Important Role of Local Angiotensin II Activity Mediated via Type 1 Receptor in the Pathogenesis of Cardiovascular Inflammatory Changes Induced by Chronic Blockade of Nitric Oxide Synthesis in Rats

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Background—The chronic inhibition of NO synthesis by N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) upregulates the cardiovascular tissue angiotensin II (Ang II)–generating system and induces cardiovascular inflammatory changes in rats.

Methods and Results—We used a rat model to investigate the role of local Ang II activity in the pathogenesis of such inflammatory changes. Marked increases in monocyte infiltration into coronary vessels and myocardial interstitial areas, monocyte chemoattractant protein-1 (MCP-1) expression, and nuclear factor-κB (NF-κB, an important redox-sensitive transcriptional factor that induces MCP-1) activity were observed on day 3 of L-NAME administration. Along with these changes, vascular superoxide anion production was also increased. Treatment with an Ang II type 1 receptor antagonist or with a thiol-containing antioxidant, N-acetylcysteine, prevented all of these changes.

Conclusions—Increased Ang II activity mediated via the type 1 receptor may thus be important in the pathogenesis of early cardiovascular inflammatory changes in this model. Endothelium-derived NO may decrease MCP-1 production and oxidative stress–sensitive signals by suppressing localized activity of Ang II. (Circulation. 2000;101:305-310.)

Key Words: endothelium-derived factors ■ nitric oxide ■ remodeling ■ proteins ■ cells

The vascular endothelium becomes dysfunctional in the early stages of atherosclerosis.\textsuperscript{1-7} Such dysfunction has been shown to lead to reduced activity of NO.\textsuperscript{1,2} A growing body of evidence suggests that endothelium-derived NO regulates the development of vascular structural changes.\textsuperscript{1,2,8,9} Recently, we observed that inhibition of NO synthesis by \textit{N}\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) induces a marked monocyte infiltration into the coronary vessels associated with induction of monocyte chemoattractant protein-1 (MCP-1) and proliferating cell nuclear antigen expression.\textsuperscript{10} The importance of our observation is supported by the well-recognized concept that the adhesion of mononuclear cells to, and their infiltration into, the blood vessel wall are assumed to be crucial early atherogenic events.\textsuperscript{11,12}

The mechanism of such inflammatory changes after blockade of NO synthesis is unknown but may be related to the increase in local activity of angiotensin II (Ang II), because an increase in activity of ACE and Ang II receptors in the heart and vessels has been observed during the first week of L-NAME administration.\textsuperscript{13,14} However, it has not been established whether increased Ang II activity is a cause or a result of cardiovascular inflammatory changes after blockade of NO synthesis. Recent in vitro studies have demonstrated that the inhibition of NO synthesis increases oxidative stress,\textsuperscript{15} activates the transcription regulatory proteins such as nuclear factor-κB (NF-κB),\textsuperscript{16,17} and induces the expression of various genes, including those encoding adhesion molecules and inflammatory cytokines\textsuperscript{18,19} in endothelial cells. Ang II has been demonstrated to promote intracellular signaling events that lead to vascular inflammation, proliferation, and chemotaxis by increasing oxidative stress through type 1 receptor stimulation.\textsuperscript{20-25}

To investigate the role of the local Ang II activity in the pathogenesis of inflammatory changes induced by blockade of NO synthesis, we used a rat model to examine whether blockade of Ang II type 1 receptor prevents such inflammatory changes.

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Hemodynamic Parameters

<table>
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<tr>
<th></th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Body Weight, g</th>
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<tr>
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<td>Day 0</td>
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<tr>
<td>Day 0</td>
<td>138±4</td>
<td>312±7</td>
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<tr>
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<td>139±6</td>
<td>310±8</td>
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<tr>
<td>L+ATRA2 group</td>
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<td>300±10</td>
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<tr>
<td>L+NAC group</td>
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<td>310±10</td>
</tr>
<tr>
<td>Day 3</td>
<td>158±8*</td>
<td>304±10</td>
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</tbody>
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Data are mean±SEM. *P<0.01 vs control group; †P<0.01 vs day 0.

