Pathogenic Role of Oxidative Stress in Vascular Angiotensin-Converting Enzyme Activation in Long-Term Blockade of Nitric Oxide Synthesis in Rats

Makoto Usui, Kensuke Egashira, Shiro Kitamoto, Masamichi Koyanagi, Makoto Katoh, Chu Kataoka, Hiroaki Shimokawa, Akira Takeshita

Abstract—Inhibition of nitric oxide (NO) synthesis with N\textsuperscript{\textdegree}-nitro-\textsuperscript{l}-arginine methyl ester (L-NAME) activates vascular angiotensin-converting enzyme (ACE) and causes oxidative stress. We investigated the role of oxidative stress in the pathogenesis of ACE activation in rats. Studies involved aortas of rats receiving no treatment, L-NAME, L-NAME plus l-arginine, or L-NAME plus an antioxidant drug (N-acetylcysteine, allopurinol, or ebselen) for 7 days. L-NAME significantly increased oxidative stress (O\textsuperscript{\textdegree}\textsuperscript{-}) and ACE activity. The increased O\textsuperscript{\textdegree} production was normalized by removal of endothelium. Immunohistochemistry showed the increased ACE activity in the endothelial layer. Treatment with antioxidant drugs did not affect the L-NAME–induced increase in systolic arterial pressure but did prevent increases in vascular O\textsuperscript{\textdegree} production and ACE activity. These results implicate oxidative stress in the pathogenesis of vascular ACE activation in rats with long-term inhibition of NO synthesis. The observed effects of antioxidant drugs on ACE activation do not appear to involve the hypertension induced by L-NAME. (Hypertension. 1999;34:546-551.)

Key Words: nitric oxide ■ stress, oxidative ■ anions ■ angiotensin-converting enzyme ■ remodeling

Reduced activity of endothelium-derived nitric oxide (NO) is a theme common to arteriosclerosis or atherosclerosis and its risk factors. \textsuperscript{1-8} Our group\textsuperscript{9-13} and others\textsuperscript{14} have reported that long-term administration of N\textsuperscript{\textdegree}-nitro-l-arginine methyl ester (L-NAME), an inhibitor of NO synthesis for 4 to 8 weeks, produces vascular structural changes (fibrosis and medial thickening) and myocardial remodeling (fibrosis and hypertrophy) in vivo in animal models. We also have found that a local renin-angiotensin system, particularly angiotensin-converting enzyme (ACE) activity, is upregulated during the first week of L-NAME administration\textsuperscript{10} and that either ACE inhibition or angiotensin II receptor blockade prevents such vascular and myocardial damage.\textsuperscript{10,11} These results support the hypothesis that a defect in NO synthesis may lead to local ACE activation and generation of angiotensin II, which in turn contributes to cardiovascular remodeling. Thus, local ACE activity is an important mediator of cardiovascular remodeling in this model. However, the mechanisms by which in vivo inhibition of NO synthesis activates vascular ACE remain to be elucidated.

Reciprocal regulation appears to exist between endothelial NO and ACE. Rieder et al\textsuperscript{15} have reported that fluid shear stress in vitro reduces ACE expression in endothelial cells in association with an increase in NO synthase activity. Hypertension\textsuperscript{16,17} and hypercholesterolemia\textsuperscript{18} are associated with decreased NO activity and increased oxidative stress in blood vessels, and ACE has been shown to be upregulated in such pathological conditions.\textsuperscript{19-21} A reduction in NO synthesis increases endothelial intracellular oxidative stress.\textsuperscript{22,23} Therefore, a defect in NO synthesis could activate local ACE via increased oxidative stress. However, solid evidence supporting such a claim is lacking.

To test the hypothesis that long-term inhibition of NO synthesis activates vascular ACE via oxidative stress in vivo, we examined the effects of antioxidant drugs on local ACE activation in the rat aorta.

