Regression by ACE inhibition of arteriosclerotic changes induced by chronic blockade of NO synthesis in rats

MAKOTO KATOH,1 KEN SUKE EGASHIRA,2 CHU KATA OKA,2 MAKOTO USUI,2 MASAMICHI KO YANAGI,2 SHI RO KITAMOTO,2 YASU SHI OHMACHI,1 AKIRA TAKE SHITA,2 AND HIROSHI NARITA1

1Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., Saitama 335-8055, Japan; and
2Department of Cardiovascular Medicine, Kyushu University Faculty of Medicine, Fukuoka, Japan

Received 5 July 2000; accepted in final form 7 December 2000.

Katoh, Makoto, Kensuke Egashira, Chu Kataoka, Makoto Usui, Masamichi Koyanagi, Shiro Kitamoto, Yasushi Ohmachi, Akira Takeshita, and Hiroshi Narita. Regression by ACE inhibition of arteriosclerotic changes induced by chronic blockade of NO synthesis in rats. Am J Physiol Heart Circ Physiol 280: H2306–H2312, 2001.—We previously reported that chronic inhibition of nitric oxide (NO) synthesis with Nω-nitro-L-arginine methyl ester (L-NAME) induces vascular inflammation at week 1 and produces subsequent arteriosclerosis at week 4 and that cotreatment with an angiotensin-converting enzyme (ACE) inhibitor prevents such changes. In the present study, we tested the hypothesis that treatment with an ACE inhibitor after development of vascular inflammation could inhibit arteriosclerosis in rats. Wistar-Kyoto rats were randomized to four groups: the control group received no drugs, the 4wL-NAME group received L-NAME (100 mg·kg⁻¹·day⁻¹) for 4 wk, the 1wL + 3wNT group received L-NAME for 1 wk and no treatment for the subsequent 3 wk, and the 1wL + 3wACEI group received L-NAME for 1 wk and the ACE inhibitor imidapril (20 mg·kg⁻¹·day⁻¹) for the subsequent 3 wk. After 4 wk, we observed significant arteriosclerosis of the coronary artery (medial thickening and fibrosis) and increased cardiac ACE activity in the 1wL + 3wNT group as well as in the 4wL-NAME group, but not in the 1wL + 3wACEI group. In a separate study, we examined apoptosis formation and found that posttreatment with imidapril (20 mg·kg⁻¹·day⁻¹) or an ANG II AT₁-receptor antagonist, CS-866 (5 mg·kg⁻¹·day⁻¹), induced apoptosis (TdT-mediated nick end-labeling) in monocytes and myofibroblasts appearing in the inflammatory lesions associated with a clear degradation in the heart (DNA electrophoresis). In conclusion, treatment with the ACE inhibitor after 1 wk of L-NAME administration inhibited arteriosclerosis by inducing apoptosis in the cells with inflammatory lesions in this study, suggesting that increased ANG II activity inhibited apoptosis of the cells with inflammatory lesions and thus contributed to the development of arteriosclerosis.

Address for reprint requests and other correspondence: M. Katoh, Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 2-2-50, Kawagishi, Toda-shi, Saitama 335-8055, Japan (E-mail: katoh-m@tanabe.co.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
sis, and 3) apoptosis is involved in the beneficial effects of ACE inhibition in rats.

METHODS

The present experiments were reviewed and approved by the Committee on Ethics in Animal Experiments, Faculty of Medicine, Kyushu University, and conducted according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and law (no. 105) and notification (no. 6) of the Japanese Government.

Protocol 1

Protocol 1 was performed to determine whether treatment with the ACE inhibitor after 7 days of L-NAME administration can attenuate vascular remodeling.

Experimental groups. Twenty-week-old male Wistar-Kyoto rats were randomly divided into four groups. The first (control) group received plain drinking water. The second group (4wL-NAME) received L-NAME in drinking water (1 mg/ml) for 4 wk. We previously demonstrated that the dose of L-NAME used in the present study sufficiently suppresses the aortic NO-generating capacity (33). The third group (1wL + 3wNT) received L-NAME in drinking water (1 mg/ml) for 1 wk and plain water for the subsequent 3 wk. The fourth group (1wL + 3wACEI) received L-NAME for 1 wk and the ACE inhibitor imidapril (0.2 mg/ml) for the subsequent 3 wk in drinking water. We monitored and confirmed that the rats drank ~30–40 ml of water and ate 20 g of chow, regardless of the treatment, and also confirmed that their drinking and eating patterns were unaffected by any treatment protocol.

