Original Article

Calcium Channel Blockers Reduce Angiotensin II-Induced Superoxide Generation and Inhibit Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 Expression in Endothelial Cells

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Calcium channel blockers have been shown to limit the progression of atherosclerosis and decrease the incidence of cardiovascular events. To investigate vasoprotective effects beyond the blood pressure-lowering effects of these agents, amlodipine (10^{-6} mol/l) and manidipine (10^{-6} mol/l) were used to pretreat angiotensin (Ang) II-stimulated rat cultured aortic endothelial cells. A 3-h period of Ang II treatment enhanced superoxide generation and the expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase protein, as detected by dihydroethidium staining and Western blotting, respectively. Pretreatment with amlodipine or manidipine attenuated the increased production of superoxide and the overexpression of NADPH oxidase. The enhanced expression of heme oxygenase-1 (HO-1) mRNA induced by Ang II was further increased by amlodipine, whereas pretreatment with manidipine led to a reduction in the expression of HO-1. Furthermore, Ang II increased vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) mRNA levels, as determined by reverse transcription (RT)–polymerase chain reaction (PCR). Pretreatment with either amlodipine or manidipine decreased the overexpression of VCAM-1, ICAM-1, and MCP-1. We also demonstrated that amlodipine or manidipine prevented the Ang II-induced increase in lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) content, thereby restoring control levels. These observations showed that amlodipine and manidipine reduced superoxide generation by the inhibition of the overexpression of NADPH oxidase in Ang II-stimulated endothelial cells. Such antioxidant effects of these agents might in turn have led to a decrease in the expression of VCAM-1, ICAM-1 and MCP-1. The salutary effects of calcium channel blockers in atherogenesis include the inhibition of the expression of LOX-1. (*Hypertens Res* 2006; 29: 105–116)

***Key Words*: calcium channel blockade, lectin-like oxidized low-density lipoprotein receptor-1, angiotensin II, atherosclerosis**

Introduction

A large body of evidence has demonstrated a significant role played by the rennin-angiotensin system in the pathogenesis of numerous cardiovascular diseases, including hypertension, hypercholesterolemia, and diabetes (*1*). The recently completed HOPE trial demonstrated a remarkable decrease in car-

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diovascular morbidity and mortality by angiotensin converting enzyme (ACE) inhibition in individuals at increased risk for cardiovascular events (*2*). Angiotensin II (Ang II), the principle effector of the renin-angiotensin system, is known to be a potent vasoconstrictor by itself, and plays an essential role in the regulation of blood pressure. Furthermore, Ang II has been shown to exert other important effects in the vasculature (*3*). Activation of the Ang II type 1 (AT_1)-receptor by Ang II leads to a variety of intracellular signaling evens, ultimately causing endothelial dysfunction, proliferation of vascular smooth muscle cells, expression of pro-inflammatory genes, and reconstruction of extracellular matrix, which are known to cause vasoconstriction and atherosclerotic plaque formation and rupture (*1*). Moreover, one of the important effects of Ang II is to promote oxidative stress by the enhancement of reactive oxygen species (ROS) such as superoxide through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major source of the production of ROS in vascular cells (*1*). The increased oxidative stress induced by Ang II contributes to vascular endothelial cell dysfunction, which has received a great deal of attention as a common feature in the development of atherosclerosis and associated complications (*1*).

Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1), which is a type-II membrane protein that belongs to a family of C-type lectin-like molecules, was identified as a novel scavenger receptor for oxidized LDL (OxLDL) in vascular endothelial cells (*4*, *5*). It binds, internalizes, and degrades OxLDL but not native LDL or acetylated LDL. The expression of LOX-1 in endothelial cells is relatively lower in the basal condition, but it can be upregulated by OxLDL, Ang II, inflammatory cytokines, vasoconstrictive peptide, and shear stress *in vitro*; and in proatherogenic conditions *in vivo*, including hypertension, diabetes mellitus, and hyperlipidemia, as well as in atherosclerosis *per se* (*6*, *7*). Moreover, free radicals have also been shown to induce the expression of LOX-1 (*7*). Recent studies have shown that the expression of LOX-1 is also upregulated in atherosclerotic tissues from rabbits and humans (*8*, *9*). Interestingly, this inducible nature of LOX-1 suggests that LOX-1 may play an active role in complex atherogenic processes, such as the induction of adhesion molecules, monocyte chemotactic protein-1, and growth factors, as well as in the further generation of superoxide (*7*).

Dihydropyridine calcium channel blockers are known to exhibit vasoprotective effects in response to cardiovascular events. Furthermore, these agents reduce cardiovascular mortality in patients after myocardial infarction (*10*). Amlodipine has been shown to limit the progression of arteriosclerosis and atherosclerosis in animals and humans (*11–14*). Although the mechanisms underlying the direct protective effects of calcium channel blockers are not fully understood, such vasoprotective effects as amelioration of endothelial dysfunction (*15*) and decreases in the proliferation of vascular smooth muscle cells (*16*), oxidative stress (*17*) and inflammation (*18*, *19*), are at least partly independent of the blood pressure-lowering effects of these agents—in other words, they are independent of the effects of these agents *via* calcium channels. Several lines of evidence suggest that calcium channel blockers also possess antioxidant activity, primarily due to their chain-breaking antioxidative action, preservation of scavengers, and direct scavenging of peroxyl radical-mediated oxidation (*20*).

To investigate the vasoprotective effects of calcium channel blockers such as amlodipine and manidipine, we analyzed the levels of ROS and the expression of molecules associated with oxidative stress and inflammation, including the expression of LOX-1, in Ang II-treated rat aortic endothelial cells.

Methods

Cell Culture

Rat aortic cells were isolated from male Wistar rats (Shimizu Laboratory Supply, Kyoto, Japan) weighing 250 g by a previously described primary explant technique (*21*). All rats were cared for in accordance with the guidelines for animal experiments at Kyoto Pharmaceutical University. 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, Two Oak Park, 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 experiments in order to induce quiescence, and then the conditioned medium was exchanged for new medium at the start of the experiments.

