Renin-Angiotensin System Blockade Improves Endothelial Dysfunction in Hypertension

Kenichi Goto, Koji Fujii, Uran Onaka, Isao Abe, Masatoshi Fujishima

Abstract——Angiotensin-converting enzyme (ACE) inhibitor improves the impaired hyperpolarization and relaxation to acetylcholine (ACh) via endothelium-derived hyperpolarizing factor (EDHF) in arteries of spontaneously hypertensive rats (SHR). We tested whether the angiotensin type 1 (AT₁) receptor antagonist also improves EDHF-mediated responses and whether the combined AT₁ receptor blockade and ACE inhibition exert any additional effects. SHR were treated with either AT₁ receptor antagonist TCV-116 (5 mg · kg⁻¹ · d⁻¹) (SHR-T), enalapril (40 mg · kg⁻¹ · d⁻¹) (SHR-E), or their combination (SHR-T&E) from 8 to 11 months of age. Age-matched, untreated SHR (SHR-C) and Wistar Kyoto (WKY) rats served as controls (n=8 to 12 in each group). Three treatments lowered blood pressure comparably. EDHF-mediated hyperpolarization to ACh in mesenteric arteries in the absence or presence of norepinephrine was significantly improved in all treated SHR. In addition, the hyperpolarization in the presence of norepinephrine was significantly greater in SHR-T&E than in SHR-E (ACh 10⁻³ mol/L with norepinephrine: SHR-C –7; SHR-T –19; SHR-E –15; SHR-T&E –22; WKY –14 mV). EDHF-mediated relaxation, assessed in the presence of indomethacin and N⁵-nitro-L-arginine, was markedly improved in all treated SHR. Hyperpolarization and relaxation to leverocimakalim, a direct opener of ATP-sensitive K⁺-channel, were similar in all groups. These findings suggest that AT₁ receptor antagonists are as effective as ACE inhibitors in improving EDHF-mediated responses in SHR. The beneficial effects of the combined AT₁ receptor blockade and ACE inhibition appear to be for the most part similar to those of each intervention. (Hypertension. 2000;36:575-580.)

Key Words: endothelium-derived factors ■ angiotensin ■ arteries ■ hypertension ■ drug therapy

Endothelial cells play an important role in the regulation of vascular tone through the release of relaxing factors such as nitric oxide (NO),³ prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF).²–⁴ EDHF relaxes underlying smooth muscle cells by producing membrane hyperpolarization through the opening of K⁺ channels.⁵–⁷ The identity of EDHF is yet to be determined, but possible candidates include cytochrome P450-derived arachidonic acid metabolites,⁸,⁹ K⁺ itself,¹⁰ or the electrical couplings via gap junctions.¹¹ Endothelium-dependent relaxation is impaired in hypertension.⁷,¹²–¹⁴ Although the mechanisms for this impairment seem to vary, it has been shown that the impaired EDHF-mediated hyperpolarization partly accounts for the decreased endothelium-dependent relaxation in mesenteric arteries of adult spontaneously hypertensive rats (SHR).⁷,¹⁴ Furthermore, antihypertensive treatment with either the angiotensin-converting enzyme (ACE) inhibitor enalapril or a combination of hydralazine and hydrochlorothiazide restores EDHF-mediated responses.¹⁵ In addition, enalapril tends to be more beneficial than the traditional combination therapy, despite a comparable blood pressure reduction,¹⁵ which raises the possibility that the renin-angiotensin system blockade, in addition to lowering blood pressure, may also be important in reversing endothelial dysfunction in hypertension.¹⁶

Although both the angiotensin type 1 (AT₁) receptor antagonist and ACE inhibitor lower blood pressure by blocking the renin-angiotensin system,¹⁷ each agent has some specific properties:¹⁸–²¹ eg, bradykinin accumulation by ACE inhibitors and possible stimulation of angiotensin type 2 (AT₂) receptors with AT₁ receptor antagonists. Furthermore, several clinical studies have demonstrated the beneficial effects of a combination of ACE inhibitors and AT₁ receptor antagonists.²²–²⁴ Although several studies have found that AT₁ receptor antagonists as well as ACE inhibitors improve endothelial function in hypertension,²⁵,²⁶ no study has evaluated the effects of AT₁ receptor antagonists on EDHF-mediated hyperpolarization per se, and little is known as to the effects of the combination of ACE inhibitor and AT₁ receptor antagonist on endothelial function. The present study tested whether the treatment of SHR with AT₁ receptor antagonists can also improve EDHF-mediated responses and whether the combination of an AT₁ receptor antagonist and an ACE inhibitor exerts any additional effects on endothelial function.

