Original Article

Calcium Channel Blockades Exhibit Anti-Inflammatory and Antioxidative Effects by Augmentation of Endothelial Nitric Oxide Synthase and the Inhibition of Angiotensin Converting Enzyme in the N^G -Nitro-L-Arginine Methyl Ester-Induced Hypertensive Rat Aorta: Vasoprotective Effects beyond the Blood Pressure-Lowering Effects of Amlodipine and Manidipine

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Long-acting dihydropyridine calcium channel blockades have been shown to limit the progression of atherosclerosis and decrease the incidence of cardiovascular events in humans and animals. To investigate the vasoprotective effects beyond the blood pressure-lowering effects of these agents, amlodipine (20 mg/kg/day) and manidipine (10 mg/kg/day) were administered by gavage to N^G -nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats for 2 weeks. L-NAME treatment (0.7 mg/ml in drinking water) significantly decreased the gene and protein expression of endothelial nitric oxide synthase (eNOS) and increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, vascular cell adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1) mRNA levels in the aorta, as determined by Western blotting and reverse transcription (RT)-polymerase chain reaction (PCR). Amlodipine and manidipine normalized the decreased expression of eNOS gene and protein, and attenuated the overexpression of NADPH oxidase, VCAM-1, and MCP-1 mRNA. Furthermore, amlodipine and manidipine prevented the L-NAME-induced increase in the angiotensin converting enzyme (ACE) mRNA content, thereby restoring control levels in the aorta. On the other hand, hydralazine treatment had no such effect in L-NAME treated rats. Furthermore, the increased expression of manganese superoxide dismutase (Mn-SOD) by L-NAME treatment was not affected by amlodipine, manidipine, or hydralazine. We concluded that the direct anti-inflammatory and antioxidative effects of calcium channel blockades in the aorta of rats with L-NAME-induced hypertension were not likely to have been mediated by the blood pressure-lowering action of these agents, but instead these beneficial effects appear to have been mediated by an augmentation of eNOS expression and by the inhibition of the expression of ACE. (*Hypertens Res* 2005; 28: 689–700)

Key Words: calcium channel blockades, atherosclerosis, angiotensin converting enzyme, endothelial nitric oxide synthase

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Received January 28, 2005; Accepted in revised form June 24, 2005.

Introduction

Endothelial-derived nitric oxide (NO), synthesized by endothelial NO synthase (eNOS), has several important anti-atherogenic effects, including controlling vascular tone, platelet aggregation, leukocyte adhesion, and modulating smooth muscle cell proliferation (1–3). Endothelial dysfunction, which has been associated with a decrease in the activity of NO, is an early feature in atherosclerosis and has been described as occurring in the context of several pathologies, including hyperlipidemia, hypertension, heart failure, and diabetes (2, 4–16). Atherosclerosis manifests as an increased expression of adhesion molecules (4, 12) and as a diminished release of NO into the arterial wall due to either impaired local production or excessive oxidative degradation (17–19). On the other hand, a reduction in NO synthesis increases endothelial intracellular oxidative stress (20, 21). Furthermore, mutant mice lacking eNOS develop hypertension and produce more severe inflammatory and proliferative vascular lesions in response to injury than do control mice (22–24).

N^G -nitro-L-arginine methyl ester (L-NAME) is a structural analog of L-arginine and is known to be a potent competitive inhibitor of NOS (25). Recently, L-NAME has been added to the drinking water of experimental animals to induce marked and persistent hypertension, with the associated impairment of endothelium-dependent arterial relaxation (2, 26–29). Chronic inhibition of NO synthesis by the administration of L-NAME is known to cause early inflammation and subsequent arteriosclerosis in experimental animals (30, 31). Some aspects of the vascular pathophysiological and pathobiologic events that have been shown to occur after the administration of L-NAME are similar to those seen in the course of human arteriosclerosis. Thus, this L-NAME-induced animal model provides a useful experimental model of hypertension for the investigation of the protective roles of therapeutic agents on arterial walls, and in particular on endothelial function.

