Endothelium-Derived Hydrogen Peroxide Accounts for the Enhancing Effect of an Angiotensin-Converting Enzyme Inhibitor on Endothelium-Derived Hyperpolarizing Factor–Mediated Responses in Mice

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Background—We have recently identified that endothelium-derived hydrogen peroxide (H$_2$O$_2$) is an endothelium-derived hyperpolarizing factor (EDHF) in animals and humans, for which endothelial nitric oxide synthase (eNOS) is an important source. Angiotensin-converting enzyme (ACE) inhibitors are known to enhance EDHF-mediated responses. In this study, we examined whether endothelium-derived H$_2$O$_2$ accounts for the enhancing effect of an ACE inhibitor on EDHF-mediated responses and, if so, what mechanism is involved.

Methods and Results—Control and eNOS$^{-/-}$ mice were maintained with or without temocapril (10 mg/kg per day orally) for 4 weeks, and isometric tensions and membrane potentials of mesenteric arteries were recorded. In control mice, temocapril treatment significantly enhanced EDHF-mediated relaxations and hyperpolarizations to acetylcholine (n=8 each). Catalase, a specific scavenger of H$_2$O$_2$, abolished the beneficial effects of temocapril, although it did not affect endothelium-independent relaxations to sodium nitroprusside or NS1619, a direct opener of K$_{Ca}$ channels (n=6 each). Western blot analysis demonstrated that the temocapril treatment significantly upregulated the expression of eNOS. By contrast, this enhancing effect of temocapril was absent in eNOS$^{-/-}$ mice (n=6).

Conclusions—These results indicate that endothelium-derived H$_2$O$_2$ accounts for the enhancing effect of temocapril on EDHF-mediated responses caused in part by eNOS upregulation, further supporting our H$_2$O$_2$ theory.

Key Words: angiotensin-converting enzyme inhibitors ■ endothelium-derived factors ■ nitric oxide synthase ■ vasodilation

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors, including prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF). It is widely accepted that EDHF plays an important role, especially in microvessels. There are several candidates for EDHF, including epoxyeicosatrienoic acids, gap junctions, K$^+$ ions, and, as we have recently identified, hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ induces vascular relaxation and hyperpolarization.

Angiotensin-converting enzyme (ACE) inhibitors are known to improve endothelial dysfunction in diabetes mellitus, aging, and patients with atherogenic risk factors. ACE inhibitors are also known to enhance EDHF-mediated endothelium-dependent relaxation even in normal arteries. However, the nature of EDHF involved in the beneficial effects of ACE inhibitors remains to be elucidated. In this study, we thus examined whether endothelium-derived H$_2$O$_2$ accounts for the enhancing effect of an ACE inhibitor on EDHF-mediated responses and, if so, what mechanism is involved to obtain further supporting evidence for our H$_2$O$_2$ theory.

Materials and Methods

This study was reviewed by the Committee of Ethics on Animal Experiments of Kyushu University and was performed according to the Guidelines for Animal Experiments of Kyushu University and of the Japanese Government.

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Animal Preparation and Vessel Isolation

A total of 98 female C57BL/6N mice, obtained from a colony at Kyushu University were used. Female mice were used because of their availability in our institute. They were treated with oral administration of vehicle, temocapril (10 mg/kg per day) or hydralazine (20 mg/kg per day) for 4 weeks, from 12 to 16 weeks of age, in their drinking water. At the end of each treatment, body weight and systolic blood pressure were measured. Systolic blood pressure was measured by tail-cuff method under conscious conditions.3,4,14

Then, the animals were anesthetized with pentobarbital (50 mg/kg, intraperitoneal) and euthanized. The mesenteric arteries were carefully isolated and were cut into 1-mm and 1.5-mm rings for the measurements of both isometric tensions and membrane potentials.3,4 To examine endothelium-independent responses, some arterial rings were stripped of the endothelium by gently rubbing. Twelve male endothelial nitric oxide synthase-deficient (eNOS−/−) mice with the same age,4 were also treated with oral administration of either vehicle or temocapril (10 mg/kg per day) for 4 weeks and used for Western blot analysis and isometric tension recordings.

