Novel anti-inflammatory actions of amlodipine in a rat model of arteriosclerosis induced by long-term inhibition of nitric oxide synthesis

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Amlodipine, a new type of long-acting calcium channel antagonist, has been shown to limit progression of arteriosclerosis in animals and humans (3, 17). A recent clinical study (17) has demonstrated that amlodipine reduces the incidence of major vascular events or procedures in patients with heart failure (9, 18, 19, 21). Thus previous studies by us and those of other investigators using mice lacking endothelial-type NO synthase (6, 13) support the notion that endothelium-derived NO is an anti-inflammatory and anti-arteriosclerotic molecule.

We (10, 11, 18, 19, 21) recently reported a rat model of chronic inhibition of NO synthesis with the administration of Nω-nitro-L-arginine methyl ester (L-NAME). This model displays two distinct stages: an early hypertensive stage associated with vascular inflammation (monocyte adhesion to the endothelium, infiltration into the blood vessel walls, increased expression of monocyte chemoattractant protein (MCP)-1), and arteriosclerosis. Here, we used the rat model to investigate the anti-inflammatory effects of amlodipine in vivo. Treatment with amlodipine markedly attenuated the L-NAME-induced increase in vascular inflammation, oxidative stress, and local ACE and Rho activity and prevented arteriosclerosis. Interestingly, amlodipine prevented the L-NAME-induced increase in MCP-1 receptor CCR2 expression in circulating monocytes. Amlodipine markedly attenuated the high mortality rate at 8 wk of treatment. These data support the hypothesis that amlodipine attenuates arteriosclerosis through inhibiting inflammatory disorders in the rat model of long-term inhibition of NO synthesis. The anti-inflammatory effects of amlodipine seem to be mediated not only by the inhibition of local factors such as MCP-1 but also by the decrease in CCR2 in circulating monocytes. Inhibition of the MCP-1 to CCR2 pathway may represent novel anti-inflammatory actions of amlodipine beyond blood pressure lowering.

MATERIALS AND METHODS

Animal Model of Chronic Inhibition of NO Synthesis

Protocol 1. Protocol 1 was performed to determine whether pretreatment with amlodipine can prevent vascular pathological changes, because prior studies conducted so far have investigated the mechanism(s) of vasculoprotective effects of amlodipine under in vitro conditions. Because the inflammatory process has received attention as a central factor for the development of arteriosclerosis and its complications (5, 14, 15), any anti-inflammatory actions of amlodipine leading to inhibition of vascular disease may have significant clinical implications. Therefore, we hypothesized that amlodipine attenuates the development of arteriosclerosis through the inhibition of inflammation in vivo.

We (10, 11, 18, 19, 21) recently reported a rat model of chronic inhibition of NO synthesis with the administration of Nω-nitro-l-arginine methyl ester (l-NAME). This model displays two distinct stages: an early hypertensive stage associated with vascular inflammation (monocyte adhesion to the endothelium, infiltration into the blood vessel walls, increased expression of monocyte chemoattractant protein (MCP)-1) within the first week of l-NAME administration, and a late decompensated stage with severe arteriosclerosis (medial thickening and fibrosis) in coronary arteries and kidney after 4–8 wk of l-NAME administration. Treatment with angiotensin-converting enzyme (ACE) or an angiotensin II type 1 receptor antagonist prevents all such inflammation and arteriosclerosis and decreases the high mortality rate, suggesting the important role of local activity of angiotensin II in the development of arteriosclerosis (9, 18, 19, 21). Thus previous studies by us and those of other investigators using mice lacking endothelial-type NO synthase (6, 13) support the notion that endothelium-derived NO is an anti-inflammatory and anti-arteriosclerotic molecule.

We consider that the rat model of chronic inhibition of NO synthesis may be useful in determining the in vivo anti-inflammatory role of amlodipine because the inflammatory process is essential in the pathogenesis of arteriosclerosis in this model (8). Some aspects of vascular pathophysiological and pathobiological events occurring after l-NAME administration are similar to those seen in the course of human arteriosclerosis. Therefore, the goal of present study was to investigate the in vivo anti-inflammatory and anti-arteriosclerotic actions of amlodipine beyond blood pressure lowering.