Calcium channel blockades exert vasculoprotective effects in cases of cardiovascular disease (32–35). Furthermore, these agents reduce cardiovascular mortality in patients after myocardial infarction (36). The calcium channel blockade amlodipine has been shown to limit the progression of arteriosclerosis and atherosclerosis in animals and humans (37, 38). Although the mechanisms underlying the direct protective effects of calcium channel blockades on endothelial cell injury are not fully understood, such vasoprotective effects as the amelioration of endothelial dysfunction (39, 40) and decreases in the proliferation of vascular smooth muscle cells (41), oxidative stress (37, 42), and inflammation (43), are at least in part independent of the blood pressure-lowering effects of these agents. The calcium channel blockade has been shown to ameliorate hypertension and improve NO production in the vasculature and kidneys. Chronic administration of the calcium channel blockade RO-5967 has been shown to attenuate hypertension, reduce intimal inflamma-

tory infiltration, and improve acetylcholine-induced vasorelaxation of the aortic rings in spontaneously hypertensive rats (44).

To investigate the antiatherosclerotic effects of calcium channel blockades, we analyzed the expression of eNOS and molecules associated with inflammatory and oxidative stress in the L-NAME-induced hypertensive rat aorta. In addition, we attempted to determine the underlying mechanism of the vasoprotective effects of calcium channel blockades.

Methods

Animals

Male Wistar rats (Shimizu Laboratory Supply, Kyoto, Japan) weighing 250 g were housed in an environmentally controlled room with a 12-h light/dark cycle, and the animals were given standard rodent chow and tap water *ad libitum*. The rats were acclimated to handling by humans before the randomization and then were divided into the following six groups of 12–16 animals each at the beginning of the study: 1) the control group received untreated drinking water; 2) the L-NAME group received drinking water containing 0.7 mg/ml L-NAME; 3) and 4) the L-NAME + amlodipine groups received drinking water containing L-NAME and amlodipine at 20 or 10 mg/kg per day by gavage, respectively; 5) the L-NAME + manidipine group received drinking water containing L-NAME and manidipine at 10 mg/kg per day by gavage; and 6) the L-NAME + hydralazine group received drinking water containing L-NAME and hydralazine at 12 mg/kg per day. After 2 weeks of treatment, each rat ($n=6-8$) was anesthetized with an intraperitoneal injection of pentobarbital sodium (35 mg/kg). The left femoral artery was exposed and then cannulated with polyethylene tubing (PE10 fused into PE50, Becton Dickinson, Franklin Lakes, USA) for measuring blood pressure and heart rate. The free ends of the catheters were tunneled subcutaneously through to the back. The mean arterial pressure (MAP) and heart rate (HR) were monitored with a pressure transducer (MP5100, Edwards Lifescience, Tokyo, Japan) and recorded on a polygraph with the animals awake after recovery. The body weight of each animal was also measured. At the end of the treatment period, the thoracic aorta was immediately excised and placed on a phosphate buffered saline (PBS)-precooled plate. The aortic tissue was dissected from the adherent fat and connective tissues on ice, and then frozen in liquid nitrogen and kept at -80°C for subsequent analysis. Extreme care was taken to avoid stretching of the arteries during dissection. This study was performed in accordance with the guidelines for animal experiments at Kyoto Pharmaceutical University.

Cell Culture

Rat aortic endothelial cells were isolated from each group of rats by a previously described primary explant technique (45,

Table 1. PCR Primers and PCR Protocols

Product size	PCR primer sequences	PCR protocols
GAPDH 308 bp	Forward: 5'-TCCCTCAAGATTGTCAGCAA-3' Reverse: 5'-AGATCCACAACGGATACATT-3'	94°C/30 s 57°C/40 s 72°C/min } 28 cycles
eNOS 96 bp	Forward: 5'-CATGTTTGTCTGCGGTGATGT-3' Reverse: 5'-GCTTCATCCAGCTCCATGCT-3'	94°C/30 s 57°C/40 s 72°C/min } 32 cycles
VCAM-1 510 bp	Forward: 5'-AGGCAGAGTACACAGACTCT-3' Reverse: 5'-ACACTCGTATACTCCGGCAT-3'	94°C/30 s 56°C/40 s 72°C/min } 36 cycles
MCP-1 259 bp	Forward: 5'-TGTTACAGTTGCTGCCTGT-3' Reverse: 5'-GATCTCACTTGGTTCTGGTC-3'	94°C/30 s 56°C/40 s 72°C/min } 36 cycles
NADPH oxidase 468 bp	Forward: 5'-TCAGATGTCATGGAACCTCCG-3' Reverse: 5'-GTCTGCAAACCACTGGAATG-3'	94°C/30 s 56°C/40 s 72°C/min } 30 cycles
ACE 599 bp	Forward: 5'-GCAGTACAAAGACTTGCTTG-3' Reverse: 5'-TGGCAGAGGCTGACATGTTA-3'	94°C/30 s 56°C/40 s 72°C/min } 32 cycles