Methods

Handling of Animals
This study was approved by the Committee on the Ethics of Animal Experimentation of the Kyushu University (Fukuoka, Japan). Male
SHR/Izm and age-matched Wistar-Kyoto rats (WKY)/Izm (Disease Model Cooperative Research Association, Kyoto, Japan) were fed a standard rat chow and had free access to tap water. At the age of 8 to 9 months, SHR were assigned to 1 control (SHR-C) and 3 treatment groups. The SHR were treated with either TCV-116 (Takeda) 5 mg·kg⁻¹·d⁻¹ (SHR-T), an AT₁ receptor antagonist, or enalapril (Sigma Chemical Co) 40 mg·kg⁻¹·d⁻¹ (SHR-E), or their combination (SHR-T&E) for 3 months. All drugs were administered through drinking water. Water intake was checked 3 times a week, and drug concentrations were adjusted to achieve the daily doses that are listed above. The dose of each agent was based on preliminary experiments in which the blood pressures of SHR were lowered to comparable extents. Untreated WKY served as normotensive controls. There were 8 to 12 rats in each group.

Systolic blood pressure was measured in conscious rats by the tail-cuff method before and at the end of the treatment. The drugs were withdrawn 2 days before the experiments. The rats were anesthetized with ether, and they were killed by decapitation. The main branch of the mesenteric artery was excised and bathed in cold Krebs solution that had the following composition (in mmol/L): Na⁺ 137.4, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.5, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134, and glucose 11.5. The artery was cut into rings of 3 mm and 1.2 mm for the electrophysiological and tension experiments, respectively.

Electrophysiological Experiments
Transverse strips cut along the longitudinal axis of the rings were placed in the experimental chamber with the endothelial layer up. Tissues were carefully pinned to the rubber base attached to the bottom of the 2-mL chamber and superfused with 36°C Krebs solution aerated with 95% O₂ -5% CO₂ (pH 7.3 to 7.4) at the rate of 3 mL/min. After equilibration for at least 60 minutes, the membrane potentials of the vascular smooth muscle cells were recorded, as described previously. Briefly, conventional glass capillary microelectrodes filled with 3 mol/L KCl and with a tip resistance of 50 to 80 mol/LΩ were inserted into the smooth muscle cell from the endothelial side. Electrical signals were amplified through an amplifier (MEZ-7200, Nihon Koden), monitored on an oscilloscope (VC-11, Nihon Koden), and recorded with a pen recorder (RJG-4002, Nihon Koden).

Each dose of acetylcholine (ACh) (Sigma Chemical Co) was applied separately after an appropriate washout period. Levocromakalim (a gift from SmithKline Beecham pharmaceuticals, Worth-ing, UK), a direct activator of ATP-sensitive K⁺ channels, was applied in a cumulative manner.

Isometric Tension Recording
Rings with intact endothelium were placed in the 5-mL organ chambers filled with 36°C Krebs solution aerated with 95% O₂ -5% CO₂ (pH 7.4). Two fine, stainless steel wires were placed through the lumen of the ring; one was anchored, and the other was attached to the mechano-transducer (UMB-203, Kishimoto). After equilibration for 60 minutes at an optimal resting tension of 1.0g, the rings were challenged with 40 mmol/L KCl until the contractions became steady. Subsequently, the rings were allocated to one of the following treatments: (1) control; (2) indomethacin (Sigma) 10⁻³ mol/L; (3) indomethacin and N⁰-nitro-L-arginine (L-NNA) (Sigma) 10⁻¹ mol/L; and (4) indomethacin, L-NNA, and 20 mmol/L KCl. The rings were contracted with 10⁻² mol/L norepinephrine (NE) (Sigma), and relaxations to ACh were observed. Relaxations to levocromakalim and sodium nitroprusside (Sigma) were studied in rings contracted with 10⁻² mol/L NE in the presence of 10⁻³ mol/L indomethacin. The extent of the relaxation was expressed as the percentage of the initial contraction.

