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# Inhibition of Renin-Angiotensin System Ameliorates Endothelial Dysfunction Associated With Aging in Rats

Yasushi Mukai, Hiroaki Shimokawa, Midoriko Higashi, Keiko Morikawa, Tetsuya Matoba, Junko Hiroki, Ikuko Kunihiro, Hassan M.A. Talukder, Akira Takeshita

**Objective**—Endothelial vasodilator functions are progressively impaired with aging, which may account in part for the increased incidence of cardiovascular events in elderly people. We examined what treatment could ameliorate the endothelial dysfunction associated with aging in rats.

**Methods and Results**—Aged (12-month-old) Wistar-Kyoto rats were treated with vehicle, temocapril, CS-866 (an angiotensin II type 1 receptor antagonist), cerivastatin, or hydralazine for 2 weeks. Endothelium-dependent relaxations (EDRs) of aortas from aged rats were markedly impaired compared with EDRs of aortas from young (3-month-old) rats. Indomethacin, NS-398 (a cyclooxygenase [COX]-2 inhibitor), and SQ-29548 (a thromboxane A<sub>2</sub>/prostaglandin H<sub>2</sub> receptor antagonist) acutely restored EDRs in aged rats, suggesting an involvement of COX-2–derived vasoconstricting eicosanoids. Tiron, a superoxide scavenger, also partially improved EDRs, suggesting an involvement of superoxide. EDRs were significantly ameliorated in aged rats after long-term treatment with temocapril or CS-866 but not after treatment with cerivastatin or hydralazine. Indomethacin induced no further improvement of EDRs after treatment with temocapril or CS-866. COX-2 protein expression and superoxide production were increased in the aortas of aged rats and were also attenuated by treatment with temocapril or CS-866.

**Conclusions**—These results demonstrate that long-term inhibition of the renin-angiotensin system ameliorates endothelial dysfunction associated with aging through the inhibition of the synthesis of COX-2–derived vasoconstricting factors and superoxide anions. (*Arterioscler Thromb Vasc Biol.* 2002;22:1445-1450.)

**Key Words:** endothelium-dependent relaxation ■ aging ■ endothelium-derived contracting factors  
■ cyclooxygenase ■ renin-angiotensin system

The endothelium plays an important role in modulating vascular tone by synthesizing various vasoactive substances, such as endothelium-derived relaxing factors and endothelium-derived contracting factors.<sup>1-3</sup> The former include NO, prostacyclin (prostaglandin I<sub>2</sub>), and endothelium-derived hyperpolarizing factor (EDHF), and the latter include cyclooxygenase (COX)-derived eicosanoids, such as thromboxane A<sub>2</sub> (TXA<sub>2</sub>)/endoperoxide (prostaglandin H<sub>2</sub> [PGH<sub>2</sub>]), endothelin-1, and superoxide anion (O<sub>2</sub><sup>-</sup>).<sup>1-3</sup> It is well known that endothelial dysfunction predisposes an individual to life-threatening cardiovascular events, such as myocardial infarction, and that impairment of endothelium-dependent relaxations (EDRs) is caused by an imbalance between relaxing and contracting factors released from the endothelium.<sup>1-3</sup> In addition to other cardiovascular risk factors (eg, hyperlipidemia, hypertension, diabetes mellitus, and smoking), aging is strongly correlated with endothelial dysfunction in animals and humans.<sup>4-9</sup> However, effective treatment that ameliorates endothelial dysfunction in aged blood vessels still remains to be developed.

It has been reported that aging-related endothelial dysfunction is caused by increased production of COX-derived endothelium-derived contracting factors<sup>4-6</sup> and superoxide,<sup>7,8</sup> both of which counteract the vasodilating and vasculoprotective effects of NO, whereas the production of NO per se may not be so impaired with aging.<sup>8,10</sup> It has been reported that ACE inhibitors,<sup>1</sup> angiotensin II type 1 (AT<sub>1</sub>) receptor antagonists,<sup>11</sup> and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins)<sup>12</sup> are all able to improve endothelial vasodilator functions under various pathological conditions.