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; eNOS, endothelial nitric oxide synthase; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; NADPH, nicotinamide adenine dinucleotide phosphate; ACE, angiotensin converting enzyme.

Table 2. Hemodynamic Parameters

Groups	MAP (mmHg)	HR (/min)	BW (g)
Control	106±3	368±6	299±13
L-NAME	163±3*	362±12	301±10
L-NAME + amlodipine (20 mg/kg/day)	130±3 [#]	358±4	288±19
L-NAME + amlodipine (10 mg/kg/day)	132±1 [#]	351±18	287±18
L-NAME + manidipine	126±5 [#]	357±11	297±14
L-NAME + hydralazine	127±3 [#]	350±6	301±13

Values are means±SEM ($n=6-8$). MAP, mean arterial pressure; HR, heart rate; BW, body weight. * $p<0.0001$ vs. control, [#] $p<0.0001$ vs. L-NAME.

46). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum, endothelial cell growth supplement (100 µg/ml), and antibiotics (50 µg/ml ampicillin, 15 µg/ml gentamicin, 1 µg/ml minomycin, and 1 µg/ml amphotericin B). Rat tail collagen (type 1)-coated tissue culture dishes (35 mm; Biocoat Cell Ware; Becton Dickinson Labware, Bedford, USA) were used for the endothelial cell culture, and cells from passage 3 were used for the experiments. After reaching 90% confluence, the endothelial cells were incubated in conditioned medium (DMEM containing 1.0% bovine serum albumin and antibiotics) for 24 h before the initiation of the experiments in order to induce quiescence before subsequent analysis. As a separate experiment, rat aortic cells were isolated from normal male Wistar rats weighing 250 g, and the cells were cultured as described above. After a 24-h period of quiescence,

the cells were incubated with amlodipine (10^{-6} mol/l) or manidipine (10^{-6} mol/l) for 4 h into the culture medium. Cultured aortic endothelial cells from each group and those from normal rats were washed three times with PBS and used for the polymerase chain reaction (PCR) analysis of angiotensin converting enzyme (ACE) mRNA expression.

RNA Isolation and Reverse Transcription (RT)-PCR

The total RNAs were isolated from rat aorta ($n=6-8$ in each group) and cultured aortic endothelial cells by the acid guanidium thiocyanate-phenol-chloroform method using Isogen reagent (NIPPON GENE, Tokyo, Japan) and were subsequently treated with RNase-free DNase. The RNA concentration was determined by ultraviolet (UV) light absor-

Fig. 1. The expression of eNOS mRNA and protein in the aorta from each type of treated rat. *A:* Expression of the mRNA for eNOS, assayed by RT-PCR. *B:* Quantitative analysis of the expression of the mRNA for eNOS, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, eNOS/GAPDH). * $p<0.005$ vs. control, # $p<0.0001$ vs. L-NAME. *C:* Expression of eNOS protein, assayed by Western blotting. *D:* Quantitative analysis of the expression of eNOS protein, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, eNOS/actin). * $p<0.0001$ vs. control, # $p<0.05$ vs. L-NAME.