Statistical Analysis
Results are given as mean±SEM. Concentration-response curves of relaxation were analyzed by a 2-way ANOVA followed by the Scheffé test for multiple comparisons. The concentrations of agonists that caused half-maximal responses (EC₅₀ value) were calculated with a nonlinear regression analysis. The EC₅₀ values were expressed as the negative logarithm of the molar concentration (pD₂ values). Other variables were analyzed by 1-way ANOVA followed by the Scheffé’s test for multiple comparisons or a paired Student’s t test. A level of P<0.05 was considered statistically significant.

Results
Systolic Blood Pressure, Heart Rate, and Body Weight
TCV-116, enalapril, or their combination significantly lowered the blood pressure of SHR to comparable extents (Table 1). Systolic blood pressure was lower in SHR-T and SHR-T&E than in WKY after treatment. Body weight was significantly smaller in SHR than in WKY both before and after treatment. Body weight was smaller in SHR-T&E than in SHR-C after treatment.

Resting Membrane Potential in Mesenteric Arteries
The resting membrane potential of the mesenteric artery was significantly less negative in SHR-C (−45.7±1.4 mV) than in WKY (−49.6±0.5 mV, P<0.05). The resting membrane potential was more negative in treated SHR (SHR-T −53.4±0.8; SHR-E −49.8±1.1 mV; SHR-T&E −53.7±0.8 mV) than in SHR-C (P<0.05, respectively). Furthermore, the
membrane was more negative in SHR-T and SHR-T&E than in WKY (P<0.05, respectively).

Endothelium-Dependent Hyperpolarization in Mesenteric Arteries

The pD2 and the maximal values of hyperpolarization to ACh applied in the resting state of the membrane are shown in Table 2. The maximal hyperpolarization to ACh was significantly less in SHR-C than in WKY (P<0.05). All treatments significantly improved the maximal hyperpolarization to ACh compared with SHR-C (P<0.05). There was no significant difference in ACh-induced hyperpolarization among treated SHR and WKY, although the maximal hyperpolarization in SHR-T&E tended to be greater than in other groups. The pD2 values did not differ among the study groups.

Representative tracings and summarized data of ACh-induced hyperpolarization in the presence of NE were shown in Figure 1. Vessels were preincubated with 10⁻⁵ mol/L indomethacin to eliminate the possible depolarizing actions of cyclooxygenase products known to be released under these conditions.⁷,¹³ ACh-induced hyperpolarization in the presence of NE was attenuated in SHR-C compared with WKY (Figure 1B, Table 3). The residual hyperpolarization was abolished by a high KCl solution (20 mmol/L). All 3 treatments markedly restored the L-NNA–resistant relaxation to ACh, and the relaxation in treated SHR was comparable to that in WKY (Figure 2C, Table 3).

When rings pretreated with indomethacin were contracted with 77 mmol/L KCl, no difference was found in ACh-induced relaxations among the five groups (pD2 values; SHR-C 6.5±0.1, SHR-T 6.5±0.1, SHR-E 6.6±0.2, SHR-T&E 6.7±0.1, and WKY 6.4±0.1, ns: maximal relaxation; SHR-C 59.2±4.0, SHR-T 62.7±3.1, SHR-E 54.9±5.7, SHR-T&E 61.6±3.5, and WKY 63.0±3.2%, ns). This relaxation was abolished by further incubation with 10⁻⁴ mol/L L-NNA (data not shown).

Endothelium-Independent Hyperpolarization and Relaxation in Mesenteric Arteries

Levcromakalim produced a comparable degree of hyperpolarization in all groups (Table 2). The levcromakalim-induced ACh-induced hyperpolarizations compared with SHR-C, and the hyperpolarization in SHR-T and SHR-E was similar to that in WKY. Furthermore, ACh-induced (10⁻⁵ mol/L) hyperpolarization in SHR-T&E was significantly greater than that in SHR-E or WKY (Figure 1B).