Thus, in the present study, we examined whether those drugs ameliorate endothelial dysfunction in aged rats and, if so, what mechanisms are involved.

## Methods

This present experiment was reviewed by the Committee of Ethics on Animal Experiments of Kyushu University and was carried out according to the Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and the Notification (No. 6) of the Japanese Government.

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### Animal Preparation and Vessel Isolation

Young (3-month-old) and aged (12-month-old) male Wistar-Kyoto rats were obtained from a colony at Kyushu University, Fukuoka, Japan. Aged rats were treated for 2 weeks with oral administration of vehicle, temocapril (10 mg/kg per day), CS-866 (5 mg/kg per day), cerivastatin (1 mg/kg per day), or hydralazine (20 mg/kg per day) in their drinking water or chow. Drug administration was stopped 2 days before the experiment to avoid any direct effects of those inhibitors. At the end of each treatment, blood pressure was measured by tail-cuff plethysmography.

Then, the animals were anesthetized with pentobarbital and exsanguinated. The thoracic aorta was carefully dissected and cleaned of any perivascular tissue in cold Krebs' solution of the following composition (mmol/L): NaCl 121, KCl 4.7, NaHCO<sub>3</sub> 24.7, MgSO<sub>4</sub> 12.2, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.2, and glucose 5.8. Krebs' solution was aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

### Force Measurements

Aortic rings (5 mm in length) were mounted vertically between 2 hooks in organ chamber myographs (Medical Supply Co) that were filled with Krebs' solution kept at 37°C (pH 7.4). Isometric tension was measured by a force transducer (Nihon Kohden Co), as previously described.<sup>13</sup> Rings were precontracted with prostaglandin F<sub>2α</sub> (3 to 10 μmol/L), and then EDRs in response to cumulative doses of acetylcholine (ACh) were measured.

To evaluate endothelial vasodilator function in aged rats, inhibitory effects of the following drugs were measured: (1) indomethacin (10 μmol/L, a nonselective COX inhibitor), (2) NS-398 (5 μmol/L, a selective COX-2 inhibitor),<sup>5,6</sup> (3) SQ-29548 (1 μmol/L, a TXA<sub>2</sub>/PGH<sub>2</sub> receptor antagonist),<sup>5,6</sup> (4) tiron (10 mmol/L, a scavenger of superoxide anion),<sup>14</sup> and (5) N<sup>ω</sup>-nitro-L-arginine (L-NNA, 100 μmol/L, an NO synthase inhibitor). Responses were normalized and expressed as a percentage of the precontraction elicited by prostaglandin F<sub>2α</sub>. Endothelium-independent relaxations in response to sodium nitroprusside (SNP) were also examined in endothelium-denuded aortic rings.

### Western Blot Analysis

Western blot analysis for COX-2 protein was performed by using a specific monoclonal antibody against COX-2 (Transduction Laboratories Co).<sup>15</sup> Extracted protein from isolated aortas was loaded for SDS-PAGE/immunoblot analysis. The regions containing COX-2

protein were visualized by using an enhanced chemiluminescence Western blotting luminol reagent (Santa Cruz Biotechnology Inc).<sup>13</sup>

### Measurement of Endothelial Superoxide Production

Vascular superoxide production in aortic rings was measured by a lucigenin-enhanced chemiluminescence assay as described previously.<sup>7,8,16</sup> Isolated aortic rings (5 mm in length) were placed in HEPES-buffered physiological salt solution containing lucigenin (bis-N-methylacridinium nitrate, 250 μmol/L) in scintillation vials, and the chemiluminescence value was measured every minute for 10 minutes at 37°C by a scintillation counter (Luminescence Reader BLR 301, Aloka). In a preliminary study, we confirmed that 5 μmol/L lucigenin was not enough for the detection of superoxide production in this model. However, we used tiron, a superoxide scavenger, in all experiments to confirm the validity of our technique with lucigenin. A recent study confirmed the validity of the use of 250 μmol/L lucigenin for the superoxide production.<sup>7</sup> Furthermore, in a preliminary experiment with simultaneous measurement of superoxide production with lucigenin chemiluminescence and electron spin resonance methods,<sup>17</sup> we confirmed the validity of the use of 250 μmol/L lucigenin in the present study (data not shown). The lucigenin count was expressed as counts per 10 minutes per milligram (dry weight) in each vessel. Background counts (without vessel) were subtracted from each value.

