the endothelial dysfunction caused in part by an impairment of endothelial NO produced by eNOS (30, 31). In addition, superoxide anion generation is increased in SHRSP, and the tissue and enzymatic sources of this excess superoxide anion appear to be the endothelium and eNOS, respectively. The increase in superoxide anion generation could explain the decreased availability of basal NO observed in SHRSP (30). In contrast, treatment with an ACE inhibitor or a calcium antagonist resulted in demonstrable improvement of endothelial dysfunction by a mechanism that is probably related to antioxidant activity (32). Our findings in the endothelium as assessed by eNOS expression were similar for the two drugs, suggesting that both drugs might preserve NO to the same extent by increasing eNOS to attenuate vascular remodeling. Although we did not measure NO activity, Park *et al.* demonstrated that a SOD mimetic prevents progression of hypertension independent of endothelial function, suggesting that oxidative stress itself plays an important role in vascular damage associated with severe hypertension in salt-loaded SHRSP (27).

In this study, we did not examine the effects of enalapril and amlodipine on the other ROS-scavenging enzymes, such as glutathione peroxidase and/or catalase, in the rat heart. Recently, Csonka *et al.* reported that the antioxidative system involving glutathione peroxidase, catalase, MnSOD, and Cu/ZnSOD in dilative cardiomyopathic hearts of SHR is induced during the process of decompensation, probably in order to help alleviate acute stress caused by ROS in the failing myocardium (33). It has also been reported that the overexpression of glutathione peroxidase inhibited LV remodeling and failure after myocardial infarction in glutathione peroxidase transgenic mice (18). Although we cannot exclude the possibility that such ROS-scavenging enzymes might affect ROS levels in the heart of SHRSP in addition to the restoration of Cu/ZnSOD activity by the administration of amlodipine, the differences in the experimental models between previous studies (18, 33) and our present study may have caused the different results concerning ROS-scavenging enzymes. Furthermore, it has been reported that rat ecSOD is lacking

in the vessel walls and is mainly present in plasma (4), and that ecSOD activity is also quite low in rat hearts (34), indicating the unlikelihood that ecSOD plays a critical role in the heart, although we cannot exclude the possibility that ecSOD in plasma could have influenced our results.

A recent large-scale clinical trial for the treatment of hypertension suggested that the ACE inhibitors lisinopril and amlodipine equally inhibited cardiovascular events (9), and the recent guideline recommends both drugs as first-line choices for the treatment of hypertension (35). Our results appear to support this recommendation, even if the mechanisms by which the two drugs inhibit oxidative stress are different.

In summary, our study demonstrated that both enalapril and amlodipine might have additional benefits for the reduction of oxidative stress, vascular remodeling, and cardiac fibrosis in the SHRSP hearts beyond blood-pressure lowering. Amlodipine inhibited vascular remodeling of intramyocardial arteries in SHRSP by efficiently modifying Cu/ZnSOD more than did enalapril. Thus, the calcium antagonist may exert an antiatherogenic antioxidative action by restoring Cu/ZnSOD activity in the heart in cases of hypertension. Further studies will be needed to clarify a plausible mechanism of the calcium antagonist and the ROS-scavenging enzyme systems in hypertension.

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