Methods

Animal Model of Chronic Inhibition of NO Synthesis

The present study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines for Animal Experiments of the Kyushu University Faculty of Medicine.

Male Wistar-Kyoto rats 20 weeks old were obtained from an established colony at the Animal Research Institute of Kyushu University Faculty of Medicine. Five groups of rats were studied. The control group received untreated chow and drinking water. The second group (L) received L-NAME in its drinking water (1 mg/mL). At this concentration, the daily intake of L-NAME for the latter group was ~30 to 40 mg/d. The third group (L+ATRA1) received L-NAME in drinking water and a high dose (75 μg/g) of a selective antagonist of the Ang II AT1 receptor CS-866 (a gift from Sankyo Pharmaceutical Co, Tokyo) in the chow. The fourth group (L+ATRA2) received L-NAME in the drinking water and a low dose (7.5 μg/g) of CS-866 in the chow. We found that the high dose of CS866 prevented the L-NAME–induced increase in systolic arterial pressure, whereas the low dose of the antagonist had no effect on such changes.1 The fifth group (L+NAC) received L-NAME in the drinking water and a thiol-containing antioxidant, N-acetylcysteine (NAC), by intraperitoneal injection (200 mg·kg⁻¹·d⁻¹). On day 3 of treatment, systolic blood pressure (by the tail-cuff method), heart rate, and body weight were measured. Then the rats were euthanized for morphometric, immunohistochemical, and biochemical analyses.

Histopathology and Immunohistochemistry

Five rats in each group were used. The animals were anesthetized with intraperitoneal pentobarbital, the heart was perfused via the aorta at a pressure of 90 mm Hg, and the coronary vasculatures were fixed for 60 minutes with methacarn solution.10,26 The heart was excised and cut into 5 pieces perpendicular to the long axis. All tissue samples were fixed in methacarn solution for 3 days, dehydrated, embedded in paraffin, and cut into slices 5 μm thick. Sections were mounted on glass slides and stained with hematoxylin-eosin solution for estimation of inflammatory cell infiltration.

Immunohistochemistry was performed as described.10,28 In brief, paraffin slices 5 μm thick were preincubated with 3% skim milk to decrease nonspecific binding. Sections were immunostained with a mouse anti-rat macrophage/monocyte antibody (ED1, Serotec Inc), a rabbit anti-rat MCP-1 antibody,29 or nonimmune mouse IgG (Zymed Laboratory Inc). Immunohistochemistry for ACE was performed as described.15 After this, tissues were embedded in OCT compound, and sections were immunostained with an antibody for human ACE (10 μg/mL, 9B9, Immunobiology Laboratory Inc) or nonimmune IgG (Zymed Laboratory Inc).

Morphometry and cell enumeration were performed by a single observer who was blind to the treatment protocols as described.10 To quantify the areas affected by inflammatory changes, the hematoxylin-eosin–stained whole-heart sections (5 per heart) were scanned at ×40 magnification with a light microscope equipped with a high-resolution video camera (Microphoto-FXA, Nicon). The sum of the areas of inflammatory cell infiltration in each section and the sum of the heart areas in the section were calculated. The areas of large arterial and venous lumen were excluded from this measurement. Then, the percentage of the areas affected by inflammatory cell infiltration in each heart (100×area affected by inflammatory change/total heart area of the section) was reported.

To quantify the number of ED1-positive monocytes, each section (5 per heart) immunohistochemically stained by an antibody against ED1 was scanned at ×40 magnification. The number of ED1-positive cells in each section was counted and summed per heart. The averaged number of the positive cells per section was reported in each animal.

Northern Blot Analysis

Five rats in each group were used. After euthanasia, the hearts were removed, the atria and great vessels were trimmed away, and the hearts were snap-frozen in liquid nitrogen and stored at −80°C.