Methods

Animal Model of Long-Term Inhibition of NO Synthesis and Antioxidant Treatment

The present experiments were reviewed and approved by the Committee on Ethics of Animal Experiments, Kyushu University Faculty of Medicine. Twenty-week-old male Wistar-Kyoto rats were housed singly in a pyrogen-free facility. Six groups of rats were studied. The first (control) group received untreated laboratory chow and drinking water. The second group (L) received L-NAME in the drinking water (1 mg/mL). At this concentration, the daily intake of L-NAME was 100 mg/kg per day.\textsuperscript{10,11} The third group (L+L-arg) received L-NAME and l-arginine (70 mg/mL) in its drinking water. The
fourth group (L+ A) received L-NAME in the drinking water and the xanthine oxidase inhibitor allopurinol (2.5 mg/g) in the chow. The fifth group (L+ E) received L-NAME in the drinking water and the antioxidant ebselen (2.5 mg/g) in the chow. Ebselen, a seleno-organic compound, has been shown to exert antioxidant activity through a glutathione peroxidase-like action.\textsuperscript{24,25} The sixth group (L+NAC) received L-NAME in the drinking water and a thiol-containing antioxidant, N-acetylcysteine (NAC), by intraperitoneal injection (200 mg/kg per day). The doses of L-arginine, allopurinol, ebselen, and NAC were determined empirically and were found to be effective in inhibiting superoxide anion (O\textsubscript{2}\textsuperscript{−}) production.

**Vessel Harvesting and Preparation**

On day 7 of treatment, we measured heart rate as well as systolic blood pressure by the tail-cuff method. The rats were anesthetized with intraperitoneally administered pentobarbital, and the chest was opened. With the heart still beating, heparin (150 IU) was given via intracardiac injection. The thoracic aorta was removed en bloc and placed in cold Krebs-Henseleit solution. Extravascular tissue was removed rapidly, and the vessel lumen was flushed with the solution. Then, in some rats the aorta was cut into three 5-mm ring segments that were used in studies of NO production, superoxide anion production, or histopathology and immunohistochemistry. In other rats, the entire block of thoracic aorta was used for measurement of ACE activity.

**Measurement of NO Production**

The 5-mm ring segments of the aorta were incubated in 2 mL of Hanks’ balanced salt solution containing a calcium ionophore A23187 (1 \(\mu\text{mol/L} \)) and L-arginine (100 \(\mu\text{mol/L} \)), as previously described.\textsuperscript{12} A chemiluminescence-based NO analyzer (270B, Sievers) was used to measure NO production. Specific NO-generating capacity was expressed as nanomoles per hour per dry weight.

**Measurement of Vascular Superoxide Anion Production**

A lucigenin chemiluminescence assay was used to measure O\textsubscript{2}\textsuperscript{−} levels in rat aortas.\textsuperscript{18} Lucigenin penetrates cell membranes and therefore can detect both intracellular and extracellular O\textsubscript{2}\textsuperscript{−}. The 5-mm ring segments of aorta were allowed to equilibrate in modified Krebs-HRPEPS buffer for 10 minutes at 37°C. Production of O\textsubscript{2}\textsuperscript{−} was measured with the use of a lucigenin (bis-N-methylacridinium nitrate; 250 \(\mu\text{mol/L} \))-enhanced chemiluminescence technique with a scintillation counter (Luminescence Reader BLR 301, Aloka). To test the specificity of the chemiluminescence reaction, counts were recorded after the intracellular superoxide scavenger tiron (4,5-dihydroxy-1,3-benzenedisulfonylic acid, 10 \(\mu\text{mol/L} \)) had been added to the vial. In all experiments, >90% of the chemiluminescence signals from the aortic rings were scavenged by tiron. Specific chemiluminescence signal was expressed as counts per minute minus the mean background counts. Signals from the aortic rings were calibrated with the use of known concentrations of xanthine and xanthine oxidase and reported as nanomoles per minute per dry weight. To assess endothelial O\textsubscript{2}\textsuperscript{−} production, the endothelium was removed from some aortic segments, as previously described.\textsuperscript{18}

**Measurement of Vascular ACE Activity**

Aortic tissue ACE activity was measured by fluorometric assay as described.\textsuperscript{10} Tissue ACE activity was calculated as nanomoles His-Leu generated per milligram tissue weight per hour.