Organ Chamber Experiments

Experiments were performed in 37°C Krebs solution bubbled with 95% O2 and 5% CO2. Arterial rings were mounted and isometric tension was measured by a force transducer (Nihon Kohden Co). Rings were precontracted with prostaglandin F2α or KCl. The extent of precontraction was adjusted to ~50% to 70% of the contractions induced by 62 mmol/L KCl.4,14 Endothelium-dependent relaxations to acetylcholine (ACh) and endothelium-independent relaxation to sodium-nitroprusside (SNP) or NS-1619, an opener of calcium-activated K channels (KCa), were examined.3,4,22 The contribution of vasodilator prostaglandins, NO, and EDHF to ACh-induced endothelium-dependent relaxation was determined by the inhibitory effects of indomethacin (10 μmol/L), Nω-nitro-L-arginine (L-NNa) (100 μmol/L), and KCl (30 to 50 mmol/L), respectively, by using area under the relaxation curves.3,4,14,21 We confirmed that coincubation of carboxy-PTIO (300 μmol/L), a scavenger of NO, with indomethacin and L-NNa did not further inhibit NO-mediated relaxation, indicating that the concentration of L-NNa was sufficient to completely inhibit NO-mediated relaxation (data not shown). To examine the involvement of endothelium-derived H2O2 in the EDHF-mediated responses, the inhibitory effect of catalase, a specific scavenger of H2O2, was examined.4,10–12.14 The effect of inactivated catalase by aminotriazole (50 mmol/L) was also examined to examine the specificity of catalase.4,10–12

Electrophysiological Experiments

The rings of small mesenteric arteries were placed in experimental chambers perfused with 37°C Krebs solution containing indomethacin (10 μmol/L) and L-NNa (100 μmol/L) bubbled with 95% O2 and 5% CO2. A fine glass capillary microelectrode was impaled into the smooth muscle from the adventitial side.3,14 Changes in membrane potentials produced by ACh were continuously recorded.4,14

Measurements of Serum Concentrations of Temocaprilat and Estradiol

Blood samples were collected for measurements of serum concentrations of temocaprilat, an active form of temocapril, with an inhibitor-binding assay using high-performance liquid chromatography22,23 and those of estradiol by radioimmunoassay. We centrifuged the blood samples for 10 minutes at 4°C and stored at −20°C until the measurements.

Western Blot Analysis

Western blot analysis for eNOS and Cu/Zn-SOD protein was performed using an antibody that specifically recognizes them.3,14 The same amount of extracted protein from whole tissue of mesenteric arteries was loaded for SDS-PAGE/immunoblot analysis. Each band was normalized by corresponding value of β-actin as an internal control.24

SOD Activity

Cu/Zn-SOD activity of mesenteric arteries was measured by nitroblue tetrazolium method, as previously described.14,25

Catalase Activity

Catalase activity of mesenteric arteries was examined by automated assay based on the peroxidatic activity of the enzyme, as previously described.14,26

Glutathione Peroxidase Activity

Glutathione peroxidase activity of mesenteric arteries was determined by oxidation of NADPH in the presence of glutathione reductase, as previously described.14

Drugs

ACh, indomethacin, L-NNa, SNP, NS1619, hydralazine hydrochloride, catalase, and aminotriazole were purchased from Sigma Chemical Co (St. Louis, Mo). Temocapril was a gift from Sankyo Pharmaceutical Co (Tokyo, Japan).

Statistical Analysis

Results are expressed as mean±SEM. Throughout the text, n represents the number of animals tested. Dose–response curves were analyzed by 2-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons. Other values were analyzed by unpaired Student t test or 1-way ANOVA. P<0.05 was considered to be statistically significant.

Results

Body Weight and Systolic Blood Pressure

After 4 weeks of maintenance, there was no significant difference in body weight (grams) among the control, temocapril, and hydralazine groups (22.2±0.5, 22.6±1.3, and 22.6±1.4, respectively). Systolic blood pressure (mm Hg) was significantly decreased in the temocapril group compared with the control group (92±3 versus 116±3; P<0.001). In the hydralazine group, systolic blood pressure was decreased to the same extent in the temocapril groups (93±3; P<0.001 versus control group).

Serum Concentrations of Temocaprilat and Estradiol

Serum concentrations of temocaprilat were detected only in the temocapril group (116±22.8 ng/mL, the detection level was 2 ng/mL). Serum concentrations of estradiol (pg/mL) were comparable among the control, temocapril, and hydralazine groups (12.8±0.7, 13.7±1.7, 12.9±0.8, respectively).

Endothelium-Dependent Relaxation

In the control group, endothelium-dependent relaxations to ACh of mesenteric arteries were sensitive to L-NNa (in the presence of indomethacin) and to KCl (in the presence of indomethacin and L-NNa), indicating an important role of NO and EDHF, respectively (Figure 1A and 1D). In the temocapril group, EDHF-mediated relaxations to ACh (in the presence of indomethacin and L-NNa) were significantly enhanced compared with the control group (Figure 1B and 1D). By contrast, the treatment with hydralazine did not enhance the EDHF-mediated relaxations (Figure 1C and 1D). The contribution of vasodilator prostaglandins was insignificant in all the groups (Figure 1).
Catalase largely inhibited the EDHF-mediated relaxation in the control and the temocapril groups, but when inactivated by aminotriazole, catalase lost its inhibitory effect, indicating a substantial involvement of H$_2$O$_2$ in the enhancing effect of temocapril on the EDHF-mediated relaxations (Figure 2).