Study Design

In preliminary studies, cells were incubated with Ang II (10^{-7} mol/l) for 1, 3, 6, or 24 h in order to determine the production of superoxide. The duration resulting in the maximal effect of Ang II was used in subsequent experiments. In parallel experiments, cells were pretreated with amlodipine (10^{-6} mol/l) or manidipine (10^{-6} mol/l) for 60 min before incubation with Ang II. Cells were washed three times with phosphate buffed saline (PBS), and were used for subsequent analysis. The concentration of Ang II was chosen on the basis of previous studies (*22*).

Oxidative Fluorescent Microtopography

The generation of superoxide was observed with dihydroethidium (DHE) fluorescence as described previously (*23*).

Table 1. PCR Primers and PCR Protocols

Product size	PCR primer seqences	PCR protocols
GAPDH	Forward: 5′-TCCCTCAAGATTGTCAGCAA-3′	94°C: 30 s
308 bp	Reverse: 5′-AGATCCACAACGGATACATT-3′	57°C: 40 s, 72°C: 1 min — 30 cycles
VCAM-1	Forward: 5′-AGGCAGAGTACACAGACTCT-3′	94°C: 30 s
510 bp	Reverse: 5′-ACACTCGTATACTCCGGCAT-3′	58°C: 40 s, 72°C: 1 min — 26 cycles
MCP-1	Forward: 5′-TGTTCAGCATTGCTGCCTGT-3′	94°C: 30 s
259 bp	Reverse: 5′-GATCTCACTTGGTTCTGGTC-3′	56°C: 40 s, 72°C: 1 min — 32 cycles
ICAM-1	Forward: 5′-AGGATCACAAACGACGCTTC-3′	94°C: 30 s
290 bp	Reverse: 5′-ACGGCACTTGTAGGTACCAT-3′	56°C: 40 s, 72°C: 1 min — 26 cycles
HO-1	Forward: 5′-GTACCATATCTATACGGCCC-3′	94°C: 30 s
583 bp	Reverse: 5′-GGTAGTATCTTGAACCAGGC-3′	58°C: 40 s, 72°C: 1 min — 30 cycles
LOX-1	Forward: 5′-CTCAACTGGAAGCTGAATGG-3′	94°C: 30 s
408 bp	Reverse: 5′-GGTGGAATGGGAAGTTGCTT-3′	62°C: 40 s, 72°C: 1 min — 30 cycles

PCR, polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intracellular adhesion molecule-1; HO-1, heme oxygenase-1; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1.

Cells cultured on glass slides were incubated in PBS containing DHE (2×10^{-6} mol/l) at 37°C for 30 min in a dark, humidified chamber. DHE is oxidized on the reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope, and fluorescence was detected with a 590-nm long-pass filter.

Protein Extraction and Western Blotting

Cells were lysed in ice-cold lysis buffer of the following composition: 50 mmol/l Tris, 150 mmol/l NaCl, 1% Triton X-100, and 50 mmol/l NaF, pH 7.5. 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 with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) by 7.5% acrylamide gel. The samples were then electrotransferred to polyviniliden difluoride (PVDF) 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 $p47^{phox}$ (1:2,500; BD Biosciences, Franklin Lakes, USA) 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). 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 the bands of actin (1:5,000, mouse anti-actin monoclonal antibody; Chemicon International, Temecula, USA; 1:2,500, Promega Corp.).

RNA Isolation and Reverse Transcription (RT)–Polymerase Chain Reaction (PCR)

The total RNAs were isolated from 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 absorbance at 260 nm. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA (1 μg) 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 sequences 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