bance at 260 nm. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA (1 μ g) with a commercially available RT kit (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen Co., Carlsbad, USA) using an Oligo (dT) 12–18 primer. Table 1 shows the primer sequence used to amplify cDNA fragments by PCR using Taq DNA polymerase (TaKaRa Ex Taq™, Takara Bio Inc., Kyoto, Japan). Database searches of GenBank were performed with BLASTN. The PCR cycles were performed in a thermal cycler (iCycler, Bio-Rad Laboratories, Inc., Hercules, USA) with the following profile after a denaturing period of 5 min

at 94°C: denaturation, 30 s at 94°C; annealing, 40 s at optimum temperature (shown in Table 1); and a 1-min extension step at 72°C. The last cycle was followed by a final extension step of 5 min at 72°C. For quantitation, preliminary studies were performed to determine the optimum annealing temperature, and a linear correlation between the PCR cycles and the densitometry intensity of the PCR products was confirmed (data not shown). Genomic DNA contamination was checked by subjecting samples to the PCR procedure without adding reverse transcriptase. The PCR products thus obtained were separated by electrophoresis on 2% agarose gel, visualized by

Fig. 2. RT-PCR assay of the expression of the mRNAs for VCAM-1 and MCP-1 in the aorta from each type of treated rat. *A*: Expression of the mRNAs for VCAM-1 and MCP-1, assayed by RT-PCR. *B*: Quantitative analysis of the expression of the mRNA for VCAM-1, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, VCAM-1/GAPDH). * $p < 0.0001$ vs. control, # $p < 0.0001$ vs. L-NAME. *C*: Quantitative analysis of the expression of the mRNA for MCP-1, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, MCP-1/GAPDH). * $p < 0.0001$ vs. control, # $p < 0.0001$ vs. L-NAME, ## $p < 0.0005$ vs. L-NAME.

ethidium bromide staining under UV light, and analyzed by scanning densitometry. The results are presented relative to the expression of the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

Protein Extraction and Western Blotting

Frozen aortas ($n=6-8$ from each group) were immersed in iced Tris buffer (5 mmol/l pH 7.4) containing the protease inhibitors leupeptin, benzamidine, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and antipain (10 μ g/ml). The tissues were homogenized for 30 s in a Polytron homogenizer (KINEMATICA AG, Luzern, Switzerland). The homogenates were then centrifuged for 10 min at $100 \times g$ in order to remove particulate matter and unbroken cells. Total protein levels were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, USA). Immunoblotting was performed according to a standard protocol. Proteins were mixed with sample buffer containing β -mercaptoethanol and were heated at 100°C for 5 min. The protein extracts were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide gel. The samples were then electrotransferred to nitrocellulose membranes and blocked overnight with 4% nonfat dry milk in PBS with 0.1% Tween 20 at room temperature. The membranes were incubated with mouse monoclonal antibody against eNOS (1:2,500, BD Biosciences, Mountain View, USA) or rabbit polyclonal antibody against manganese superoxide dismutase (Mn-SOD, 1:5,000, Stressgen Biotechnologies, Victoria, Canada) overnight and then were incubated for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,500, anti-mouse IgG (H+L) HRP conjugate, Promega Corp., Madison, USA; 1:2,500, anti-rabbit IgG, HRP-linked antibody, Cell Signaling Technology, Beverly, USA). The detection of chemiluminescence was carried out with enhanced chemiluminescence (ECL, Amersham Biosciences Corp., Piscataway, USA). The bands were quantified by scanning densitometry, and the amount of each product was normalized with respect to the amount of actin (1:5,000, mouse anti-actin monoclonal antibody, Chemicon International, Temecula, USA; 1:2,500, Promega Corp.).

Statistics

Data are expressed as mean $\pm$ SEM and were analyzed with the use of Bonferroni's test for analysis of variance (ANOVA) for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Blood Pressure, HR, and Body Weight

The changes in MAP, HR, and body weight are summarized in Table 2. L-NAME treatment alone markedly increased MAP levels, as compared to those of the control group. The increase in MAP induced by L-NAME was suppressed by the administration of amlodipine, manidipine, or hydralazine. No significant differences in HR or body weight were observed among any of the six groups.

Fig. 3. RT-PCR assay of the expression of the mRNA for NADPH oxidase in the aorta from each type of treated rat. *A*: Expression of the mRNA for NADPH oxidase, assayed by RT-PCR. *B*: Quantitative analysis of the expression of the mRNA for NADPH oxidase, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n = 6-8$, NADPH oxidase/GAPDH). * $p < 0.0001$ vs. control, # $p < 0.0001$ vs. L-NAME.