### Table 2. Hyperpolarization to Acetylcholine and Levcromakalim in the Mesenteric Artery of SHR and WKY

<table>
<thead>
<tr>
<th></th>
<th>pD2</th>
<th>Max, mV</th>
<th>pD2</th>
<th>Max, mV</th>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td></td>
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<tr>
<td>SHR-C</td>
<td>7.1±0.1</td>
<td>−6.4±0.8†</td>
<td>6.8±0.0</td>
<td>−27.3±1.1</td>
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<tr>
<td>SHR-T</td>
<td>7.2±0.1</td>
<td>−11.2±0.8*</td>
<td>6.8±0.1</td>
<td>−23.5±1.0</td>
</tr>
<tr>
<td>SHR-E</td>
<td>7.0±0.1</td>
<td>−10.9±0.7*</td>
<td>6.8±0.1</td>
<td>−26.8±0.8</td>
</tr>
<tr>
<td>SHR-T&amp;E</td>
<td>7.1±0.1</td>
<td>−12.6±0.9*</td>
<td>6.9±0.1</td>
<td>−26.2±1.2</td>
</tr>
<tr>
<td>WKY</td>
<td>7.1±0.1</td>
<td>−10.1±1.3*</td>
<td>6.8±0.1</td>
<td>−26.1±1.1</td>
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</table>

pD2 indicates negative logarithm of molar concentration of the drug causing half-maximal hyperpolarization; Max, maximal hyperpolarization to drugs. Values are mean±SEM. There were 6 to 12 rats in each group. *P<0.05 vs SHR-C; †P<0.05 vs WKY.

Additional incubation with 10⁻⁴ mol/L L-NNA virtually abolished the relaxation in SHR-C but not in WKY (Figure 2C, Table 3). The residual relaxation was abolished by a high KCl solution (20 mmol/L). All 3 treatments markedly restored the L-NNA–resistant relaxation to ACh, and the relaxation in treated SHR was even more pronounced than that in WKY (Figure 2C, Table 3).

### Figure 1

A. Representative tracings showing hyperpolarization to 10⁻⁵ mol/L ACh under conditions of depolarization with NE (10⁻⁴ mol/L) in the presence of indomethacin (10⁻⁶ mol/L) in endothelium-intact mesenteric arteries of SHR-C, SHR-T, SHR-E, SHR-T&E, and WKY. B. Hyperpolarization to 10⁻⁷ and 10⁻⁵ mol/L ACh on the basis of A. Values are mean±SEM. There were 8 to 11 rats in each group. *P<0.05 vs SHR-C; †P<0.05 vs SHR-C; †P<0.05 vs WKY; ‡P<0.05 vs SHR-E.
relaxation in rings precontracted with 10^{-5} mol/L NE was also similar among the 5 groups (Table 3).

The maximum relaxations to sodium nitroprusside, an NO-donor, in rings precontracted with 10^{-5} mol/L NE did not differ significantly among the 5 groups, although the sensitivity to sodium nitroprusside tended to be lower in SHR-C than in other groups (Table 3).

**Relationships Between the Amplitude of Acetylcholine-Induced (10^{-5} mol/L) Hyperpolarization and Systolic Blood Pressure**

There was a significant negative relationship between the amplitude of ACh-induced (10^{-5} mol/L) hyperpolarization in the presence of NE and systolic blood pressure when all study groups were included in the analysis (Figure 3A). However, no significant relationship was observed between these parameters in the subgroup of treated SHR and WKY (Figure 3B).

**Discussion**

The present study has for the first time demonstrated that AT1 receptor antagonists block the action of Ang II regardless of its physiological significance is yet to be elucidated; and AT1 receptor antagonists also improve EDHF-mediated responses.