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inflammatory/proliferative changes, and high mortality rate. Four groups of 20-wk-old Wistar-Kyoto (WKY) rats were studied. The control group received untreated chow and drinking water. The second group (L-NAME group) received the NO synthase inhibitor l-NAME in drinking water (1 mg/ml). The third group (L+AM1 group) received l-NAME in drinking water and amlodipine at a dose of 1 mg·kg⁻¹·day⁻¹ by an osmotic minipump. The fourth group (L+AM3 group) received l-NAME in drinking water and 3 mg·kg⁻¹·day⁻¹ amlodipine by osmotic minipump. We used two doses of amlodipine because the high dose attenuated the l-NAME-induced increases in systolic arterial pressure, whereas the low dose had no effect on such changes (Table 1). This study design allowed us to investigate the potential in vivo effects of amlodipine beyond blood pressure lowering.

Protocol 2. Protocol 2 was performed to determine whether post-treatment with amlodipine after 7 days of l-NAME administration can attenuate vascular pathological changes. Four groups of WKY rats were studied. The control group received untreated chow and drinking water. The second group (1wL+3wNT group) received l-NAME in drinking water (1 mg/ml) for 1 wk and untreated water for the subsequent 3 wk. The third group (1wL+3wAM1 group) received l-NAME for 1 wk and amlodipine at a dose of 1 mg·kg⁻¹·day⁻¹ for the subsequent 3 wk. The fourth group (1wL+3wAM3 group) received l-NAME for 1 wk and amlodipine at a dose of 3 mg·kg⁻¹·day⁻¹ for the subsequent 3 wk.

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core, Kyushu University School of Medical Sciences.

Measurement of Tissue ACE Activity
Cardiac tissues were isolated, and the ACE activity was measured by fluorometric assay as previously described. Tissue ACE activity was calculated as nanomoles of His-Leu generated per milligram of tissue weight per hour (19).

Histopathology and Immunohistochemistry
Five paraffin-embedded sections were prepared from each heart as previously described (10, 11, 21). In brief, the heart was perfused via the aorta at a pressure of 90 mm Hg, and the coronary vasculature was fixed with methacarn solution. The heart was excised and cut into five pieces perpendicular to the long axis. The left ventricular sections were either stained with hematoxylin-eosin and Masson trichrome (Takara Shuzo), and Northern blot hybridization was then performed by 10 min at 37°C. Scintillation vials containing 2 ml of Krebs-HEPES buffer with 250 μmol/l lucigenin (250 μmol/l Bis-N-methylacridinium nitrate) were placed into a scintillation counter switched to the out-of-coincidence mode. After 15 min, background counts were recorded, and a vascular segment was then added to the vial. Scintillation counts then were recorded for 10 min, and the respective background counts were subtracted with a scintillation counter (Luminescence Reader BLR 301, Aloka; Tokyo, Japan). To test the specificity of the chemiluminescence reaction, the counts were recorded after an intracellular superoxide scavenger, tiron (10 μmol/l 4,5-dihydroxy-1,3-benzedisulfonic acid), was added to the vial. In all experiments, >90% of the chemiluminescence signals from the aortic rings were scavenged by tiron. The specific chemiluminescence signal was expressed as counts per minute minus the mean background counts.

Northern Blot and PCR Analysis
Total RNA was extracted from the heart by the acid guanidium thiocyanate-phenol-chloroform method (ISOGENE, Nippon Gene). Poly(A)+ RNA was purified using an oligo(dT)-cellulose column (Takara Shuzo), and Northern blot hybridization was then performed as previously described. A rat MCP-1 cDNA probe, rat transforming growth factor-β1 (TGF-β1) probe, and a mouse GAPDH cDNA probe were used. The relative amount of MCP-1 mRNA was normalized against the amount of GAPDH mRNA.

Transcripts from 1 μg of total RNA were reverse transcribed, and the resultant cDNA was amplified by PCR with the following primers for detecting the CCR2 gene: sense primer 5'-GCCACCGAACCCACCAACTAT-3' and antisense primer 5'-GGAATCTCCTCA-