Endothelium-Dependent Hyperpolarization

Resting membrane potentials were significantly more negative in the temocapril group than in the control group, and the ACh-induced hyperpolarization was significantly larger in the temocapril group (Figure 3A and 3B). The enhancing effect of temocapril on the ACh-induced hyperpolarization was markedly inhibited by catalase (Figure 3B). There was no significant difference in the resting potential or the ACh-induced hyperpolarization between the control and the hydralazine groups (Figure 3A and 3B).

Figure 1. Enhancing effect of temocapril on EDHF-mediated relaxation of mouse mesenteric arteries. Endothelium-dependent relaxation to ACh in the control (A), temocapril (B), and hydralazine (C) groups and the relative contribution of vasodilator prostaglandins, NO, and EDHF to the ACh-induced relaxations in the 3 groups (D). EDHF-mediated relaxations to ACh (in the presence of indomethacin and L-NNA) were enhanced in the temocapril group but not in the hydralazine group. Results are expressed as means±SEM.

Figure 2. Involvement of H$_2$O$_2$ in the enhancing effect of temocapril on the EDHF-mediated relaxations. Catalase (6250 U/mL) markedly inhibited the EDHF-mediated relaxation in both the control and temocapril groups, whereas it lost its inhibitory effect when inactivated by aminotriazole (50 mmol/L). Results are expressed as means±SEM.

Figure 3. Enhancing effect of temocapril on the EDHF-mediated hyperpolarizations under resting conditions (A) and in response to ACh (B). Results are expressed as means±SEM.

Endothelium-Independent Relaxation

There was no significant difference in the endothelium-independent relaxations to SNP or NS1619 between the control and the temocapril groups (Figure 1, available online at http://atvb.ahajournals.org). Furthermore, those relaxations to SNP or NS1619 were unaltered in the presence of indomethacin, L-NNA, or catalase (data not shown).

Protein Expression of eNOS and Cu/Zn-SOD

The expression of eNOS protein in the mesenteric arteries was significantly higher in the temocapril group compared with the control group (Figure 4A), whereas the expression of Cu/Zn-SOD protein was comparable between the 2 groups (Figure 4B).

Enzyme Activities

The vascular activity of Cu/Zn-SOD, catalase, or glutathione peroxidase was comparable between the control and the temocapril groups (Figure II, available online at http://atvb.ahajournals.org).

Endothelium-Dependent Relaxations in eNOS$^{-/-}$ Mice

After 4 weeks of maintenance, there was no significant difference in body weight (grams) between the control (untreated) and the temocapril-treated eNOS$^{-/-}$ mice (25.7±1.2 and 22.3±0.9, respectively). The treatment with
Enhancing Effect of Temocapril on EDHF-Mediated Responses in Normal Mice

The major findings of the present study were as follows: (1) the long-term treatment with temocapril significantly enhanced EDHF-mediated relaxations and hyperpolarizations to ACh of mouse mesenteric arteries; (2) the enhancing effects of temocapril on the EDHF-mediated responses were mediated primarily by H2O2; (3) the enhanced expression of eNOS may be involved in the effect of temocapril, whereas vascular enzyme activities, including those of Cu/Zn-SOD, catalase, or glutathione peroxidase, were unaltered by the temocapril treatment; and (4) the enhancing effects of temocapril on the EDHF-mediated responses were not observed in eNOS−/− mice. These results demonstrate that endothelium-derived H2O2 accounts for the enhancing effect of an ACE inhibitor on the EDHF-mediated responses, further confirming our EDHF/H2O2 theory.4,10-12,14

Discussion

The mechanisms of the beneficial effects of ACE inhibitors include an inhibition of bradykinin degradation,28 suppression of oxidative stress,29,30 and enhancement of eNOS activity.31,32 ACE inhibitors upregulate eNOS mRNA expression that may result from the inhibition of kininase II that catalyzes the degradation of bradykinin.31 In the present study, the expression of eNOS protein in the temocapril group was significantly enhanced (∼1.4-fold) as compared with the control group. This is consistent with the previous study in which eNOS protein expression was significantly enhanced in nondiabetic rats with an ACE inhibitor.33

ACE inhibitors are known to increase eNOS expression and its activity via increasing bradykinin content,30 and a bradykinin B2 receptor antagonist, HOE-140, inhibits the effect of ACE inhibitors even in normal animals.35 It is reported that the vascular effects of temocapril are also mediated through the bradykinin pathway.36 In the present study, because eNOS expression was increased and this process is resistant to L-arginine analogues,37 eNOS consists of 2 domains, oxygenase and reductase domains, and the coupling of the 2 domains is not so tight as compared with the other 2 isoforms, nNOS and iNOS.37 Thus unlike nNOS or iNOS, eNOS produces superoxide anions from reductase domain even under physiological conditions, and this process is resistant to L-arginine analogues.37 eNOS produces superoxide anions from the oxygenase domain only