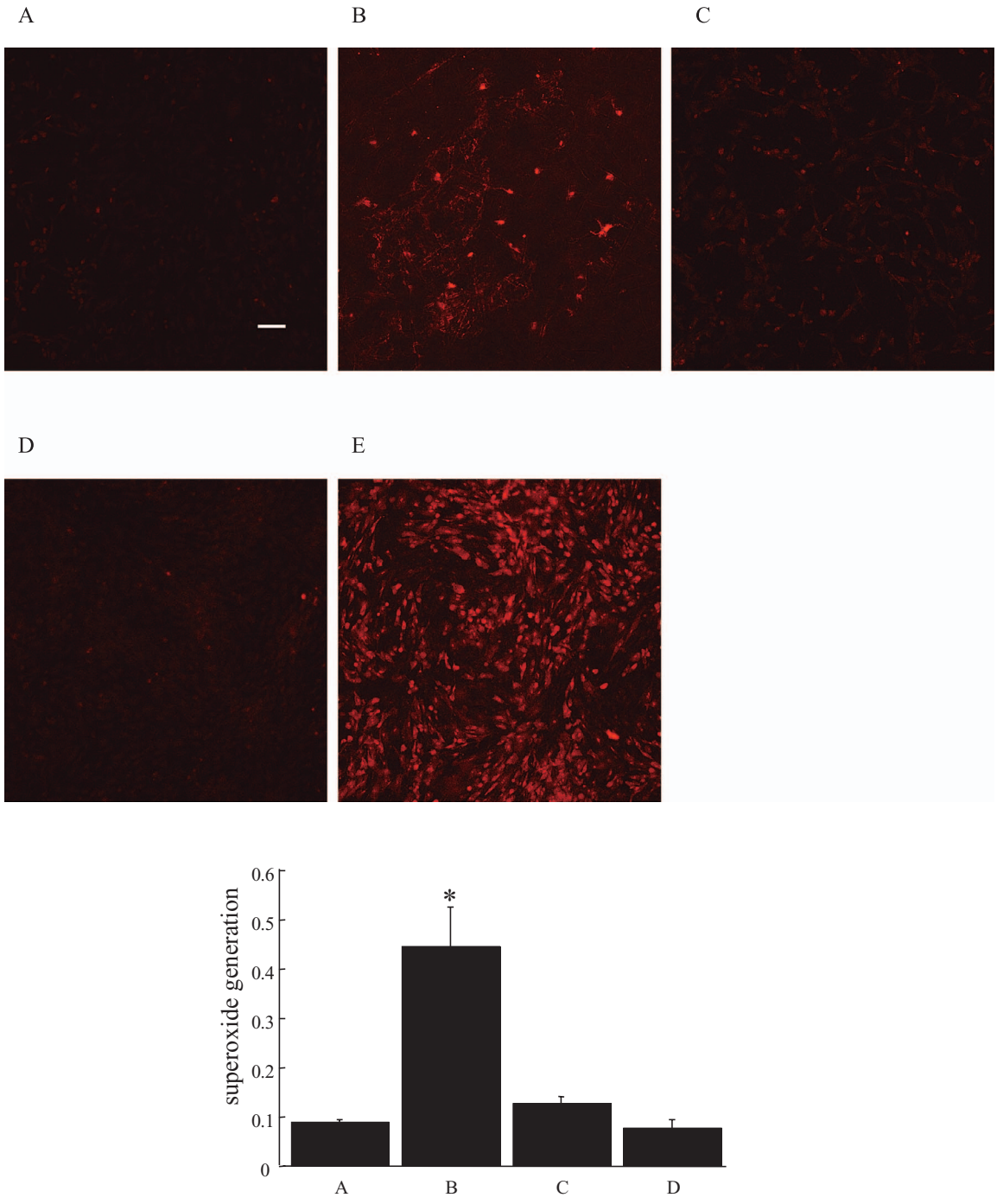

Fig. 1. *Confocal fluorescent images of cells stained with DHE. Superoxide levels in rat aortic endothelial cells treated with angiotensin II (10^{-7} mol/l) for 1 (A), 3 (B), 6 (C), or 24 (D) h in culture medium. E represents cells treated with H_2O_2 as a positive control. The bar indicates 100 μm. The graph shows the quantitative analysis of superoxide generation, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8). *$p < 0.0005$ vs. others.*