Total RNA was extracted from each sample by the acid guanidinium thiocyanate–phenol-chloroform method (ISOGEN, Nippon Gene), and poly(A)⁺ RNA was purified on an oligo (dT)–cellulose column (Takara Shuzo). Northern blot hybridization of rat MCP-130 was performed as described.10 Relative amounts of MCP-1 mRNA were normalized against the amounts of GAPDH mRNA.

Electrophoretic Mobility Shift Assays

To determine activation of NF-κB binding to the nucleus, electrophoretic mobility shift assays were performed. Five rats in each group were used. Nuclear protein extracts were prepared from the aorta as described.31 The NF-κB oligodeoxynucleotides corresponding to putative consensus sequences (NF-κB: 5'-AGTTGAGGGG-AGTTCCCCAGGC-3') were column-purified on a Sephadex G-25 M column (Pharmacia Biotech Inc). Labeled probe (50 000 cpm) was added to 5 μg of nuclear protein in the presence of a nonspecific blocker, salmon testes DNA (0.1 μg/μL). The binding reaction also contained 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.2% Nonidet P-40, and 0.5 mmol/L DTT. This mixture was incubated at 25°C for 20 minutes and separated by electrophoresis on a 5% polyacrylamide gel in 1×Tris-glycine-EDTA buffer. Gels were dried and subjected to autoradiography. Autoradiographs were later subjected to laser densitometry. Specificity was determined by the addition of excess cold oligonucleotide (Santa Cruz Biotechnologies Inc) to the nuclear extracts 10 minutes before addition of radiolabeled probe.

Measurements of Vascular Superoxide Anion Production

We used the lucigenin chemiluminescence assay to measure O₂⁻ levels in rat aorta.52 The thoracic aorta was removed en bloc and placed in cold Krebs-Henseleit solution. The aorta was cut into 5-mm ring segments and allowed to equilibrate in modified Krebs-HEPES buffer for 10 minutes at 37°C. The O₂⁻ production was measured by the lucigenin (bis-N-methylacridinium nitrate,
250 μmol/L)--enhanced chemiluminescence technique with a scintillation counter (Luminescence Reader BLR 301, Aloka Co). To test the specificity of chemiluminescence reaction, the counts were recorded after an intracellular superoxide scavenger, Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, 10 μmol/L), was added to the vial. Signals from the aortic rings were calibrated by use of known concentrations of xanthine and xanthine oxidase and calculated as nmol min^{-1} mg^{-1}.

Statistical Analysis
Data are expressed as mean±SEM. Changes in parameters of a group over time were compared by 1-way ANOVA and Bonferroni’s multiple comparison test. Differences between groups were determined with 2-way ANOVA and a multiple comparison test. A level of P<0.05 was considered statistically significant.

Results
Systolic Arterial Pressure, Heart Rate, and Body Weight
Compared with the control group, the L, L+ATRA2, and L+NAC groups showed a rise in systolic arterial pressure on day 3 of treatment. In the L+ATRA1 group, systolic arterial pressure did not change (Table).

Histopathology and Immunohistochemistry
Compared with the control group, tissue sections from rats of the L group exhibited a marked infiltration of mononuclear leukocytes and fibroblast-like cells into the perivascular areas that immediately surrounded the coronary vessels (Figure 1). Attachment of mononuclear leukocytes to the coronary arterial and venous lumen and their invasion to the vascular medial layer were also observed. Infiltration of inflammatory cells into myocardial interstitial spaces was observed.

We observed no evidence of inflammation in the control rats (Figures 1 and 2). The increase in the areas of inflammation seen in the L group was significantly inhibited by the
treatment with the low (L+ATRA2 group) and high (L+ATRA1 group) doses of Ang II AT1 receptor antagonist or with NAC (L+NAC group).