Table 1. Systolic blood pressure and tissue ACE activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 56</th>
<th>ACE Activity, nmol/mg⁻¹·h⁻¹</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Left ventricle</td>
<td>Aorta</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>128±4</td>
<td>128±3</td>
<td>126±4</td>
<td>128±3</td>
<td>133±5</td>
<td>0.80±0.05</td>
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<tr>
<td>L-NAME</td>
<td>125±4</td>
<td>175±3</td>
<td>175±3*</td>
<td>180±2*</td>
<td>192±2*</td>
<td>1.11±0.11</td>
</tr>
<tr>
<td>L+ AM1</td>
<td>130±4</td>
<td>169±3</td>
<td>172±2*</td>
<td>184±2*</td>
<td>188±3*</td>
<td>1.03±0.07</td>
</tr>
<tr>
<td>L+ AM3</td>
<td>124±4</td>
<td>131±3*</td>
<td>140±4*</td>
<td>144±3†</td>
<td>142±3†</td>
<td>0.68±0.04</td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 7–9 rats. The control group received untreated chow and drinking water. The L-NAME group received N'-nitro-l-arginine methyl ester (l-NAME) in the drinking water (1 mg/ml). The L+ AM1 group received l-NAME in the drinking water and 1 mg·kg⁻¹·day⁻¹ amlodipine by osmotic minipump. The L+ AM3 group received l-NAME in the drinking water and 3 mg·kg⁻¹·day⁻¹ amlodipine by osmotic minipump. *P < 0.05 vs. control; †P < 0.05 vs. l-NAME.
CAACCTGTTC-3'. GAPDH mRNA was amplified in the same way with the following primers: sense primer 5'-ACCACAGTCCACGTAC-3' and antisense primer 5'-TCCACACCGCTGGCT-3'. The relative gene expression was expressed as the ratio of CCR2 mRNA to GAPDH mRNA. The RT-PCR analysis was performed by comparing the relative change in CCR2 mRNA expression with GAPDH. The numbers of cycles used, 27 and 23, were within the linear range of the amplification response for the CCR2 and GAPDH genes, respectively.

**Flow Cytometry Analysis**

Peripheral blood mononuclear cells (PBMC) were purified by centrifugation and were washed with ice-cold PBS supplemented with 1% BSA and 0.1% sodium azide. The isolated PBMC (1 × 10⁶) preincubated with 4 μg of goat IgG for 15 min at room temperature and then incubated with 4 μg of biotin anti-rat mononuclear phagocyte (Becton-Dickinson) and 4 μg of goat anti-rat CCR2 antibody (Santa Cruz Biotechnology) for 30 min at 4°C. After being washed, cells were stained with 4 μg of phycoerythrin-conjugated streptavidin (BD Biosciences) and 4 μg of FITC-labeled mouse anti-goat IgG (Santa Cruz Biotechnology) for 30 min at 4°C. Stained cells were analyzed by FACS Calibur instrument using CELL QUEST software (Becton-Dickinson). In control experiments, FITC-conjugated nonspecific goat IgG was used to measure nonspecific binding.

**Serum NOx Concentration**

Serum nitrate/nitrite (NOx) concentration was measured by a fluorometric assay using a commercially available NOx assay kit (NO2 / NO3 Assay Kit-F, Wako) (16). Data were expressed as micromoles per liter.

**Determination of Rho Translocation**

Proteins were prepared from the heart and separated by SDS-PAGE as previously described (7, 16). Membrane and cytosolic proteins in cardiac tissue were isolated. Immunoblotting for RhoA in the membrane and cytosolic fractions was performed.

**Statistical Analysis**

Data are expressed as means ± SE. Statistical differences were determined by ANOVA and Bonferroni’s multiple-comparison tests. Survival curves were evaluated by the Kaplan-Meier method. A level of P < 0.05 was considered statistically significant.

**RESULTS**

**Protocol 1**

**Systolic arterial pressure.** Compared with the control group, the 1-NAME, L+AM1, and L+AM3 groups had greater systolic arterial pressures on weeks 1, 2, 4, and 8 of treatment (Table 1). Treatment with the high dose of amloidipine significantly decreased the 1-NAME-induced rise in systolic arterial pressure, whereas the low dose of amloidipine had no effect.

**Survival curve.** As reported by others (2), compared with the control group, rats who received 1-NAME displayed a high mortality rate at 8 wk (Fig. 1). Amlodipine at the low dose markedly attenuated and amloidipine at the high dose normalized the survival rate.

**Tissue ACE activity on day 3.** Compared with the control group, cardiac and aortic tissue ACE activities were significantly greater in the 1-NAME group (Table 1). Treatment with the low and high doses of amloidipine prevented the increases in cardiac and aortic tissue ACE activities.