Expression of mRNA and Protein for eNOS

The expression of eNOS mRNA and protein significantly decreased in the L-NAME group, as compared with the levels in the control group. The decreased expression of eNOS was reversed by treatment with amlodipine or manidipine; in particular, the mRNA level in the L-NAME group recovered more than did that of the control by treatment with amlodipine at 20 mg/kg per day or manidipine. The administration of hydralazine did not affect the L-NAME-induced decrease in the expression of either eNOS mRNA or protein (Fig. 1).

Expression of mRNAs for Vascular Cell Adhesion Molecule-1 (VCAM-1) and Monocyte Chemoattractant Protein-1 (MCP-1)

The VCAM-1 mRNA level was significantly greater in the L-NAME group. The increased expression of VCAM-1 mRNA was prevented by treatment with either amlodipine or manidipine. The aortic MCP-1 mRNA level was markedly increased in the L-NAME group. The increased expression of MCP-1 mRNA was reduced in L-NAME + amlodipine and L-NAME + manidipine groups, whereas treatment with a low dose of amlodipine (10 mg/kg per day) did not lead to a reduction of MCP-1 mRNA to the control level. The administration of hydralazine did not affect the L-NAME-induced

increase in the expression of either VCAM-1 or MCP-1 mRNA (Fig. 2).

Expression of mRNA for Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase

Compared with the control group, the L-NAME group had higher levels of expression of NADPH oxidase mRNA. Treatment with amlodipine or manidipine prevented the L-NAME-induced increase in the expression of NADPH oxidase mRNA. The administration of hydralazine did not affect the L-NAME-induced increase in the expression of NADPH oxidase mRNA (Fig. 3).

Expression of Protein for Mn-SOD

The expression of Mn-SOD protein was significantly increased by L-NAME treatment, and this increase was not prevented by either calcium channel blockades or by treatment with hydralazine (Fig. 4).

Expression of mRNA for ACE

As shown in Fig. 5, the level of ACE mRNA in the aortic tissue from the L-NAME group was higher than that of the control. The L-NAME-induced increase in the ACE mRNA level

Fig. 4. Western blotting assay of the expression of Mn-SOD protein in the aorta from each type of treated rat. *A*: Expression of Mn-SOD protein, as determined by Western blot analysis. *B*: Quantitative analysis of the expression of the protein for Mn-SOD, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, Mn-SOD/actin). * $p < 0.005$ vs. control.

Fig. 5. RT-PCR assay of expression of the mRNA for ACE in the aorta of each type of treated rat. *A*: Expression of the mRNA for ACE, assayed by RT-PCR. *B*: Quantitative analysis of the expression of the mRNA for ACE, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, ACE/GAPDH). * $p < 0.0001$ vs. control, # $p < 0.0001$ vs. L-NAME.

was restored to the control level by treatment with calcium channel blockades, but not by treatment with hydralazine.

Similarly, higher levels of ACE mRNA expression were observed in the cultured aortic endothelial cells from the L-

Fig. 6. RT-PCR assay of the expression of the mRNA for ACE in cultured aortic endothelial cells from each group of rats (groups: control, L-NAME, L-NAME+amlodipine (10), L-NAME+manidipine, and L-NAME+hydralazine). A: Expression of the mRNA for ACE assayed by RT-PCR. B: Quantitative analysis of the expression of the mRNA for ACE, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, ACE/GAPDH). * $p<0.05$ vs. control, # $p<0.05$ vs. L-NAME.

NAME group than was observed in the control, and this increase was prevented by calcium channel blockades, but not by hydralazine (Fig. 6). Our *in vitro* study also revealed that treatment with either amlodipine or manidipine suppressed the expression of ACE mRNA in the cultured endothelial cells from the normal rat aorta (Fig. 7).