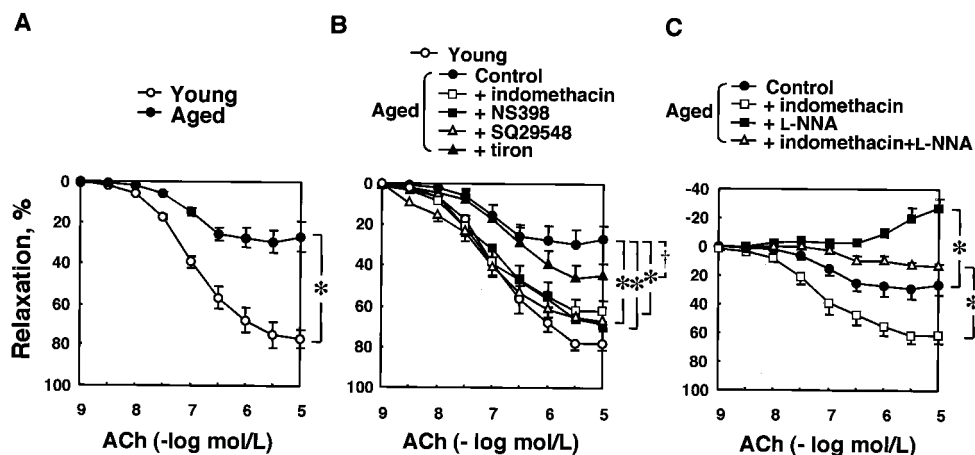
The measurement was also performed in the presence of tiron (30 mmol/L) to confirm the specificity of the assay and in the presence of the NAD(P)H oxidase inhibitor, diphenyleneiodonium (DPI, 10 μmol/L) or apocynin (100 μmol/L), to detect the source of superoxide in blood vessels as previously described.<sup>18</sup>

### Drugs

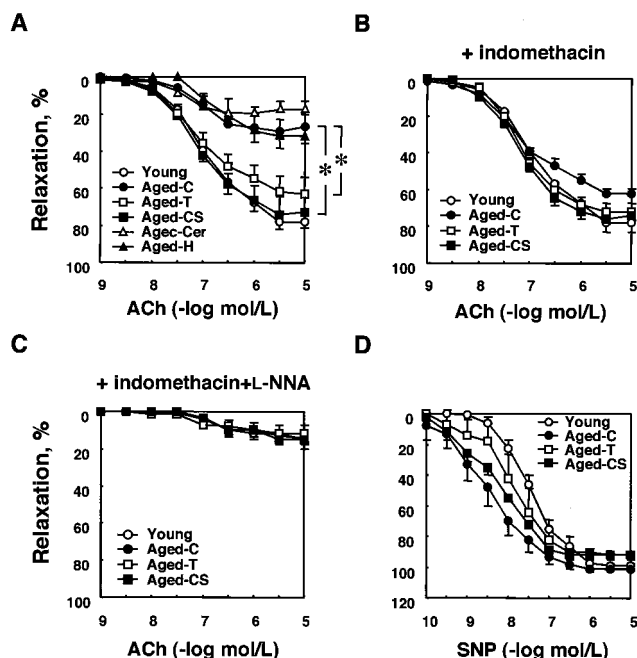
ACh, indomethacin, L-NNA, NS-398, and hydralazine hydrochloride were purchased from Sigma Chemical Co. Temocapril and CS-866 were gifts from Sankyo Pharmaceutical Co, Tokyo, Japan.