**Histopathology and Immunohistochemistry**

For histopathology, the 5-mm ring segments of aorta were fixed for a few days with 6% formaldehyde solution and then dehydrated and embedded in paraffin. The aorta was transversely sectioned at a thickness of 5 \(\mu\text{m} \). Sections were mounted on slides and stained with hematoxylin and eosin for morphometric analysis. The thickness of media was measured with a Nikon microscope equipped with a video camera and an online computer. Ten aortic ring sections from each random animal were evaluated. The means of 3 separate measurements for each rat were used for analysis. For immunohistochemistry, paraffin-embedded sections (thickness, 5 \(\mu\text{m} \)) were preincubated with 3% skim milk to decrease nonspecific binding. Sections were incubated overnight at 4°C with affinity-purified antibodies against mouse anti-rat monocyte antibody (1 to 3 \(\mu\text{g/mL} \), ED1, Serotec), human von Willebrand factor (5 \(\mu\text{g/mL} \)), 9B9, Immunobiology Laboratories), or nonimmune mouse IgG (Zymed Laboratories). Biotinylated and affinity-purified goat anti-rabbit IgG was used as the secondary antibody. Avidin-biotin application was followed by incubation with the substrate (3',3'-diaminobenzine). As a final step, sections were counterstained with hematoxylin. The number of cells positive for monocyte antigen was counted per section. Ten sections were selected for each rat. The average number of positive cells per section was calculated.

**Statistical Analysis**

Data are expressed as mean±SEM. Differences between 2 experiments were compared by Student’s t tests. Differences between \(n\geq3\) experiments were determined by 2-way ANOVA and a Bonferroni’s multiple comparison test. A \(P\) value of \(\leq0.05\) was considered statistically significant.

**Results**

**Hemodynamic Parameters**

During the 7-day treatment period, the L group exhibited a significant rise in systolic arterial pressure compared with the control group (Table 1). Increases in systolic arterial pressure were similar in the L group and in the L+L-arg, L+ A, L+ E, and L+NAC groups (Table 1). While heart rate did not change significantly in the control group, a reduction in heart rate was seen in the L, L+L-arg, L+ A, L+ E, and L+NAC groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>Body Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>136±5</td>
<td>413±10</td>
<td>313±11</td>
</tr>
<tr>
<td>Day 7</td>
<td>139±4</td>
<td>410±12</td>
<td>326±10</td>
</tr>
<tr>
<td>L group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>145±2</td>
<td>414±9</td>
<td>322±5</td>
</tr>
<tr>
<td>Day 7</td>
<td>172±4†</td>
<td>332±14†</td>
<td>322±6</td>
</tr>
<tr>
<td>L+L-arg group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>136±3</td>
<td>426±10</td>
<td>314±7</td>
</tr>
<tr>
<td>Day 7</td>
<td>161±5†</td>
<td>310±11†</td>
<td>318±8</td>
</tr>
<tr>
<td>L+NAC group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>130±3</td>
<td>424±14</td>
<td>314±10</td>
</tr>
<tr>
<td>Day 7</td>
<td>171±3†</td>
<td>344±19†</td>
<td>320±10</td>
</tr>
<tr>
<td>L+ A group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>130±3</td>
<td>424±14</td>
<td>314±10</td>
</tr>
<tr>
<td>Day 7</td>
<td>168±3†</td>
<td>344±19†</td>
<td>320±10</td>
</tr>
<tr>
<td>L+E group (n=12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>132±3</td>
<td>426±14</td>
<td>314±10</td>
</tr>
<tr>
<td>Day 7</td>
<td>166±5†</td>
<td>355±22†</td>
<td>324±10</td>
</tr>
</tbody>
</table>

Table 1. Hemodynamic Parameters

Data are mean±SE. *\(P<0.01\) vs control group. †\(P<0.01\) vs day 0.
Aortic NO and O\textsubscript{2}\textsuperscript{-} Production

NO production was much lower in the L group than in the control group (Figure 1). Removal of the endothelium markedly decreased aortic NO production in the control group, to the level shown by the L group with intact endothelium. Removal of endothelium did not affect NO production in the L group. Treatment with L-arginine attenuated the L-NAME–induced decrease in NO production (Figure 2). Treatment with NAC, allopurinol, or ebselen did not affect the L-NAME–induced decrease in NO production.