The systolic arterial pressure (the tail-cuff method) of each rat was measured on days 0, 7, and 28 of treatment. On day 28, the rats were killed for morphometric and biochemical analysis.

Histopathology and morphometry. Histopathology and morphometry were performed by a single observer who was blind to all treatment protocols (30, 31). Findings were evaluated in seven to eight rats of each group as previously described. Excised hearts were perfused at a pressure of 90 mmHg, and then the coronary vasculature was fixed for 30 min in 6% formaldehyde solution and the heart was cut perpendicularly to the long axis at the papillary muscle level. The tissues were fixed in 6% formaldehyde for a few days and then dehydrated and embedded in paraffin. The paraffin slices were stained with Masson’s trichrome staining solution.

To evaluate the thickening of coronary arterial walls and perivascular fibrosis, short-axis images of the coronary arteries (30–200 μm ID) were studied (30, 31). The inner border of the lumen and the outer border of the tunica media were traced in each arterial image with Masson’s trichrome staining solution and the area of fibrosis (collagen deposition stained with aniline blue) immediately surrounding the blood vessels were then calculated; i.e., perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. In each heart, ~30 coronary arteries were examined. Average values for vessels of each size were used for analysis.

Biochemical analysis. Assays of ACE activity were performed on five to eight rats from each group. Serum and tissue ACE activities were measured at the 4th wk of treatment using a spectrophotometric assay as described previously (31). Cardiac ACE was extracted from the homogenized left ventricle, and the reaction product hippuric acid from the substrate Hip-His-Leu was isolated from the reaction mixture by HPLC and detected at 225 nm using a spectrophotometer. Cardiac and serum ACE activities were calculated to give the rate of hippuric acid generation from Hip-His-Leu as nanomoles of Hip-His-Leu turning over per milligram of tissue weight per hour and as nanomoles of Hip-His-Leu turning over per milliliter of serum per hour, respectively.

Protocol 2

Protocol 2 was performed to examine whether the beneficial effects of ACE inhibition seen in protocol 1 can be attributed to the induction of apoptosis in the inflammatory cells appearing in the cardiovascular lesions.

Experimental groups. Four groups of rats were studied. The first group (control) received plain drinking water and chow. The second group (1wL + 4dNT, n = 11) received L-NAME in drinking water (1 mg/ml) for 1 wk and plain water for the subsequent 4 days. The third group (1wL + 4dACEI, n = 11) received L-NAME for 1 wk and the ACE inhibitor imidapril (0.2 mg/ml) for the subsequent 4 days in drinking water. The fourth group (1wL + 4dAT1RA, n = 11) received L-NAME for 1 wk and an ANG II AT1-receptor antagonist, CS-866 (Sankyo Pharmaceutical, Tokyo, Japan; 75 μg/g), for the subsequent 4 days in chow. This dose of CS-866 has been used successfully by other investigators (21). After treatment, the systolic arterial pressure of each rat was measured. Animals were killed for determination and characterization of apoptosis.

TdT-mediated dUTP nick end-labeling and immunohistochemistry. Paraffin blocks were cut into 5-μm-thick slices and mounted on slides. TdT-mediated dUTP nick end-labeling (TUNEL) was performed using an in situ apoptosis detection kit (Takara Shuzo) according to the manufacturer’s instructions. For immunohistochemistry, the slices were preincubated with 10% normal horse serum to decrease nonspecific binding and incubated with mouse anti-rat macrophage/macrophage antibody (ED1, Serotec) at a dilution of 1:1,000, mouse anti-human α-smooth muscle (α-SM) actin antibody (Dako) at 1:500, or nonimmune mouse IgG (Zymed) at 1:500 overnight at 4°C. The samples were subsequently incubated with biotinylated, affinity-purified horse anti-mouse IgG (Vector). In indirect immunoperoxidase techniques, the labeled antibody was visualized with 3',3'-diaminobenzidine and hydrogen peroxide to appear brownish-black. The tissue samples were counterstained with hematoxylin.

To quantify the number of positive cells in hearts, two sections per heart were stained by the TUNEL detection kit or immunohistochemically by antibodies against ED1 and α-SM actin and scanned at ×100 magnification. The number of cells that stained positive for TUNEL, ED1, or α-SM actin was counted; the average number of positive cells per section was reported for each animal.