### Statistical Analysis

Results are expressed as mean±SEM. Throughout the text, n represents the number of animals tested. Concentration-response curves were analyzed by a 2-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. Differences among groups were compared by 1-way ANOVA followed by a Bonferroni post



**Figure 1.** Endothelial dysfunction in aged rats and its characterization. A, Endothelium-dependent relaxations of the isolated aorta in response to ACh were significantly reduced in aged rats compared with young rats. B, Impaired endothelium-dependent relaxations in aged rats were acutely and significantly restored in the presence of indomethacin (10 μmol/L), NS-398 (5 μmol/L), or SQ-29548 (1 μmol/L) to levels comparable to those seen in young rats and were partially but significantly improved in the presence of tiron (10 mmol/L). C, In the presence of indomethacin (10 μmol/L) and L-NNA (100 μmol/L), endothelium-dependent relaxations to ACh were abolished. In the presence of L-NNA (100 μmol/L) alone, dose-dependent contractions in response to ACh were noted. The ACh-induced contractions in the presence of L-NNA alone were absent in endothelium-denuded vessels (data not shown), indicating that the contractions were endothelium dependent. Results are expressed as mean±SEM (n=6 each). \*P<0.01; †P<0.05.



**Figure 2.** Amelioration by treatment of EDRs of aortas from aged rats. A, EDRs to ACh were significantly ameliorated in aged rats treated with temocapril (aged-T) or CS-866 (aged-CS) but not in those treated with hydralazine (aged-H) or cerivastatin (aged-Cer). B, EDRs were not further augmented in aged rats treated with temocapril (aged-T) or CS-866 (aged-CS) in the presence of indomethacin (10  $\mu\text{mol/L}$ ). C, The improved components of EDRs in animals treated with temocapril (aged-T) or CS-866 (aged-CS) were abolished in the presence of indomethacin (10  $\mu\text{mol/L}$ ) and L-NNA (100  $\mu\text{mol/L}$ ). D, In aortic rings without endothelium, endothelium-independent relaxations to SNP were not enhanced but were slightly reduced in animals treated with temocapril (aged-T) or CS-866 (aged-CS) compared with control animals (aged-C). Results are expressed as mean  $\pm$  SEM (n=6 each). \* $P < 0.01$ .

hoc test for multiple comparisons. A value of  $P < 0.05$  was considered to be statistically significant.

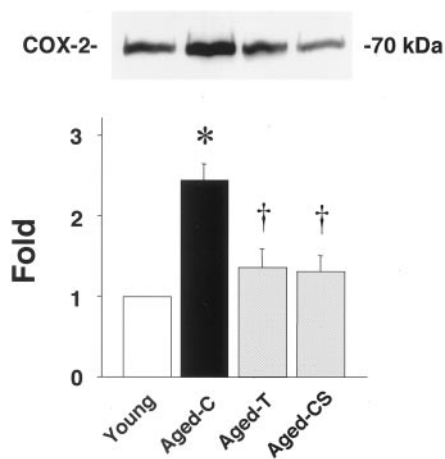
## Results

### Endothelial Dysfunction With Aging

EDRs in response to ACh were markedly impaired in aged rats compared with young rats (Figure 1A). By contrast, endothelium-independent relaxations to SNP were augmented in aged rats compared with young rats ( $IC_{50}$  was  $3.8 \times 10^{-8}$  mol/L for young rats and  $3.6 \times 10^{-9}$  mol/L for aged rats;  $P < 0.01$ ), whereas the extent of maximal relaxation was comparable ( $99 \pm 2\%$  for young rats and  $101 \pm 1\%$  for aged rats).

### Acute Restoration of Aging-Related Endothelial Dysfunction

The impaired EDRs in aged rats were acutely restored in the presence of either indomethacin, NS-398, or SQ-29548 to the levels in young rats and were also partially, but significantly, improved in the presence of tiron (Figure 1B). In the presence of L-NNA alone, ACh caused dose-dependent contractions (Figure 1C), which were absent in rings without endothelium (data not shown). In the presence of indomethacin and



**Figure 3.** Western blot analysis for COX-2 protein expression in the aorta. Expression of COX-2 protein in the aorta was significantly upregulated in aged rats (aged-C) compared with young rats. The upregulation of COX-2 protein in aged rats was significantly attenuated by the long-term treatment with either temocapril (aged-T) or CS-866 (aged-CS). Results are expressed as mean  $\pm$  SEM (n=6 each). \* $P < 0.01$  vs young; † $P < 0.01$  vs aged-C.