Although both ACE inhibitors and AT1 receptor antagonists block the renin-angiotensin system, there are certain differences in their pharmacological profiles: ACE inhibition may lead to bradykinin accumulation, a peptide that causes endothelium-dependent relaxation; in the presence of AT1 receptor antagonists, Ang II may stimulate the unopposed AT2 receptors whose physiological significance is yet to be elucidated; and AT1 receptor antagonists block the action of Ang II regardless of its forming pathway, eg, ACE, chymase, or other enzymes.

**Figure 2.** Concentration-response curves of relaxation to ACh in endothelium-intact mesenteric arterial rings precontracted with NE (10^{-5} mol/L) in SHR-C, SHR-T, SHR-E, SHR-T&E, and WKY. A, Without indomethacin and L-NNA. B, Effects of 10^{-5} mol/L indomethacin. C, Effect of 10^{-5} mol/L indomethacin and 10^{-6} mol/L L-NNA. Values are mean±SEM. There were 8 to 12 rats in each group. *P<0.05 vs SHR-C; †P<0.05 vs WKY.

**TABLE 3. Relaxation to Acetylcholine, Sodium Nitroprusside, and Levcromakalim in the Mesenteric Arteries of SHR and WKY**

<table>
<thead>
<tr>
<th>Acetylcholine</th>
<th>Sodium Nitroprusside</th>
<th>Levcromakalim</th>
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<tbody>
<tr>
<td>pD2</td>
<td>Max, %</td>
<td>pD2</td>
</tr>
<tr>
<td>SHR-C</td>
<td>6.5±0.1</td>
<td>15.7±3.9†</td>
</tr>
<tr>
<td>SHR-T</td>
<td>6.7±0.1</td>
<td>75.4±6.3*</td>
</tr>
<tr>
<td>SHR-E</td>
<td>6.7±0.1</td>
<td>70.8±6.9*</td>
</tr>
<tr>
<td>SHR-T&amp;E</td>
<td>6.7±0.1</td>
<td>84.3±2.6*</td>
</tr>
<tr>
<td>WKY</td>
<td>6.7±0.2</td>
<td>59.6±7.7*</td>
</tr>
</tbody>
</table>

pD2 indicates negative logarithm of molar concentration of the drug causing half-maximal relaxation in the norepinephrine (10^{-5} mol/L)-precontracted arterial rings; Max, maximal relaxation to drugs; and ND, not determined. Values are mean±SEM. There were 8 to 12 rats in each group. *P<0.01 vs SHR-C; †P<0.05 vs WKY.
In the present study, the AT$_1$ receptor antagonist TCV-116 improved EDHF-mediated hyperpolarization and relaxation to a similar extent as the ACE inhibitor enalapril in mesenteric arteries of SHR. The EDHF-mediated relaxation after treatment with TCV-116 even exceeded that of WKY, as is the case with enalapril. It thus appears that AT$_1$ receptor antagonists and ACE inhibitors are equally effective in restoring EDHF-mediated responses in SHR. These findings suggest that their effects are primarily due to the inhibition of the actions of Ang II as well as the reduction of blood pressure, and the specific actions of each drug may not play a major role in improving EDHF-mediated responses. However, caution should be exercised in extrapolating the present findings to humans, because substantial heterogeneity may exist among species concerning the involvement of kinins in the action of ACE inhibitors, and the role of alternate pathways for Ang II formation.

Several recent clinical studies have demonstrated the possible benefits of a combination ACE inhibitor and AT$_1$ receptor antagonist compared with either intervention alone: In sodium-depleted normotensives, combined ACE inhibition and AT$_1$ receptor blockade exerts additive effects on blood pressure; an AT$_1$ receptor antagonist produces additional hemodynamic and hormonal effects when given to patients with chronic heart failure that was treated with an ACE inhibitor; and the combination of candesartan (TCV-116) and enalapril was more beneficial for preventing left ventricular dilatation than either therapy alone in patients with congestive heart failure.

In the present study, the beneficial effects of the combined AT$_1$ receptor blockade and ACE inhibition on endothelial function appeared to be for the most part similar to those of each intervention alone, except that ACh-induced hyperpolarization under stimulated conditions was significantly greater in the combined treatment group than in the enalapril-treated SHR or WKY. ACh-induced hyperpolarization under unstimulated conditions showed similar trends, but the difference among the treated SHR was not statistically significant.