Production of O\textsubscript{2}\textsuperscript{-} in the aortic segments with intact endothelium was greater in the L group than in the control group (Figure 1). In the L group segments without endothelium, O\textsubscript{2}\textsuperscript{-} production was similar to that in the control group segments without endothelium (Figure 1). Treatment with L-arginine reduced the L-NAME–induced decrease in O\textsubscript{2}\textsuperscript{-} production (Figure 2). Treatment with NAC, allopurinol, or ebselen did not affect the L-NAME–induced decrease in O\textsubscript{2}\textsuperscript{-} production.

Effects of Antioxidant Drugs or L-Arginine on Aortic O\textsubscript{2}\textsuperscript{-} Production and ACE Activity

Aortic ACE activity was significantly increased in the L group compared with the control group (Figure 3). Treatment with NAC, allopurinol, or ebselen also prevented the L-NAME–induced increases in aortic ACE activity (Figure 3).

Histopathology and Immunohistochemistry

No morphometrically evident differences in medial thickness occurred among the aortas in the 6 groups (Table 2). When leukocytes were examined with the use of immunohistochemistry, the number of ED1-positive monocytes infiltrating the intima did not significantly differ among the 6 groups (Table 2).

Immunostaining for ACE or von Willebrand factor was performed in the 6 groups (Figure 4). In the control and L+L-arg groups, ACE immunoreactivity was weakly present in the intimal layer of the aorta. In the L group, the intimal layer was intensely immunoreactive to ACE antibody. Von Willebrand factor immunoreactivity was present to the same extent in the intima of all 6 groups. No immunoreactivity was noted when ACE or von Willebrand factor antibody was
Long-term inhibition of NO synthesis also was found to increase endothelial generation of \( \text{O}_2^- \). This inhibition of NO synthesis increases intracellular oxidative stress in endothelial cells in vitro.22,23 Several novel findings emerged from this study. First, long-term inhibition of NO synthesis increased aortic \( \text{O}_2^- \) generation. Second, antioxidant therapy prevented the increase in vascular ACE activity. These findings suggest an important role of oxidative stress in the pathogenesis of vascular ACE activation in this animal model.

**Increased \( \text{O}_2^- \) Production After Inhibition of NO Synthesis**

Endothelial NO production in the aorta was greatly reduced after 7 days of L-NAME administration. This inhibition of NO production was reversed by treatment with L-arginine. Long-term inhibition of NO production also was found to increase endothelial generation of \( \text{O}_2^- \) in the aorta. This extends prior observations that short-term (0.5 to 4 hours) inhibition of NO synthesis increases intracellular oxidative stress in endothelial cells in vitro and in vivo.22,23

In diseased blood vessels, \( \text{O}_2^- \) may be overproduced by the endothelium,18 smooth muscle cells,16,27 adventitial fibroblasts,28 or inflammatory cells that have migrated to the vessel.29 Recently, Kato et al30 reported that long-term administration of L-NAME for 18 days increased medial thickness in the rat aorta and monocyte infiltration into the intima. Luvara et al31 reported that blockade of NO synthesis for 4 weeks induced a proinflammatory phenotype (expression of adhesion molecules) in the aortic wall. However, no significant increase in medial thickness or monocyte infiltration was found in the present study. The different observations can be explained by the more limited duration (7 days) of L-NAME administration in the present study. Thus, monocytes in the intima are not likely to have contributed to overproduction of \( \text{O}_2^- \) in the present results. Several oxidase systems in the blood vessel wall can generate \( \text{O}_2^- \).32 Recent evidence suggests that NO may downregulate xanthine oxidase or NADPH oxidase gene expression and activity.33–35 However, investigation of the mechanism of increased \( \text{O}_2^- \) production after blockade of NO synthesis is beyond the scope of the present study.