L-NNA, endothelium-dependent contractions to ACh were almost abolished (Figure 1C).

### Changes in Blood Pressure After Treatment With Drugs

Systolic blood pressure (mm Hg) measured by tail-cuff plethysmography in aged rats ( $159 \pm 5$  mm Hg) was slightly but significantly higher than that in young rats ( $141 \pm 4$  mm Hg,  $P < 0.01$ ). Treatment with temocapril ( $121 \pm 3$  mm Hg), CS-866 ( $122 \pm 3$  mm Hg), or hydralazine ( $118 \pm 3$  mm Hg, all  $P < 0.01$ ) but not with cerivastatin ( $157 \pm 3$ ) significantly lowered systolic blood pressure in aged rats.

### Amelioration of Endothelial Dysfunction by Long-Term Treatment With Drugs

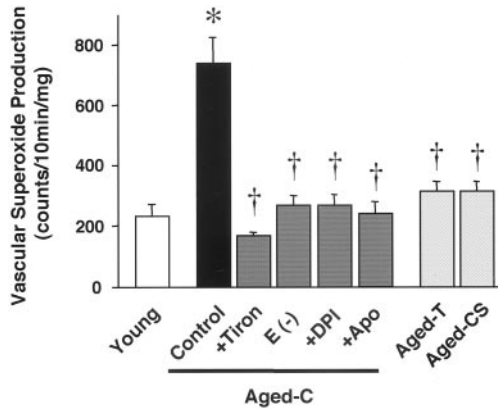
In the treatment groups, EDRs to ACh were significantly ameliorated in rats treated with temocapril or CS-866 but not in rats treated with cerivastatin or hydralazine (Figure 2A). The relaxations were no longer augmented in rats treated with temocapril or CS-866 in the presence of indomethacin (Figure 2B). The improved components of EDRs in rats treated with temocapril or CS-866 were almost abolished in the presence of indomethacin and L-NNA (Figure 2C). In endothelium-denuded rings, endothelium-independent relaxations to SNP were not augmented but were slightly reduced in rats treated with temocapril or CS-866 compared with EDRs in aged control rats (Figure 2D).

### COX-2 Protein Expression

Expression of COX-2 protein in the aorta was significantly upregulated in aged rats compared with young rats (Figure 3). The upregulated COX-2 protein expression was significantly attenuated by treatment with temocapril or CS-866 (Figure 3).

### Vascular Superoxide Production

Vascular superoxide production in the aorta was significantly increased in aged rats compared with young rats (Figure 4).