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

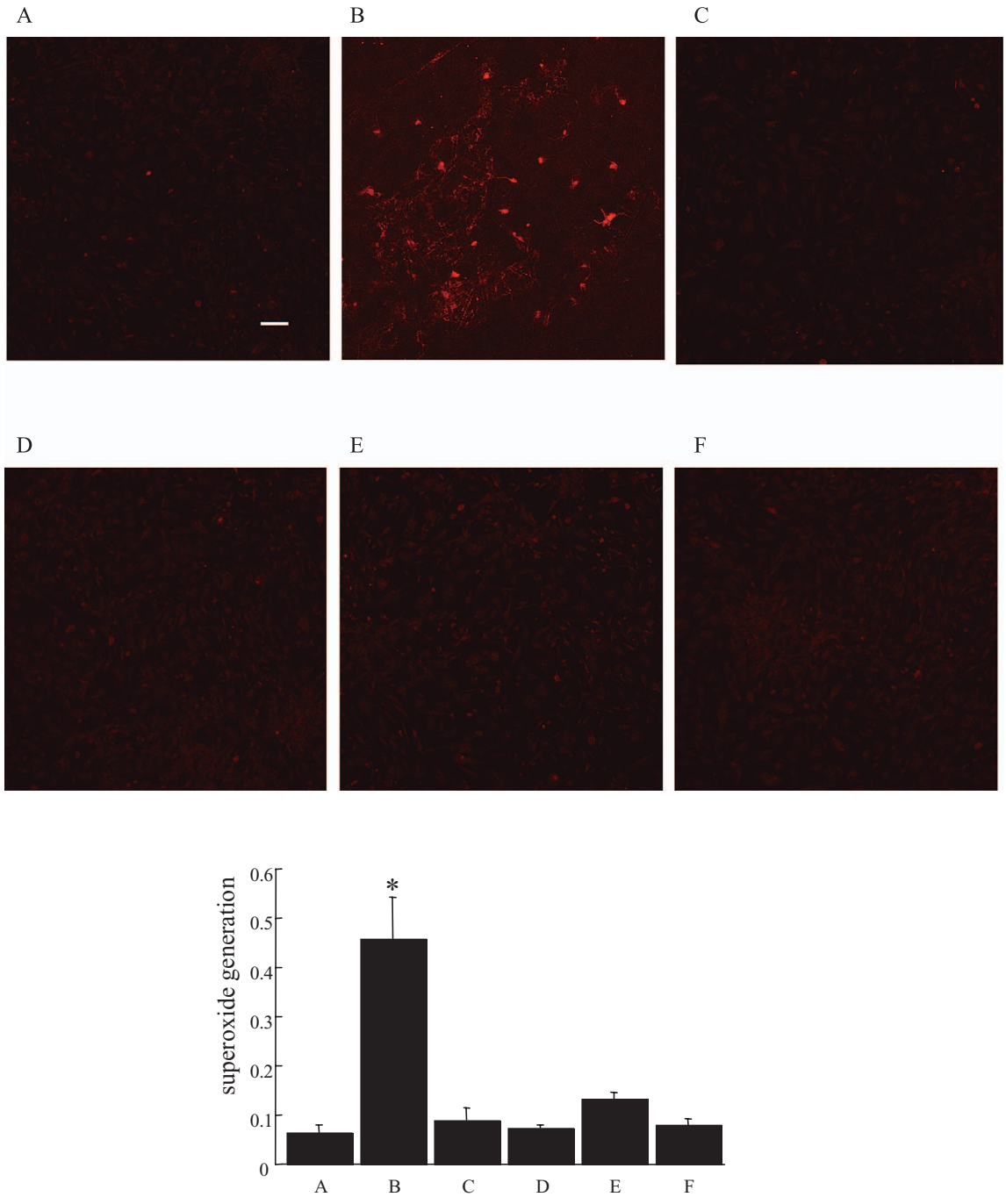

Fig. 2. *Confocal fluorescent images of cells stained with DHE. Superoxide levels in rat aortic endothelial cells. Images show non-treated cells (A), cells incubated with angiotensin II (Ang II) (10^{-7} mol/l) for 3 h (B), and cells treated for 60 min with amlodipine (10^{-6} mol/l) or manidipine (10^{-6} mol/l), followed by stimulation with Ang II (10^{-7} mol/l) for 3 h in culture medium (C and D, respectively). E and F show cells treated with amlodipine (10^{-6} mol/l) or manidipine (10^{-6} mol/l) alone, respectively. The bar indicates 100 μm. The graph shows the quantitative analysis of superoxide generation, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8). *p < 0.0001 vs. others.*

by electrophoresis on 2% agarose gel, visualized by 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.

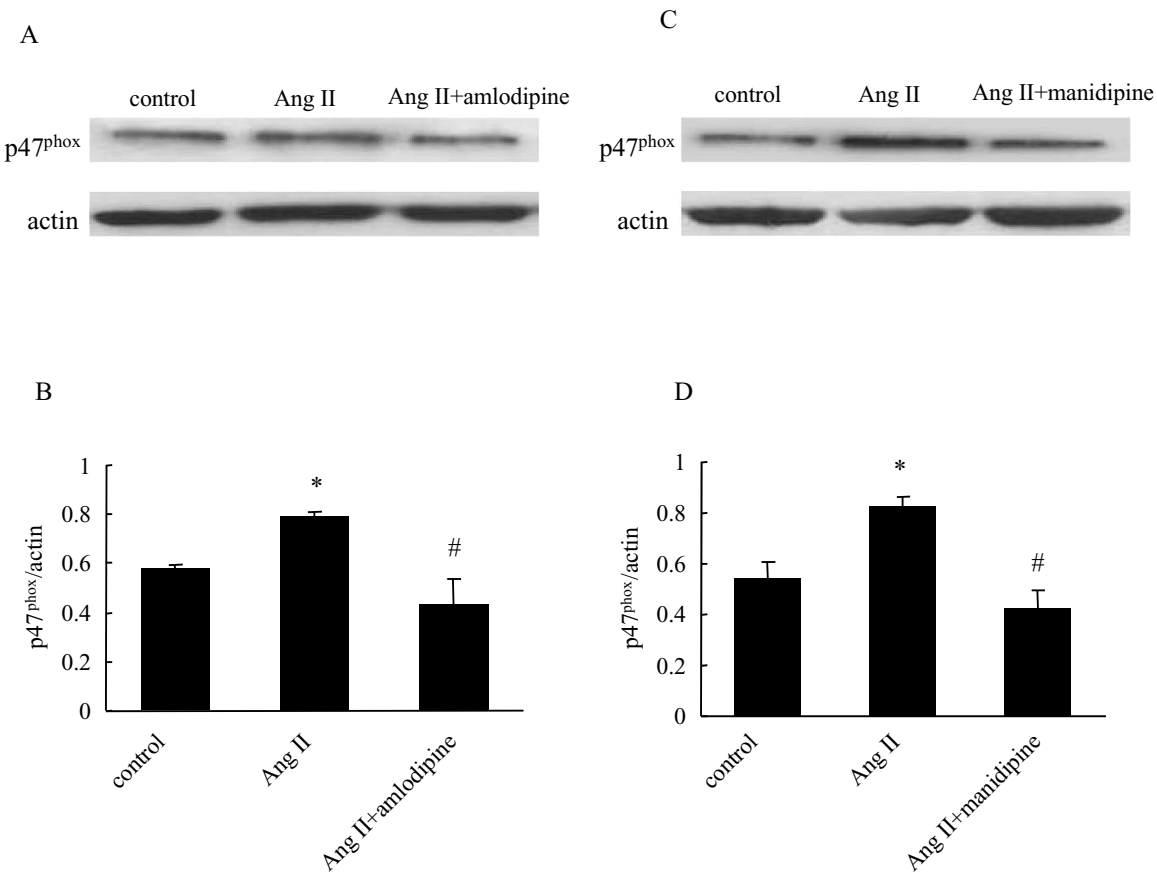

Fig. 3. *Western blotting assay of the expression of p47^{phox} protein in cultured rat aortic endothelial cells treated for 60 min with amlodipine (10^{-6} mol/l; A and B) or manidipine (10^{-6} mol/l; C and D), followed by stimulation with angiotensin II (Ang II) (10^{-7} mol/l) for 3 h in culture medium. A and C: Expression of p47^{phox} protein, as determined by Western blotting. B and D: Quantitative analysis of the expression of p47^{phox} protein, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8, p47^{phox}/actin). *$p < 0.05$ vs. control; #$p < 0.005$ vs. Ang II.*

Statistics

Data are expressed as the mean±SEM. Results were analyzed using one way analysis of variance (ANOVA) for multiple comparisons followed by Fisher's Protected Least Significant Difference. A value of $p < 0.05$ was considered statistically significant.

Results

Superoxide Generation

Incubation of rat aortic endothelial cells with Ang II caused an increase in the generation of superoxide, which reached its maximum after 3 h (Fig. 1). Therefore, in all subsequent experiments, cells were incubated with Ang II for 3 h.

Pretreatment with amlodipine and manidipine markedly decreased the generation of superoxide. Amlodipine or manidipine alone had no effect on the basal levels of superoxide formation (Fig. 2).

Expression of Protein for NADPH Oxidase

As shown in Fig. 3, the endothelial expression of p47^{phox} protein was significantly increased by a 3-h period of stimulation with Ang II. Pretreatment with amlodipine or manidipine prevented the Ang II-induced increase in the expression of p47^{phox} protein.