To determine the cell type of the TUNEL-positive cells, immunohistochemical double staining was performed. The slides were stained first with TUNEL as described above and incubated with a monoclonal antibody against α-SM actin (1:250) or EDI (1:500) overnight at 4°C. The samples were subsequently incubated with goat anti-mouse IgG, and then with mouse alkaline phosphatase anti-alkaline phosphatase immune complex. Bound alkaline phosphatase was visualized with Fast red and levamisole to yield a red reaction product.

Assessment of DNA fragmentation. Five individual frozen hearts per group were homogenized on dry ice and lysed with
Table 1. Systolic blood pressure and ACE activity

<table>
<thead>
<tr>
<th></th>
<th>Systolic Blood Pressure, mmHg</th>
<th>ACE Activity Serum, nmol·ml⁻¹·h⁻¹</th>
<th>Heart, nmol·mg⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>137 ± 3</td>
<td>1.07 ± 0.03</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td>Week 1</td>
<td>134 ± 3</td>
<td>1.04 ± 0.04</td>
<td>2.20 ± 0.21</td>
</tr>
<tr>
<td>Week 4</td>
<td>133 ± 5</td>
<td>1.16 ± 0.03</td>
<td>1.64 ± 0.30</td>
</tr>
<tr>
<td>4wL-NAME</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>141 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>182 ± 4†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>198 ± 4†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1wL + 3wNT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>134 ± 4</td>
<td>1.16 ± 0.03</td>
<td>0.02 ± 0.003*</td>
</tr>
<tr>
<td>Week 1</td>
<td>185 ± 6†</td>
<td>1.64 ± 0.30</td>
<td>0.21 ± 0.06*</td>
</tr>
<tr>
<td>Week 4</td>
<td>133 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3wACEI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–10. Control group received plain drinking water; 4wL-NAME group received N- nitro-L-arginine methyl ester (L-NAME) in drinking water (1 mg/ml) for 4 wk; 1wL + 3wNT group received L-NAME in drinking water (1 mg/ml) for 1 wk and untreated water for subsequent 3 wk; 1wL + 3wACEI group received L-NAME for 1 wk and the angiotensin-converting enzyme (ACE) inhibitor imidapril (0.2 mg/ml) for subsequent 3 wk in drinking water. *P < 0.01 vs. control. †P < 0.01 vs. day 0.

Protocol 1

Systolic blood pressure. On day 7, all rats in the 4wL-NAME, 1wL + 3wNT, and 1wL + 3wACEI groups showed a rise in systolic arterial pressure (Table 1). On day 28, the 4wL-NAME group showed a sustained increase in systolic arterial pressure. In contrast, the systolic arterial pressure in the 1wL + 3wNT or 1wL + 3wACEI group returned to the level comparable to that in the control group (Table 1).

Serum and cardiac tissue ACE activity at week 4. Serum ACE activity was comparable among the control, 1wL + 3wNT, and 1wL + 3wACEI groups. Cardiac tissue ACE activity was markedly increased in the 4wL-NAME and 1wL + 3wNT groups. Serum and tissue ACE activities were significantly reduced in the 1wL + 3wACEI group compared with the control group (Table 1).

Coronary vascular remodeling. Micrographs of the coronary arteries obtained from the four groups are shown Fig. 1. The wall-to-lumen ratios and perivascular fibrosis in the coronary arteries were significantly greater in the 4wL-NAME than in the control group (Figs. 1 and 2). These vascular structural changes (remodeling) in the 4wL-NAME group did not significantly differ from those in the 1wL + 3wNT group. In contrast, such vascular structural changes were not evident in the 1wL + 3wACEI group (Figs. 1 and 2).

 Protocol 2

Determination and characterization of apoptotic cells. As we reported previously (16, 18, 33, 36), infiltration of ED1-positive monocytes and α-SM actin-positive myofibroblasts was observed in the perivascu-

![Fig. 1. Micrographs of coronary artery sections stained with Mason's trichrome stain in the control group (A) and in rats treated with N-nitro-l-arginine methyl ester (L-NAME) for 4 wk (4wL-NAME group; B), L-NAME for 1 wk and no treatment for the subsequent 3 wk (1wL + 3wNT group; C), and L-NAME for 1 wk and imidapril for the subsequent 3 wk (1wL + 3wACEI group; D). Compared with control, increases in wall-to-lumen (medial thickness-to-internal diameter) ratio and area of fibrosis (collagen deposition stained with aniline blue) are observed to similar extents in the 4wL-NAME and 1wL + 3wNT groups. M and C, medial thickening and collagen deposition, respectively. Scale bar, 100 μm.](http://www.ajpheart.org/content/185/4/1981/F1.large.jpg)
lar and interstitial tissues in the 1wL + 4dNT, 1wL + 4dACEI, and 1wL + 4dAT1RA groups, whereas no such cells were observed in the hearts from control rats (Fig. 3A). The number of such immunopositive cells was significantly lower in the 1wL + 4dACEI and 1wL + 4dAT1RA groups than in the 1wL + 4dNT group (Fig. 3A).