Discussion

The present study showed that calcium channel blockades such as amlodipine and manidipine normalized decreased eNOS expression and attenuated the overexpression of molecules associated with inflammation and oxidative stress in aortas from rats with chronic, L-NAME-induced inhibition of NO synthesis. Interestingly, these anti-inflammatory and antioxidant effects of calcium channel blockades were found to be mediated by the inhibition of the expression of ACE, and were also shown to be independent of the blood pressure-lowering effects of these agents. Although the vasoprotective effects of amlodipine have been reported by a number of groups (37–39), we were able to exclude the possibility that the beneficial effects of the calcium channel blockades were derived from the direct effects of amlodipine; these findings

Fig. 7. RT-PCR assay of the expression of the mRNA for ACE in cultured rat aortic endothelial cells treated with amlodipine (10^{-6} mol/l) or manidipine (10^{-6} mol/l) for 4 h in culture medium. A: Expression of the mRNA for ACE assayed by RT-PCR. B: Quantitative analysis of the expression of the mRNA for ACE, analyzed by scanning densitometry. Values are means $\pm$ SEM ($n=6-8$, ACE/GAPDH). * $p<0.01$ vs. control.

were obtained by a simultaneous study involving treatment with manidipine.

We hypothesized that the beneficial effects of the calcium channel blockades seen in this study involved an increase in the expression of NO synthase. Ding and Vaziri demonstrated that the calcium channel blockade felodipine increased NO production, eNOS activity, and eNOS protein expression in cultured endothelial cells from the rat aorta; in that study, it was suggested that the enhancement of endothelial NO production could account for the reported improvement in endothelial function associated with calcium channel blockade in the case of both renal and hypertensive disorders (47). We found that treatment with calcium channel blockades augmented eNOS expression. Such an increase in the expression of eNOS might in turn have led to an increase in eNOS activity and NO production; this interpretation of the findings would be in agreement with the *in vitro* study mentioned above. Moreover, it is thought that this increase in the expression of eNOS might elicit the anti-inflammatory and antiatherosclerotic effects of a calcium channel blockade in the L-NAME-treated rat aorta. It should be noted this putative mechanism is also indicated by evidence demonstrating that manidipine improves endothelial dysfunction by increasing NO production in the hypertensive rat aorta (45). However, a previous study of L-NAME-treated rats showed that although amlodipine did inhibit early inflammation and late arterio-

sclerosis, it did not restore the concentration of plasma nitrogen oxides (NOx) (48). To clarify this discrepancy, further research (e.g., studies on eNOS activity and NO production) will be necessary.

MCP-1 is known to be a potent chemokine for monocytes (49). VCAM-1 is an inducible form of adhesion molecule, as compared with the more constitutive intercellular adhesion molecule-1 (ICAM-1). Chronic inhibition of NO synthesis by L-NAME enhanced the expression of MCP-1, VCAM-1, and ICAM-1 in arteries from this rat model (48, 50). Consistent with these reports, we showed that VCAM-1 and MCP-1 mRNA levels were increased in the aorta of L-NAME-treated rats; moreover, an important result of the present study was that calcium channel blockades prevented the overexpression of these molecules. We also found that the calcium channel blockades used here normalized the increased expression of ICAM-1 mRNA induced by L-NAME in the aorta (data not shown). In addition, Kataoka *et al.* demonstrated that treatment with amlodipine attenuated the L-NAME-induced increase in inflammatory changes (including increased gene expression of MCP-1) in coronary arteries (48). Our results, taken together with the findings of the previous reports, indicate that calcium channel blockades may prevent inflammation, the mechanism of which has received a great deal of attention as a central factor in the development of atherosclerosis and associated complications. Furthermore, it has been shown that NO downregulates VCAM-1 and ICAM-1 expression *in vitro* (51, 52). It is therefore considered to be supporting evidence that calcium channel blockades were found to normalize the L-NAME-induced decrease in eNOS content, which may in turn be related to the prevention of the overexpression of adhesion molecules observed in this study.