Expression of mRNA for Heme Oxygenase-1 (HO-1)

Ang II induced a significant increase in the expression of HO-1 mRNA in endothelial cells. Pretreatment with amlodipine caused a further increase in the expression of HO-1, whereas pretreatment with manidipine suppressed the overexpression of HO-1 mRNA induced by Ang II. Amlodipine or manidipine alone had no effect on the basal expression of HO-1 mRNA (Fig. 4).

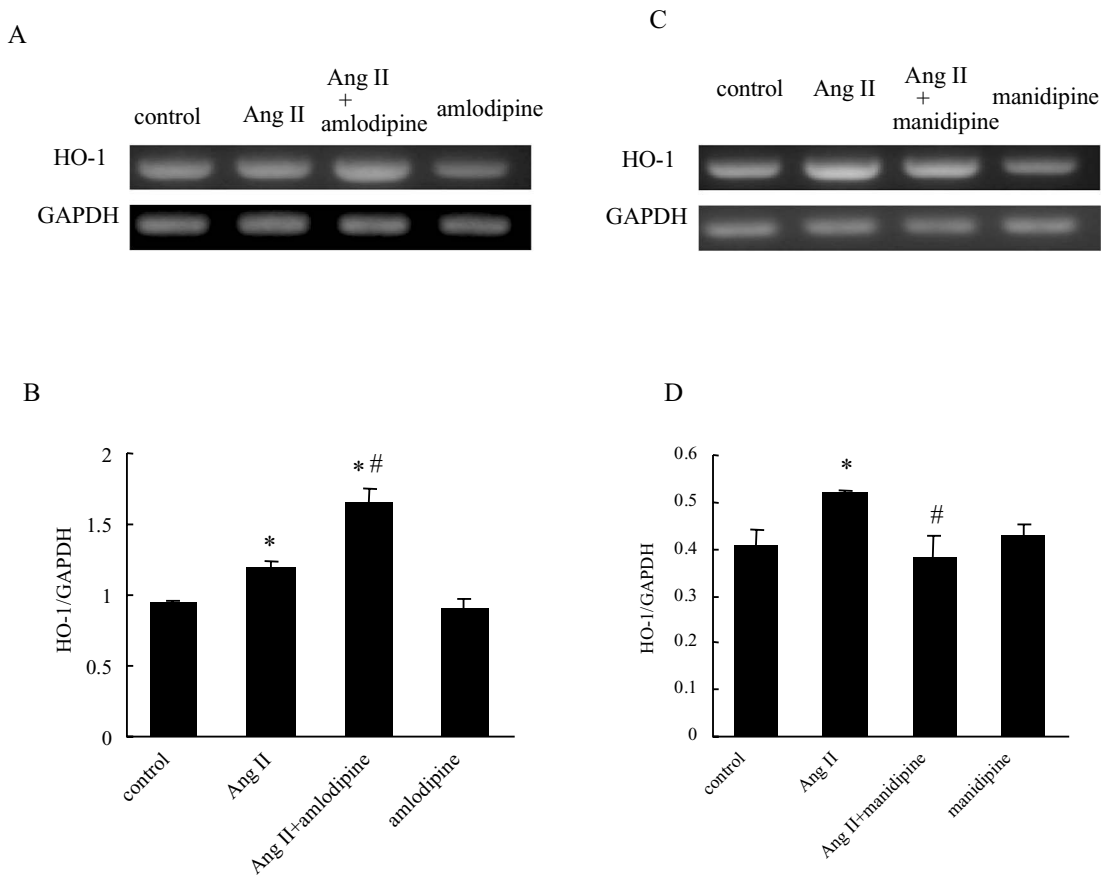

Fig. 4. *RT-PCR assay of the expression of the mRNA for HO-1 in cultured rat aortic endothelial cells treated for 60 min with amlodipine (10^{-6} mol/l; A and B) or manidipine (10^{-6} mol/l; C and D), followed by stimulation with angiotensin II (Ang II) (10^{-7} mol/l) for 3 h in culture medium. A and C: Expression of the mRNA for HO-1, assayed by RT-PCR. B and D: Quantitative analysis of the expression of the mRNA for HO-1, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8, HO-1/GAPDH). *$p < 0.05$ vs. *control;* #$p < 0.05$ vs. *Ang II.*

Expression of mRNA for Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 mRNA level in endothelial cells incubated with Ang II was higher than that in non-treated cells. The increased expression of MCP-1 mRNA was prevented by pretreatment of amlodipine or manidipine. The basal expression of MCP-1 mRNA was not affected by a single treatment with either amlodipine or manidipine alone (Fig. 5).

Expression of mRNAs for Adhesion Molecules

Treatment with Ang II resulted in higher levels of expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) mRNA, as compared with those in non-treated cells. Treatment with amlodipine or manidipine did not affect the basal expression of mRNA for these adhesion molecules (Fig. 6).

Expression of mRNA for LOX-1

Incubation with Ang II significantly increased the expression of LOX-1 mRNA. This Ang II-induced increase in LOX-1 mRNA level was restored to the control level by pretreatment with either amlodipine or manidipine. Amlodipine or manidipine alone had no effect on the basal expression of LOX-1 mRNA (Fig. 7).