Figure 5. Effect of temocapril on EDHF-mediated relaxations of rat mesenteric arteries.27 Because we used female mice in the present study because of the availability of animals, we measured serum concentrations of estradiol and confirmed that the concentrations were comparable among the control, temocapril, and hydralazine groups. Therefore, it is unlikely that the enhanced EDHF-mediated relaxation in the temocapril group was caused by menstrual cycle of the animals or to the increased concentration of estradiol by temocapril. Because in the temocapril group catalase markedly inhibited the enhanced EDHF-mediated responses and inactivated catalase lost its inhibitory effect, increased endothelial production of H2O2 appears to play a primary role in the enhancing effects of temocapril on the EDHF-mediated responses.

Possible Mechanism Involved in the Enhancing Effect of an ACE Inhibitor on EDHF-Mediated Responses

Long-term treatment with an ACE inhibitor temocapril is known to improve endothelial dysfunction in diabetes mellitus,15 aging,16 and hypertension.17 It has been reported that the mechanisms of the beneficial effects of ACE inhibitors include an inhibition of bradykinin degradation,28 suppression of oxidative stress,29,30 and enhancement of eNOS activity.31,32 ACE inhibitors upregulate eNOS mRNA expression that may result from the inhibition of kininase II that catalyzes the degradation of bradykinin.31 In the present study, the expression of eNOS protein in the temocapril group was significantly enhanced (≈1.4-fold) as compared with the control group. This is consistent with the previous study in which eNOS protein expression was significantly enhanced in nondiabetic rats with an ACE inhibitor.33

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when the uncoupling of the 2 domains is accelerated by tetrahydrobiopterin or L-arginine deficiency, the process of which is sensitive to L-arginine analogues. Because we examined endothelial function under physiological conditions with and without an ACE inhibitor treatment, it is not surprising that endothelial production of superoxide is resistant to L-arginine analogues. Thus, eNOS could serve as a source of H₂O₂ even in the presence of L-NNA. In our previous study, we confirmed that endothelial production of H₂O₂ was unaffected by indomethacin or L-NNA. Thus, H₂O₂ derived from eNOS can be synthesized and act as an EDHF in the presence of indomethacin and L-NNA. It is thus conceivable that H₂O₂ released from upregulated eNOS by an ACE inhibitor is able to cause vasodilatation and hyperpolarization.

Heat shock protein 90 (hsp90), one of the eNOS-associated proteins, is known to be coupled with eNOS for NO generation. Because inhibition of hsp90 is sufficient to uncouple eNOS activity and lead to increased superoxide generation, it remains to be examined whether this mechanism is involved in the present finding with temocapril.

To confirm the involvement of eNOS upregulation in the effect of an ACE inhibitor, we further examined the effect of temocapril in eNOS−/− mice. In those mice, there was no eNOS protein expression in control or temocapril-treated animals. Importantly, temocapril failed to enhance EDHF-mediated relaxations in those mice, indicating that the enhancing effect of temocapril on EDHF-mediated responses is mediated, at least in part, by eNOS upregulation by the ACE inhibitor.

Recently, we have demonstrated that endothelial Cu/Zn-SOD plays an important role in producing H₂O₂ as an EDHF synthase in mouse mesenteric arteries. In the present study, we found no significant difference in the expression of Cu/Zn-SOD or its activity, as well as other vascular enzyme activities. H₂O₂ might be produced not only by SOD but also by spontaneous conversion from superoxide, and this point remains to be examined in future studies.

ACE inhibitors are known to have radical scavenging effects. Captopril, for example, has the protective action on endothelium-dependent relaxations, at least in part, by eNOS upregulation by the ACE inhibitor. Temocapril, for example, has the protective action on endothelium-dependent and -independent coronary arteriolar dilation: role of cyclooxygenase and potassium channels. Am J Physiol Heart Circ Physiol. 2003;285:H2255–H2263.

Clinical Implications
EDHF plays an important role in human arteries to maintain endothelium-dependent relaxations, especially in patients with multiple risk factors. ACE inhibitors are now widely used for the treatment of patients with hypertension, acute and chronic heart failure, myocardial infarction, and diabetes mellitus. ACE inhibitors also prevent endothelial dysfunction in patients with coronary artery disease. In the present study, the serum concentration of temocaprilat in mice was equivalent to that of elderly patients receiving daily dose of temocapril. We and others have demonstrated that endothelium-derived H₂O₂ is an EDHF in human mesenteric and coronary arteries. Thus, our present findings may contribute to our better understanding of the beneficial effects of ACE inhibitors on EDHF/H₂O₂-mediated responses in the clinical setting.

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References


Mediated Responses in Mice
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