The reason for the greater hyperpolarization under stimulated conditions in SHR-T&E than in SHR-E remains unclear. Systolic blood pressure tended to be lower in SHR-T&E than in SHR-E, SHR-T, or WKY. However, such differences in blood pressure within normotensive ranges may not be sufficient to explain the differences in the response, because there was no significant relationship between blood pressure levels and ACh-induced hyperpolarization among the groups of treated SHR and WKY (Figure 3B). At present, we can only speculate that a more complete inhibition of the renin-angiotensin system, which is expected to be achieved with the combination of AT$_1$ receptor antagonists and ACE inhibitors, may lead to a greater restoration of EDHF-mediated hyperpolarization. In any case, because no statistical difference was found in ACh-induced hyperpolarization between SHR-T&E and SHR-T, present findings alone are not sufficient to conclude that the combination therapy is more beneficial than each therapy regarding EDHF-mediated hyperpolarization.

ACh-induced relaxation in the rat mesenteric artery is determined by the balance of NO, EDHF, and cyclooxygenase-derived contracting factors. ACh-induced relaxation resistant to the combined blockade of cyclooxygenase and NO synthase can be attributable to EDHF. In the present study, ACh-induced, EDHF-mediated relaxation was markedly improved in treated SHR compared with untreated SHR, which probably reflected the improved ACh-induced hyperpolarization. The EDHF-mediated relaxation in all treated SHR was even better than that in WKY, a finding consistent with our previous study.

On the other hand, EDHF-mediated relaxations were similar in vessel rings precontracted with norepinephrine among the 3 treated SHR groups and did not appear to reflect a certain difference in ACh-induced hyperpolarization obtained under similar conditions among these rats. The reason for such a discrepancy between hyperpolarization and relaxation is unclear, but one possible explanation might be that the membrane potential induced by 10$^{-5}$ mol/L ACh was more negative than −45 mV, the threshold level for contraction induced by depolarization, in all treated SHR. In this case, further hyperpolarization might not evoke further relaxation.
NO-induced relaxation can be assessed by the relaxation to ACh in KCl-contracted rings in which EDHF-mediated hyperpolarization is absent.\textsuperscript{13,29} Indeed, such relaxation is abolished by pretreatment with NO synthase inhibitor L-NNA.\textsuperscript{15} In the present study, ACh-induced relaxation in a high KCl solution was comparable in all groups, indicating that the NO-mediated relaxation in mesenteric arteries of SHR is preserved and not modulated by drug therapy, including AT\textsuperscript{1} receptor antagonists. On the other hand, it has been reported that AT\textsuperscript{1} receptor antagonists or ACE inhibitors prevent the deterioration of endothelial function in coronary arteries of SHR, presumably through preserving the availability of NO.\textsuperscript{25} It thus appears that the underlying mechanisms of endothelial dysfunction and its improvement by drug therapy may differ depending on the vascular bed, the vessel size used, or the timing of the initiation of drug therapy.

The EDHF system does exist in human arteries,\textsuperscript{30,31} and its contribution to relaxation may increase in smaller vessels.\textsuperscript{31} Hypertension is a major cardiovascular risk factor and is often associated with endothelial dysfunction. Endothelial dysfunction may also work as an aggravating factor in atherosclerotic cardiovascular diseases.\textsuperscript{32} It is conceivable that the improvement in endothelial function, including that involving EDHF, by AT\textsuperscript{1} receptor antagonists as well as ACE inhibitors contributes to their clinical benefits. It remains to be determined whether the combined AT\textsuperscript{1} receptor blockade and ACE inhibition further improve endothelial function in humans.

In conclusion, AT\textsuperscript{1} receptor antagonists as well as ACE inhibitors improve EDHF-mediated hyperpolarization and relaxation in SHR, and the combined AT\textsuperscript{1} receptor blockade and ACE inhibition appears to exert similar effects on endothelial function to those of each intervention. The clinical relevance of our findings remains to be determined.

Acknowledgments

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References