In general, oxidative stress is considered to participate to the development of vascular inflammation (53, 54). Previous studies (48, 54–56) have suggested that L-NAME treatment enhances oxidative stress in the aorta. We demonstrated here that the expression of NADPH oxidase, of which activation the production of reactive oxygen species is mainly due to in the vascular wall, was increased in the aorta from L-NAME-treated rats. More importantly, we also demonstrated that either of two calcium channel blockades, amlodipine or manidipine, effectively suppressed this type of overexpression, suggesting that these agents acted as the antioxidant in the present experiments. The antioxidant effects of amlodipine have been previously reported (37, 57). It is likely that calcium channel blockades exert their anti-inflammatory effects by blocking the main source of oxidative stress, without affecting the antioxidant system, which was found to be upregulated by L-NAME-treatment in the present study (as shown by the representative data from studies of Mn-SOD). However, another study (37) using high cholesterol-fed animals suggested that the preservation of SOD may be a mechanism of the antiatherosclerotic effects of amlodipine. In addition, it has been reported that nifedipine exhibits its anti-oxidative effects by the upregulation of Mn-SOD (58). Fur-

ther examination will still be necessary to clarify this mechanism.

Recently, the critical role played by the local activity of the renin-angiotensin system in the development of atherosclerosis in L-NAME-treated animals has been a focus of attention (30, 59, 60). It has not only been reported that ACE expression and activity are increased at the tissue level, but also that ACE inhibitor or angiotensin II type 1 (AT₁) receptor blockades can prevent most of the effects induced by L-NAME treatment, such as the production of reactive oxygen species and inflammation, associated with atherosclerosis and cardiovascular pathological changes (30, 48, 54–56, 61, 62). Previous reports have compellingly supported the notion that the beneficial effects of calcium channel blockades observed in this model may have been mediated at least in part by their effects on the local renin-angiotensin system. To investigate this hypothesis, we examined the effects of calcium channel blockades on the expression of ACE mRNA; here, we found for the first time that treatment with calcium channel blockades prevented the L-NAME-induced increase in the levels of ACE mRNA in the rat aorta. More interestingly, Kataoka *et al.* demonstrated that a low dose of amlodipine, which had no effect on blood pressure, prevented an increase in ACE activity, as well as suppressed the production of superoxides in the L-NAME-induced hypertensive rat aorta (48). The findings of that report, taken together with those of the present study strongly support our hypothesis that the anti-inflammatory and antioxidative effects of calcium channel blockades might be due to the inhibition of the local renin-angiotensin system.

To further confirm this interpretation of the results of our investigation of ACE mRNA expression, we also conducted a series of experiments on cultured aortic endothelial cells. Thus, we were able to reproduce the results regarding ACE mRNA expression *in vivo* and *in vitro* (Fig. 6). Furthermore, we analyzed the direct effects of amlodipine and manidipine on cultured endothelial cells, and we reconfirmed the ability of calcium channel blockades on the suppression of ACE expression (Fig. 7).

In the present study, we demonstrated that amlodipine and manidipine could prevent inflammation and oxidative stress in the L-NAME-treated rat aorta after a 2-week period of administration. However, it remains unclear at which point in time these beneficial effects of calcium channel blockades emerge. Therefore, we also investigated the effects of shorter-term administration (*i.e.*, 1 week) of calcium channel blockades using the same strain of L-NAME-treated rats (data not shown). We found that amlodipine and manidipine exhibited vasoprotective effects in 1 week, just as seen in the 2-week examination. This result suggests that calcium channel blockades exert antiatherogenic effects independent of the time course.

In the present study, we demonstrated that hydralazine treatment normalized L-NAME-induced hypertension to the same level as that observed with calcium channel blockades, and this blood pressure-lowering effect by hydralazine was

not found to inhibit the pathological changes induced by L-NAME; it is therefore unlikely that the anti-hypertensive effects of calcium channel blockades contribute largely to the prevention of cardiovascular pathological changes. However, the possibility that some of the beneficial effects of the calcium channel blockades might have been due to the normalization of blood pressure cannot be ruled out.

In conclusion, we demonstrated here the direct anti-inflammatory and antioxidative effects of calcium channel blockades in a rat model of chronic inhibition of NO synthesis induced by L-NAME treatment. Such vasoprotective effects of calcium channel blockades are not likely to be primarily mediated by their blood pressure-lowering effects, but instead appear to be mediated by the augmentation of eNOS expression, as well as by the inhibition of ACE expression. The present study might therefore provide novel insights into the mechanism by which calcium channel blockades reduce the incidence of cardiovascular events.

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