**Inflammatory and proliferative changes on day 3.** Three days after 1-NAME treatment was started, the rats in the 1-NAME group had marked infiltration of ED1-positive monocytes into the intima and adventitia of their coronary arteries (Figs. 2A and 3A). Nuclear staining for PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, and lesional monocytes in the 1-NAME group. No such inflammatory and proliferative changes were observed in the control group. In rats treated with 1-NAME plus amloidipine, such inflammatory and proliferative changes were markedly suppressed. When ED1-positive monocytes or PCNA-positive cells were counted, the number of immunopositive cells per section was significantly greater in the 1-NAME group than in the control group (Fig. 3A). The increases in ED1-positive cells and PCNA-positive cells were both significantly reduced by treatment with the low and high doses of amloidipine.

**Arteriosclerosis and glomerular injury on day 56.** Compared with the control group, medial thickening (the wall-to-lumen ratio) and perivascular fibrosis of coronary arteries were significantly greater in the 1-NAME group. Cotreatment with the low and high doses of amloidipine inhibited the 1-NAME-induced medial thickening and perivascular fibrosis (Figs. 2B and 3B).

Compared with the control group, the glomerular injury score was significantly greater in the 1-NAME group. Cotreatment with the low and high doses of amloidipine significantly but partly inhibited the 1-NAME-induced glomerulosclerosis (Fig. 3C).

**Expression of TGF-β1 and MCP-1 mRNA on day 3.** As we have previously shown, cardiac TGF-β1 and MCP-1 mRNA levels were significantly greater in the 1-NAME group (Fig. 4). The increased expressions of TGF-β1 and MCP-1 mRNA were both prevented by treatment with the low and high doses of amloidipine.

**RhoA translocation on day 3.** Compared with the control group, the 1-NAME group had greater RhoA expression in the membrane fraction (Fig. 5). Treatment with amlodipine prevented the 1-NAME-induced increase in the membranous RhoA expression. Cytosol RhoA expression levels did not differ among groups (Fig. 5).

**Plasma NOx concentration on day 3.** The plasma NOx concentration was significantly decreased in the 1-NAME group.
group (1.0 ± 0.1 μmol/l, n = 8, P < 0.01 vs. control) compared with the control group (2.6 ± 0.3 μmol/l, n = 8). Treatment with amloidipine did not affect the L-NAME-induced decrease in serum NO\(_x\) levels in the L+AM1 (1.1 ± 0.1 μmol/l, n = 8, P < 0.01 vs. control) and L+AM3 groups (1.1 ± 0.1 μmol/l, n = 7, P < 0.01 vs. control).

Oxidative stress on day 3. Superoxide anion production by the aortic segments with endothelium was greater in the L-NAME group than in the control group, as we have previously reported (20). Treatment with the low and high doses of amloidipine normalized the L-NAME-induced increase in aortic superoxide anion production (Fig. 4C).

Immunohistochemical analysis of HNE-modified protein reveals the cellular localization of lipid peroxidation. Lipid peroxides were positively stained in mainly the coronary arteries in the L-NAME-treated rats (Fig. 2A). The increased immunoreactivity for HNE was not noted in rats from the L+AM1 and L+AM3 groups. No immunoreactivity was noted when the antibody against HNE-modified protein was replaced with nonimmune IgG (negative control).

PCR and flow cytometry in PBMCs on days 3 and 7. CCR2 mRNA and protein levels in PBMCs were assessed by PCR and flow cytometry, respectively. Compared with the control group, the CCR2 mRNA levels in cardiac and vascular tissues did not increase in the L-NAME group or in the L-NAME plus amloidipine groups (data not shown). However, the CCR2 mRNA levels in PBMCs were greater in the L-NAME group than in the control group (Fig. 6A). Treatment with amloidipine at the low and high doses prevented the L-NAME-induced increase in CCR2 gene expression. In keeping with the increase in CCR2 mRNA levels, CCR2 antigen levels on PBMCs increased in the L-NAME group, which was prevented by treatment with amloidipine (Fig. 6B).

Protocol 2

Effects of posttreatment with amloidipine on histopathological changes of coronary arteries were determined on day 28. As we (8) have previously reported, the wall-to-lumen ratios and perivascular fibrosis were significantly greater in the
1wL/H1/1001 3wNT group than in the control group (Fig. 7). Such vascular structural changes were not evident in the 1wL/H1/1001 3wAM1 and 1wL/H1/1001 3wAM3 group.

DISCUSSION

We have demonstrated herein that treatment with amlodipine normalized the high mortality rate and attenuated the L-NAME-induced increase in inflammatory and proliferative changes in coronary arteries and the kidney. Interestingly, amlodipine prevented the L-NAME-induced increase in the MCP-1 receptor CCR2 expression in circulating monocytes. Our present data suggest novel anti-inflammatory effects of amlodipine beyond blood pressure lowering.