Reaction of cells with antibodies directed against ED1 or MCP-1 was rarely observed in the control group (Figure 1). We found that the number of monocytes that had infiltrated into the coronary vessels and myocardium was markedly increased in the L group (Figure 2). This increase in monocyte infiltration was significantly reduced by treatment with the low and high doses of the Ang II AT1 receptor antagonist or with NAC. Cells positive for MCP-1 were observed in the vascular intima and media and in some mononuclear cells that had infiltrated into the lesion (Figure 1).

In the control group, ACE immunoreactivity was weakly present in the intimal layer of the aorta (Figure 3). In the L group, the intimal layer and the perivascular and myocardial inflammatory lesions were intensely immunoreactive to ACE antibody (Figure 3). No immunoreactivity was noted when ACE antibody was replaced with nonimmune IgG (data not shown). In the L+ATRA1 group, no such intense ACE immunostaining activity was noted (Figure 3).

Expression of MCP-1 mRNA

In concert with mononuclear cell infiltration, the cardiac MCP-1 mRNA level was markedly increased in the L group (Figure 4). The increased expression of MCP-1 mRNA was reduced in the L+ATRA1 and L+ATRA2 groups.

**O$_2^-$ Production**

O$_2^-$ production by the aortic segments with endothelium was greater in the L group than in the control group (Figure 5). Treatment with the low and high doses of Ang II AT1 receptor antagonist or with NAC normalized the L-NAME–induced increase in aortic O$_2^-$ production.

**NF-κB Activity**

Compared with the control group, NF-κB binding was increased in the heart from the L group (Figure 6). The shifted bands were specific for NF-κB, because the addition of 100-fold excess cold corresponding oligonucleotide to the nuclear extract abolished the band. The L-NAME–induced increase in NF-κB activity in the L group was prevented by the Ang II AT1 receptor blockade and by antioxidant treatment with NAC.

**Discussion**

This study demonstrates for the first time that Ang II type 1 receptor blockade prevents monocyte infiltration into the coronary vessels and myocardial tissues, MCP-1 expression, NF-κB activation, and aortic O$_2^-$ production induced by inhibition of NO synthesis in rats in vivo. We interpret these findings to suggest that an increase in local activity of Ang II mediates such early inflammatory changes.
Recent in vitro evidence suggests that inhibition of NO synthesis increases NF-κB activity through increased oxidative stress. In the present study, we found that antioxidant treatment with NAC markedly attenuated the increases in inflammatory changes and NF-κB activity, suggesting that oxidative stress may be involved in the pathogenesis of NF-κB activation in our in vivo model.

Because inflammatory changes in coronary vessels have been reported in animal models with hypertension, inflammatory changes seen in our experimental model might result at least in part from the rapid increase in systolic arterial pressure induced by L-NAME administration. In the present study, however, a low dose of the AT1 receptor antagonist did not affect the increase in systolic loading conditions but rather produced an inhibitory effect identical to that observed with an antihypertensive high dose of the AT1 receptor antagonist. We recently found that AT1 receptor blockade prevented inflammatory changes and the increase in NF-κB activity induced by a lower dose of L-NAME that had no effect on systolic blood pressure (unpublished data, 1999). Thus, it is unlikely that the increase in systolic arterial pressure contributed greatly to the induction of inflammatory changes in our experimental model.

In conclusion, our present observations suggest that the increase in the localized Ang II--generating system plays a primary role in mediating cardiovascular inflammatory changes, MCP-1 expression, oxidative stress, and NF-κB activity induced by chronic inhibition of NO synthesis. We have recently found that NO directly downregulates Ang II AT1 receptor in vascular smooth muscle cells. Thus, it appears that endogenous NO that is physiologically produced by vascular endothelial cells may decrease MCP-1 production and oxidative stress–sensitive signals by suppressing localized activity of Ang II in the blood vessels. The antiartherosclerotic and antiatherosclerotic effects of endothelium-derived NO could be explained by this mechanism.

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