When TUNEL-positive apoptotic cells were examined, the positive cells were detected in the 1wL + 4dNT, 1wL + 4dACEI, and 1wL + 4dAT1RA groups (Figs. 3B and 4A), whereas no such positive cells were observed in the hearts from control rats (Fig. 3B). The number of TUNEL-positive cells was significantly greater in the 1wL + 4dACEI and 1wL + 4dAT1RA groups than in the 1wL + 4dNT group (Figs. 3B and 4A). The number of positive cells was comparable between the 1wL + 4dACEI and 1wL + 4dAT1RA groups.

DNA electrophoresis showed a clear degradation (180–200 bp) in the hearts from the 1wL + 4dACEI and 1wL + 4dAT1RA groups that was more marked than in those from the 1wL + 4dNT group, whereas no such degradation was observed in the hearts from control rats (Fig. 5).

Immunohistochemical double staining revealed that the fragmented DNA was colocalized with ED1-positive macrophages or α-SM actin-positive myofibroblasts but not with cardiomyocytes (Fig. 4B). Systolic arterial pressure was comparable between the control (135 ± 4 mmHg), 1wL + 4dNT (137 ± 4 mmHg), 1wL + 4dACEI (126 ± 5 mmHg), and 1wL + 4dAT1RA groups (133 ± 1 mmHg).

**DISCUSSION**

We demonstrated in the present study that treatment with the ACE inhibitor after 7 days of L-NAME administration inhibited subsequent arteriosclerosis of coronary arteries induced by inhibition of NO synthesis in rats. Our data suggest that enhanced disappearance of cells with inflammatory lesions due to apoptotic

![Figure 2](http://example.com/image2.png)

**Fig. 2.** Wall-to-lumen ratio in coronary arteries (A) and perivascular fibrosis (B) in control, 4wL-NAME, 1wL + 3wNT, and 1wL + 3wACEI groups. Wall-to-lumen (medial thickness-to-internal diameter) ratio and area of fibrosis (collagen deposition stained with aniline blue) immediately surrounding the blood vessels were calculated; i.e., perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. Values are means ± SE; n = 7. **P < 0.01 vs. control.

![Figure 3](http://example.com/image3.png)

**Fig. 3.** (A): number of TdT-mediated dUTP nick end-labeling (TUNEL)-positive cells in hearts from control, 1wL + 4dNT, 1wL + 4dACEI, and 1wL + 4dAT1RA groups. Data are expressed as the number of positive cells per heart cross-sectional area; n = 6. **P < 0.01 vs. control. ††P < 0.01 vs. 1wL + 4dNT. (B): number of monocyte antibody (ED1)-positive cells (solid bars), and α-smooth muscle (α-SM) actin-positive cells (open bars) in hearts from control, 1wL + 4dNT, 1wL + 4dACEI, and 1wL + 4dAT1RA groups. Data are expressed as the number of positive cells per heart cross-sectional area; n = 6. **P < 0.01 vs. control. ††P < 0.01 vs. 1wL + 4dNT.
cell death may be involved in the observed effects of the ACE inhibitor.

In this study, we showed that vascular inflammatory changes induced by 7 days of l-NAME administration (1wL + 3wNT group) result in arteriosclerosis at day 28. Such arteriosclerotic changes were comparable between the 1wL + 3wNT and 4wL-NAME groups. In addition, we had observed that rats did not develop vascular structural changes after the 1st wk of l-NAME administration (31) and that cotreatment with ACE inhibitors or AT1-receptor antagonists prevented l-NAME-induced early vascular inflammation and subsequent vascular structural changes (16, 30, 36). Thus these findings suggest that the early inflammatory changes are responsible for the development of arteriosclerosis in this study. Despite only 7 days of l-NAME administration, tissue ACE activity was increased in the 1wL + 3wNT group as much as in the 4wL-NAME group. The latter observation suggests that continuous activation of local ACE may contribute to the vascular structural changes seen in the 1wL + 3wNT group, because we previously reported that the early rise in local ANG II activity plays a key role in the development of early inflammation and subsequent arteriosclerosis in rats (16, 18, 33, 36).