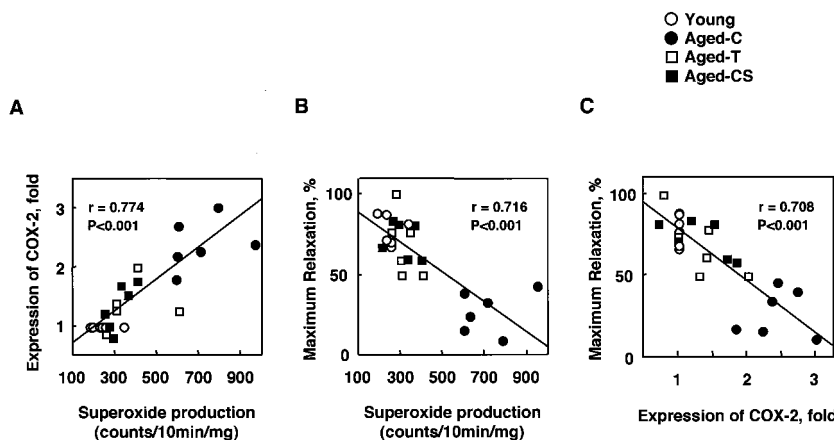


**Figure 4.** Vascular superoxide production. Vascular superoxide production in the aorta was significantly increased in aged rats (aged-C) compared with young rats (young). The increased superoxide production was acutely normalized in the presence of the superoxide scavenger tiron (Tir, 30 mmol/L). The increased superoxide production in aged rats was also acutely and significantly attenuated by endothelium removal [(-)E] or in the presence of the NAD(P)H oxidase inhibitor, DPI (10  $\mu$ mol/L) or apocynin (Apo, 100  $\mu$ mol/L). The long-term treatment with temocapril (aged-T) or CS-866 (aged-CS) also normalized vascular superoxide production in aged rats. Results are expressed as mean  $\pm$  SEM (n=6 each). \* $P$ <0.01 vs young; † $P$ <0.01 vs aged-C (control).

The superoxide production was almost abolished in the presence of the superoxide scavenger tiron (Figure 4). The increased vascular superoxide production in aged rats was significantly attenuated by endothelium removal or by the presence of the NAD(P)H oxidase inhibitor, DPI or apocynin (Figure 4). Vascular superoxide production was significantly suppressed in aged rats treated with temocapril or CS-866 compared with aged control rats (Figure 4).

**Correlation Among Endothelial Superoxide Production, COX-2 Expression, and Impairment of EDRs**

A possible correlation among endothelial superoxide production, COX-2 expression, and impairment of EDR was examined. As shown in Figure 5, there was a highly significant correlation among those parameters, suggesting a cause/effect relationship among them.



**Discussion**

The major findings of the present study were that (1) endothelial dysfunction in aged rats was caused primarily by an increased production of vasoconstricting eicosanoids derived from COX-2 and of superoxide anion, (2) inhibition of the renin-angiotensin system (RAS) with either an ACE inhibitor or an AT<sub>1</sub> receptor antagonist effectively ameliorated endothelial dysfunction in aged blood vessels by decreasing the production of vasoconstricting eicosanoids and superoxide, and (3) inhibition of RAS suppressed the increased expression of COX-2 in the blood vessels of aged rats. To the best of our knowledge, this is the first study that demonstrates the effectiveness of RAS inhibition in ameliorating the aging-related endothelial dysfunction with special reference to COX-2.

**Mechanisms of Endothelial Dysfunction With Aging in Rats**

In the present study, ACh-induced EDRs were markedly impaired in the aortas of aged rats, a finding consistent with previous reports.<sup>4-6</sup> Our results suggest that increased production of COX-2-derived vasoconstricting factors (such as TXA<sub>2</sub> and PGH<sub>2</sub>) has a crucial role in aging-related endothelial dysfunction, inasmuch as the nonselective COX inhibitor indomethacin, the selective COX-2 inhibitor NS-398, and the TXA<sub>2</sub>/PGH<sub>2</sub> receptor antagonist SQ29548 equally ameliorated the endothelial dysfunction in aged rats. Similar results have recently been reported in mesenteric arteries of aged rats.<sup>5</sup> Furthermore, in the present study, the superoxide scavenger tiron slightly but significantly restored EDRs in aged rats, suggesting an involvement of the radical in the endothelial dysfunction in aged rats through an increased breakdown of NO.

ACh-induced EDRs in the rat aorta were mediated primarily by NO because the relaxations were almost abolished in the presence of the NO synthase inhibitor L-NNA in young and aged rats, a finding consistent with our previous observations.<sup>19</sup> Thus, the contribution of prostaglandin I<sub>2</sub> and EDHF to EDRs may be minimal in the rat aorta.<sup>19</sup>

The impaired EDRs with aging were not due to smooth muscle hyporesponsiveness to NO, because the vasodilating response of vascular smooth muscle to SNP was augmented in aged rats. Indeed, we and others have previously observed

**Figure 5.** Correlation among endothelial superoxide production, COX-2 expression, and impairment of EDRs. There was a highly significant correlation among those parameters. Young indicates young rats; aged-C, aged-T, and aged-CS, aged rats treated with vehicle, temocapril, and CS-866, respectively (n=6 each [total n=24]).

that vascular smooth muscle response to NO is augmented either after pharmacological inhibition of NO synthesis<sup>20</sup> or in mice deficient in endothelial NO synthase.<sup>21,22</sup> However, the mechanism(s) for the augmentation remains to be elucidated.