Discussion

The present study demonstrated that dihydropyridine calcium channel blockers such as amlodipine and manidipine restored increased superoxide production and NADPH oxidase expression and attenuated the overexpression of mRNA associated with inflammation in Ang II-stimulated rat aortic endothelial cells. Interestingly, these antioxidant and anti-inflammatory effects of calcium channel blockers were mediated by inhibition of the expression of LOX-1. In this study, we were able to demonstrate that the beneficial effects of cal-

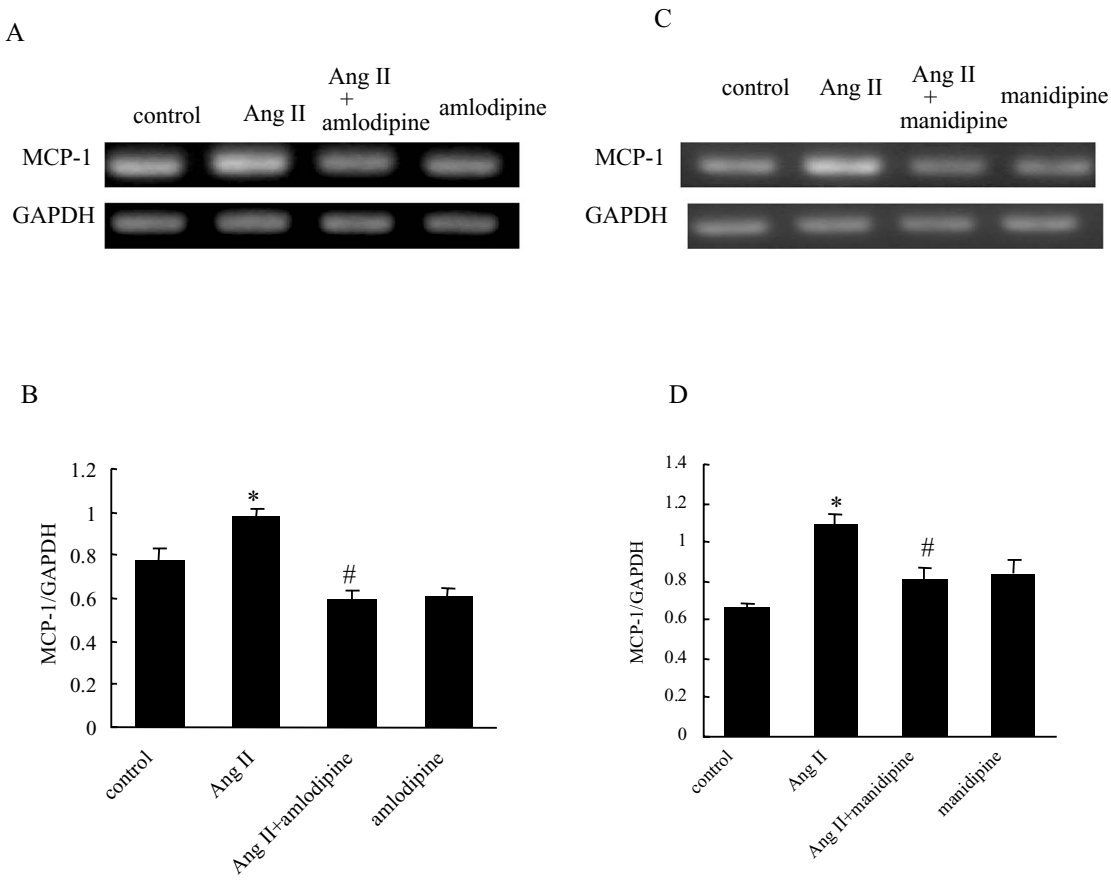

Fig. 5. *RT-PCR assay of the expression of the mRNA for MCP-1 in cultured rat aortic endothelial cells treated for 60 min with amlodipine (10^{-6} mol/l; A and B) or manidipine (10^{-6} mol/l; C and D), followed by stimulation with angiotensin II (Ang II) (10^{-7} mol/l) for 3 h in culture medium. A and C: Expression of the mRNA for MCP-1, assayed by RT-PCR. B and D: Quantitative analysis of the expression of the mRNA for MCP-1, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8, MCP-1/GAPDH). *$p < 0.01$ vs. *control;* #$p < 0.005$ vs. *Ang II.*

cium channel blockers were independent of the effects of these agents *via* calcium channels, because macrovascular endothelial cells have been reported to lack voltage-operated L-type calcium channels (*24*, *25*).

In general, oxidative stress induces endothelial dysfunction and increases subsequent vascular remodeling in patients with atherosclerosis (*26*). In order to determine the molecular source of endothelial superoxide, we investigated the expression of the NADPH oxidase subunit; we found that the expression of the NADPH oxidase subunit $p47^{phox}$ showed a significant correlation with the generation of superoxide in Ang II-treated rat aortic endothelial cells. Indeed, Zhang *et al*. demonstrated that Ang II induced a release of superoxide in endothelial cells *via* activation of NADPH oxidase (*27*). More importantly, we also demonstrated that either of two calcium channel blockades, amlodipine or manidipine, effectively suppressed this type of increase in superoxide generation and the expression of NADPH oxidase, suggesting that these agents acted as antioxidants by downregulating NADPH oxidase expression. It has been reported that Ang II increases HO-1 expression in hypertensive rats, suggesting a possible tissue protective effect of HO-1 in response to Ang II-induced tissue injury (*14*). An Ang II-induced increase in the expression of the HO-1 gene was also seen in our study; this type of increase was further enhanced by coincubation with amlodipine, but not by coincubation with manidipine. It has been reported that amlodipine exerts its antioxidant effects by the preservation of superoxide dismutase (SOD) (*28*). Our results, taken together with the findings of the previous reports, indicate that amlodipine may prevent oxidative stress not only by blocking the production of superoxide, but also by stimulating superoxide-quenching activity. On the other hand, it is likely that manidipine exerts its antioxidant effects by blocking the main source of oxidative stress, without affecting the antioxidant enzymes. However, another study using monocytes suggested that the preservation of HO-1 might be a mechanism of the antioxidant effects of manidipine (*29*). Further examination will be necessary to clarify this mechanism.