We had demonstrated that simultaneous treatment with l-NAME and the ACE inhibitor inhibited the rat arteriosclerotic changes at day 28 (15, 30, 31). Thus we wanted to examine whether posttreatment with an ACE inhibitor could attenuate the arteriosclerotic changes in the present study. We found that ACE inhibition with imidapril after 7 days of l-NAME administration reversed such changes, indicating that ACE inhibition induced regression of the arteriosclerotic process. It is unlikely that the decrease in systolic blood pressure by the ACE inhibitor imidapril contributed to the inhibition of arteriosclerosis, because the systolic blood pressure was similar between the 1wL + 3wNT and 1wL + 3wACEI groups.

To explore mechanisms of the regression of arteriosclerosis, we examined the effect of inflammatory changes 4 days after ACE inhibitor treatment was started. We found that ACE inhibition markedly decreased the number of infiltrated monocytes and myofibroblasts as early as 4 days after the start of ACE inhibition. Thus it seems that ACE inhibition accelerated the disappearance of the inflammatory changes in this study. In addition, the number of cells with inflammatory lesions was comparable between the 1wL + 4dACEI and 1wL + 4dAT1RA groups, suggesting that inhibition of ANG II activity, mediated via the AT1 receptors, is responsible for the beneficial effects of the ACE inhibitor. Therefore, these results indicate that increased ANG II activity may be important not only for the acceleration of recruitment of monocytes/mac-

Fig. 4. A: micrograph of TUNEL-positive cells (arrowheads) in coronary arteries (a, c, and e) and myocardium (b, d, and f) from the 1wL + 4dNT (a and b), 1wL + 4dACEI (c and d), and 1wL + 4dAT1RA (e and f) groups. Scale bar, 50 μm. B: immunohistochemical double staining with TUNEL and monocyte antibody (ED1) and TUNEL and α-SM actin in hearts from the 1wL + 4dACEI group. a: Co-localization of TUNEL (brown nucleus) with ED1 antibody (red cytoplasm) in myocardial inflammatory lesions (arrow). b: Co-localization of TUNEL (brown nucleus) with α-SM actin antibody (red cytoplasm) in myocardial inflammatory lesions (arrows). Scale bar, 10 μm.

Fig. 5. Agarose gel electrophoresis of DNA extracted from the heart in the control (lane 1), 1wL + 4dNT (lane 2), 1wL + 4dACEI (lane 3), and 1wL + 4dAT1RA (lane 4) groups. Lane 5, 100-bp molecular marker. Gel is typical of 5 experiments.
nuclear factor-κB (12) or Akt/protein kinase B (29, 35).

Recent evidence suggests that ANG II may activate the macrophages and myofibroblasts were examined. Recent evidence suggests that ANG II may activate nuclear factor-κB (12) or Akt/protein kinase B (29, 35) in vascular cells. We previously showed that treatment with the AT1-receptor antagonist prevented the increase in cardiac nuclear factor-κB activity induced by chronic administration of L-NAME (36). Activation of nuclear factor-κB (2, 38) or Akt (5, 10, 17) has been shown to inhibit apoptosis. Thus it is possible that ACE inhibition or AT1-receptor blockade increased apoptotic cell death in cells with inflammatory lesions by inhibiting ANG II-induced antiapoptotic signals. Further studies are needed to prove this possibility.

In conclusion, we have shown that treatment with the ACE inhibitor after the development of vascular inflammation can inhibit arteriosclerosis in rats. Our data suggest that, in addition to previously reported proinflammatory actions, an antiapoptotic action on cells with inflammatory lesions induced by local ANG II activity through the AT1 receptor might contribute to the development of arteriosclerosis in rats. The observed effects of the ACE inhibitor may be mediated at least by increased disappearance of cells with inflammatory lesions due to apoptotic cell death. Thus it appears that endothelium-derived NO may promote apoptosis of inflammatory cells that infiltrate into the blood vessels by suppressing ANG II-induced antiapoptotic signals. Antiapoptosis-promoting effects on cells with inflammatory lesions can be added to a list of antiarteriosclerotic actions of ACE inhibitors.

The authors thank Dr. Shigeyuki Takeyama for suggestions to improve the manuscript.

This study was supported by Ministry of Education, Science, and Culture (Tokyo, Japan) Grants-in-Aid for Scientific Research 11470164, 11158216, 11557056, 10307019, and 10177226, the Ryouuchi Naito Foundation for Medical Research (Osaka, Japan), and a research grant from Kanae Foundation of Research for New Medicine (Osaka, Japan).

REFERENCES