### Beneficial Effect of RAS Inhibition on Endothelial Dysfunction With Aging

In the present study, long-term treatment with the ACE inhibitor temocapril or the AT<sub>1</sub> antagonist CS-866 effectively and equally ameliorated the endothelial dysfunction in aged blood vessels. Furthermore, indomethacin caused no further improvement of EDRs in aged blood vessels after treatment with temocapril or CS-866. To further elucidate the mechanism involved in the beneficial effects of those drugs, we examined the expression of COX-2 in aged blood vessels. The results demonstrated that COX-2 was upregulated in aged blood vessels, a finding consistent with the previous study,<sup>5</sup> and that the COX-2 upregulation was effectively attenuated by the treatment with temocapril or CS-866. Indeed, RAS is involved in the COX-2 expression *in vitro*<sup>23,24</sup> and *in vivo*.<sup>25,26</sup> Thus, it is highly possible that the inhibition of RAS with temocapril or CS-866 downregulates the expression of COX-2, decreases the production of vasoconstricting eicosanoids, and thus ameliorates the endothelial dysfunction in aged blood vessels. Recently, it has also been demonstrated that ACE inhibitors suppress the production of vasoconstrictor eicosanoids and thus ameliorate endothelial dysfunction in a rat model of heart failure.<sup>27</sup>

Furthermore, increased superoxide production in aged rats, which was derived mainly from endothelial NADPH oxidase,<sup>7,28</sup> was significantly attenuated by treatment with temocapril or CS-866. This result indicates that suppression of endothelial superoxide production is also involved in the beneficial effect of temocapril and CS-866. Indeed, it is well known that superoxide rapidly reacts with NO and decreases its bioavailability.<sup>1–3,7,8,10,16</sup> In addition, superoxide is also involved in the regulation of COX-2 expression *in vitro*,<sup>29</sup> and COX-derived vasoconstricting factors are involved in the impaired vasomotion in a rat model of increased oxidative stress caused by vitamin E deficiency.<sup>30</sup> In the present study, there was a highly significant correlation among endothelial superoxide production, COX-2 expression, and the impairment of EDRs, suggesting a cause/effect relationship among those responses. It is conceivable that long-term treatment with an antioxidant would also ameliorate endothelial dysfunction with aging; however, this point remains to be examined in a future study.

The amelioration of EDRs induced by RAS inhibition in the present study may not be a consequence of the blood pressure-lowering effect because hydralazine, which exerted a comparable extent of the blood pressure-lowering effect, did not improve the relaxations. In addition, the amelioration of EDRs induced by RAS inhibition may not be a consequence of increased responsiveness of vascular smooth muscle to NO inasmuch as endothelium-independent relaxations to SNP were unaltered by the treatment.

It has been recently reported that ACE inhibitors improve EDHF-mediated hyperpolarizations and thus relaxations of

mesenteric arteries of aged rats.<sup>31</sup> However, this mechanism may not significantly contribute to the beneficial effects of RAS inhibition because the contribution of EDHF to endothelium-dependent relaxations of the rat aorta may be minimal.<sup>19</sup> The effect of the RAS inhibition on EDHF-mediated responses of resistance vessels remains to be examined.

Statins may improve endothelial function through an increase in the bioavailability of NO by multiple mechanisms.<sup>12,32,33</sup> However, in the present study, cerivastatin failed to improve EDRs in aged rats. This result may be explained in part by the fact that the expression and function of endothelial NO synthase are not necessarily blunted with aging.<sup>8,10</sup>

In conclusion, we were able to provide the evidence that long-term inhibition of RAS ameliorates endothelial dysfunction in aged rats through an inhibition of the production of COX-2-derived vasoconstricting factors and of superoxide anion. Thus, inhibition of RAS may be an important strategy to treat aging-related endothelial dysfunction in humans.

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