Oxidative stress is considered to participate in the develop-

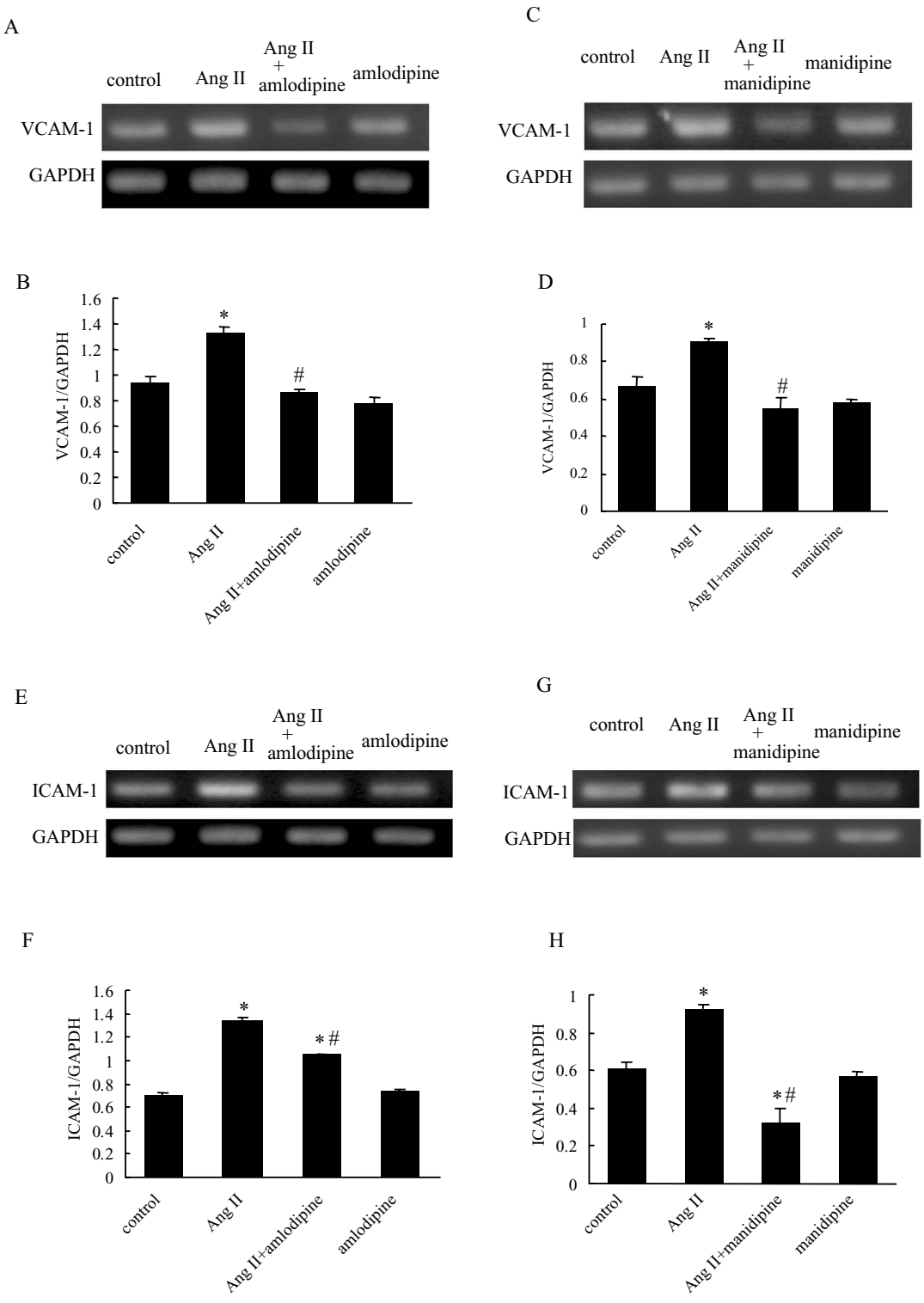

Fig. 6. *RT-PCR assay of the expression of the mRNA for adhesion molecules in cultured rat aortic endothelial cells treated for 60 min with amlodipine (10^{-6} mol/l; A, B, E, and F) or manidipine (10^{-6} mol/l; C, D, G, and H), followed by stimulation with angiotensin II (Ang II) (10^{-7} mol/l) for 3 h in culture medium. A and C: Expression of the mRNA for VCAM-1, assayed by RT-PCR. B and D: Quantitative analysis of the expression of the mRNA for VCAM-1, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8, VCAM-1/GAPDH). E and G: Expression of the mRNA for ICAM-1, assayed by RT-PCR. F and H: Quantitative analysis of the expression of the mRNA for ICAM-1, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8, ICAM-1/GAPDH). *$p < 0.05$ vs. *control*; #$p < 0.001$ vs. *Ang II*.