The beneficial effects of amlodipine might result from the decrease in systolic arterial pressure after L-NAME administration. In the present study, however, the low dose of amlodipine did not affect the systolic loading conditions but did inhibit early inflammation as well as late arteriosclerosis. Amlodipine did not affect the L-NAME-induced inhibition of NO synthesis. Thus it is likely that the cardiovascular protective effects of amlodipine may not be explained by its antihypertensive effect or by restoration of NO production.

An important feature that emerged in the present study is that amlodipine prevented inflammatory (monocyte infiltration, increased gene expression of MCP-1 and TGF-β1, and increased Rho activity) and proliferative (appearance of PCNA-positive cells) disorders. Activated monocytes, endothelial cells, and/or smooth muscle cells are capable of producing growth-promoting factors. We have previously demonstrated that 1) oxidative stress participates in the development of vascular inflammation (20, 21), NF-κB activation (10), and MCP-1 expression (11) at early stages (within 7 days) of L-NAME treatment; 2) MCP-1 mediates inflammation and arteriosclerosis (11); and 3) TGF-β1 mediates fibrosis (12) in this rat model. In the present study, we have shown that superoxide anion formation is increased in aortic tissues of the L-NAME-treated group compared with controls and that immunohistochemically demonstrable lipid peroxidation, induced possibly by increased superoxide anion formation, can be detected in the vicinity of coronary arteries. Treatment with amlodipine reduced the markers of oxidative stress, suggesting that amlodipine acted as an antioxidant in the present experiments. Therefore, it is likely that pretreatment with amlodipine

![Fig. 3](http://ajpheart.physiology.org/)

1wL + 3wNT group than in the control group (Fig. 7). Such vascular structural changes were not evident in the 1wL + 3wAM1 and 1wL + 3wAM3 group.

![Fig. 4](http://ajpheart.physiology.org/)
might prevent such pathological inflammatory disorders by blocking oxidative stress and biological activity of MCP-1 and TGF-β1. Interestingly, amlodipine prevented L-NAME-induced translocation of Rho, suggesting a contribution of increased Rho activity to the anti-inflammatory actions of amlodipine (7). Furthermore, we showed that posttreatment with amlodipine attenuates arteriosclerosis, suggesting that amlodipine might have accelerated the disappearance of inflammation even after vascular inflammatory changes had established.

We (9, 18, 19) have previously shown the critical role of local activity of angiotensin II (increased activity of tissue ACE and angiotensin II type 2 receptor) in the development of arteriosclerosis induced by L-NAME. An increase in angiotensin II activity mediated via type 1 receptors has been shown to cause vascular inflammation, oxidative stress, Rho activation, and arteriosclerosis (7, 9–11, 19, 21). Therefore, the beneficial effects of amlodipine seen in the present study may be explained by the decrease in inflammatory changes in cardiovascular tissues caused by increased angiotensin II activity through angiotensin II type 1 receptors.

Previous studies that investigated the inflammatory aspects of vascular disease focused on lipid- or stress-induced changes in inflammation driving factors, such as MCP-1 in cells of the arterial wall, whereas pathobiological changes in peripheral circulating monocytes have not attracted much attention. We found here, for the first time, that treatment with amlodipine
prevented the L-NAME-induced increase in CCR2 expression in circulating monocytes, suggesting that the anti-inflammatory effects of amlodipine may be mediated at least in part by the decrease in expression and activity of CCR2 in circulating monocytes.

A caveat of interpreting our present data is that the mechanism of improvement of the survival rate by amlodipine remains to be elucidated. This is because autopsies of animals shown in Fig. 1 were not performed. Although the occurrence of heart failure, fatal arrhythmia, renal failure, and/or stroke resulting from inflammatory changes might be the cause of death, there are no mechanistic data indicating that the inflammation changes did contribute to the effects of amlodipine on survival curves.

In summary, the present data suggest that amlodipine attenuated arteriosclerosis through inhibition of inflammatory disorders in a rat model of long-term inhibition of NO synthesis. The anti-inflammatory effects of amlodipine may be mediated by the inhibition of local factors, such as MCP-1, TGF-β, and Rho, and oxidative stress and by the decrease in CCR2 in circulating monocytes. Inhibition of the MCP-1 to CCR2 pathway may represent novel anti-inflammatory actions of amlodipine beyond blood pressure lowering.

GRANTS

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