At least 3 caveats are important in interpreting our present observation. First, lucigenin itself has been reported to generate \( \text{O}_2^- \) in a cell-free system.36 Using electron-spin resonance, however, we could not detect \( \text{O}_2^- \) production when lucigenin was added to rat aortic tissues (M. Usui et al, unpublished data, 1998). In addition, we tested the specificity of the chemiluminescence reaction using the superoxide scavenger tiron in all experiments. Thus, auto-oxidation of lucigenin is unlikely to have contributed materially to our chemiluminescence data. Second, Miller et al37 have found lucigenin to detect \( \text{O}_2^- \) within the endothelium but to be less sensitive in measuring \( \text{O}_2^- \) throughout the wall thickness of the rabbit aorta. Thus, we cannot exclude the possibility that lucigenin detected \( \text{O}_2^- \) generated mostly by the endothelium rather than by smooth muscle cells in the media. Third, the methodology used to detect NO in this study does not distinguish free NO from a variety of nitrosylated compounds.

**Role of \( \text{O}_2^- \) in the Mechanism of Local ACE Activation**

Immunohistochemistry demonstrated increased ACE activity in the intima (possibly in endothelial cells) of aorta in the L group. This localized ACE activation was prevented by treatment with L-arginine, suggesting NO regulation of local ACE activity in vivo. Importantly, antioxidant drugs used in this study, including allopurinol (a xanthine oxidase inhibitor), ebselen (a seleno-organic free radical scavenger), and NAC (a thiol-containing free radical scavenger), were found to prevent increases in ACE activity. These findings suggest that oxidative stress is important in the pathogenesis of vascular tissue ACE activation in the rat aortic model.

**TABLE 2. Medial Thickness and Immunohistochemically Demonstrated Inflammation in Rat Aorta**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>L Group</th>
<th>L+L-arg Group</th>
<th>L+NAC Group</th>
<th>L+A Group</th>
<th>L+E Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial thickness, ( \mu \text{m} )</td>
<td>94±6</td>
<td>100±3</td>
<td>97±6</td>
<td>93±4</td>
<td>99±3</td>
<td>99±3</td>
</tr>
<tr>
<td>ED1-positive monocytes per section</td>
<td>0.2±0.3</td>
<td>0.4±0.3</td>
<td>0.4±0.2</td>
<td>0.5±0.3</td>
<td>0.4±0.2</td>
<td>0.4±0.3</td>
</tr>
</tbody>
</table>

Data are mean±SEM.
ACE has been shown to be induced by fibroblast growth factor,\(^\text{38}\) endothelin-1,\(^\text{39}\) or protein kinase C\(^\text{40}\) in vitro. Because these growth-promoting factors are upregulated after L-NAME administration in rat hearts and vessels (M.U. et al, unpublished data, 1998), they might contribute to the pathogenesis of vascular ACE activation in our model. Recent evidence suggests that reactive oxygen species such as O\(_2^-\) act as intracellular second messengers in response to such growth-promoting factors.\(^\text{32}\) Further studies are needed to elucidate the molecular and cellular mechanism by which oxidative stress increases local ACE activity after chronic blockade of NO synthesis.

Conclusions

Our present findings suggest that ACE is upregulated by redox-sensitive mechanisms in the rat aorta induced by inhibition of NO synthesis in vivo. The observed effects of antioxidants appear independent of the arterial hypertension induced by L-NAME. These data should provide new insight into the mechanism of the ACE activation under conditions of deficient NO synthesis in vivo. Deficient endothelium-derived NO synthesis may result in vascular remodeling in concert with local angiotensin II activity and/or oxidative stress.

Acknowledgments

This study was supported by grants-in-aid for scientific research (10307019, 10177226, 09877136, 08457212) from the Ministry of Education, Science, and Culture, Tokyo, Japan; by the Ryouichi Naito Foundation for Medical Research, Osaka, Japan; and by a research grant from Kimura Memorial Foundation, Osaka, Japan.

References


Figure 4. Immunohistochemistry in the rat aorta. Shown are sections stained with ACE antibody in a control rat (top left), an L group rat (top middle), and an L + L-arg group rat (top right) and sections stained with von Willebrand factor (vWF) antibody in a control rat (bottom left), an L group rat (bottom middle), and an L + L-arg group rat (bottom right). Bar=50 μm.


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Hypertension. 1999;34:546-551
doi: 10.1161/01.HYP.34.4.546

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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