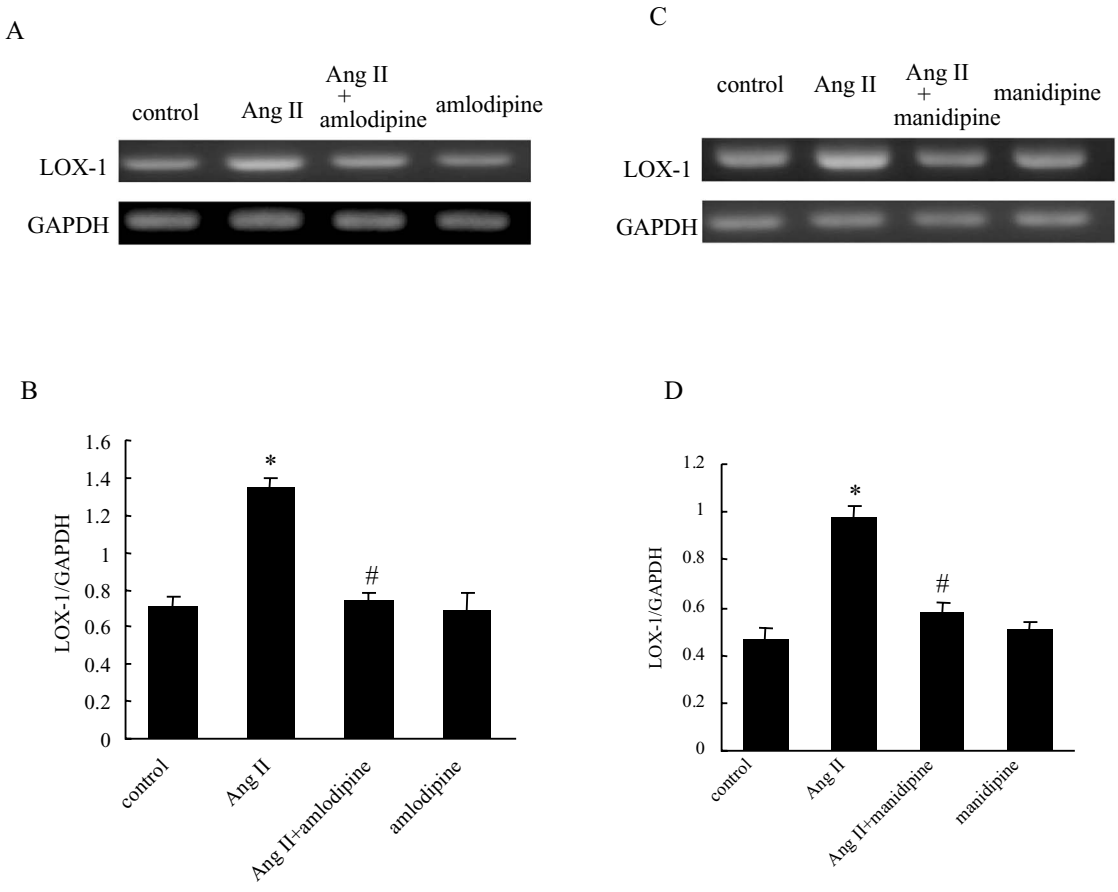

Fig. 7. *RT-PCR assay of the expression of the mRNA for LOX-1 in cultured rat aortic endothelial cells treated for 60 min with amlodipine (10^{-6} mol/l; A and B) or manidipine (10^{-6} mol/l; C and D), followed by stimulation with angiotensin II (Ang II) (10^{-7} mol/l) for 3 h in culture medium. A and C: Expression of the mRNA for LOX-1, assayed by RT-PCR. B and D: Quantitative analysis of the expression of the mRNA for LOX-1, analyzed by scanning densitometry. Values are the means±SEM (*n = 6–8, LOX-1/GAPDH). *$p < 0.001$ vs. *control;* #$p < 0.005$ vs. *Ang II.*

ment of atherosclerosis, which is known to be a final stage of vascular inflammation. One of the earliest detectable cellular responses in the formation of atherosclerotic lesions is the focal recruitment of leukocytes to the endothelium (*30, 31*). Upregulation of adhesion molecules and MCP-1 occurs at sites of lesion formation in the endothelial cells in early atherosclerosis (*32*). Ang II has been shown to enhance the expression of VCAM-1, ICAM-1, and MCP-1 in vascular endothelial cells (*1*). Consistent with these reports, we demonstrated that the mRNA levels of these inflammatory molecules were increased in Ang II-treated rat aortic endothelial cells; moreover, an important result of the present study was that calcium channel blockers prevented the overexpression of these molecules. In addition, it has been reported that calcium channel blockers suppress the overexpressions of these molecules both *in vivo* (*33, 34*) and *in vitro* (*18*). Our results, taken together with the findings of the previous reports, indicate that calcium channel blockers may prevent endothelial inflammation, the mechanism of which has been received a great deal of attention as a central factor in the development of atherosclerosis and associated complications.

LOX-1, a receptor for OxLDL, plays a critical role in endothelial dysfunction and atherosclerosis (*7*). Ang II has been shown to increase the expression of LOX-1 in the endothelium (*7*). Based on studies by Nagase *et al*., it has been suggested that oxidative stress is involved in Ang II-mediated upregulation of the expression of LOX-1 (*35*). Furthermore, activation of LOX-1 may cause an additional increase in superoxide production, resulting in a vicious cycle in which the increased expression of LOX-1 further increases oxidative stress in Ang II-treated rat aortic endothelial cells. In addition, it has been demonstrated that activation of LOX-1 causes upregulation of the expression of adhesion molecules (*7*). In the present study, we found for the first time that treatment with calcium channel blockers prevented the Ang II-induced increase in the levels of LOX-1 mRNA in rat aortic endothelial cells *in vitro*. Along these lines, Zhou *et al*. demonstrated that amlodipine prevented an increase in the expres-

sion of LOX-1 mRNA, in addition to suppressing the production of superoxide in Ang II-infused rat aortae *in vivo* (*36*). Previous reports have compelling supported the notion that the beneficial effects of calcium channel blockers observed in Ang II-treated rat aortic endothelial cells may have been mediated at least in part by their effects on LOX-1. We preliminarily found that the effect of calcium channel blockers on superoxide production was attenuated in the presence of an NADPH oxidase inhibitor, apocynin (data not shown). Because it has been reported that LOX-1-induced superoxide production may be related to the increased activity of NADPH oxidase (*37*), we suggest that the antioxidative and anti-inflammatory effects of calcium channel blockers might be due to the inhibition of LOX-1 expression.

In conclusion, we here demonstrated the direct antioxidative and anti-inflammatory effects of calcium channel blockers in Ang II-treated cultured rat aortic endothelial cells, including the inhibition of LOX-1 expression. Such vasoprotective effects of calcium channel blockers are not likely to be mediated by their effects *via* calcium channels, which vascular endothelial cells have been reported to lack. The present study might therefore provide novel insights into the protection conferred by calcium channel blockers against cardiovascular events.

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