Age and Hypertension Promote Endothelium-Dependent Contractions to Acetylcholine in the Aorta of the Rat

Tokushi Koga, Yutaka Takata, Kazuo Kobayashi, Shuichi Takishita, Yoshiaki Yamashita, and Masatoshi Fujishima

This study was undertaken to compare age-related changes in endothelium-dependent vascular responses in both hypertensive and normotensive rats. Aorta from normotensive Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) aged 4–6 weeks (young), 3–6 months (adult), and 12–25 months (old) were examined for relaxation to acetylcholine, adenosine 5'-triphosphate (ATP), and sodium nitroprusside. Rubbed (endothelium denuded) aorta from all groups displayed neither relaxation nor contraction to acetylcholine. Maximal relaxation responses to acetylcholine were reduced progressively with increasing age in unrubbed aorta of both SHR and WKY rats. In addition, acetylcholine caused not only dose-dependent relaxations at lower concentrations but also increases in tension at higher concentrations in unrubbed aorta of old WKY rats as well as adult and old SHR. However, indomethacin completely inhibited the tension development. As a result, aorta treated with indomethacin demonstrated similar acetylcholine-induced, endothelium-dependent relaxations in all groups. The thromboxane A₂ synthetase inhibitor (E)-7-phenyl-7-(3-pyridyl)-6-heptanoic acid (CV-4151) partially but significantly depressed the increases in tension in aorta of old WKY rats. The degrees of endothelium-dependent relaxations to ATP and endothelium-independent relaxations to sodium nitroprusside were almost similar in all groups. These findings suggest that the release of or vascular responsiveness to endothelium-derived relaxing factor in the aorta is well maintained through senescence in both strains and that, in the aorta of not only SHR but also old normotensive WKY rats, the endothelium releases contracting factors that may be thromboxane A₂ and other vasoconstrictor prostanoids. (Hypertension 1989;14:542–548)

The endothelium may play an obligatory role in the local regulation of vascular function, as endothelial cells produce some biologically active substances such as endothelium-derived relaxing factor (EDRF), prostaglandin I₂, and endothelium-derived contracting factors (EDCF). It is well known that, in the course of hypertension and aging, the endothelium changes both morphologically and functionally. It has been demonstrated that endothelium-dependent responses are altered with various types of hypertension. However, the alterations of endothelium-dependent vascular responses with increasing age are still controversial. Some authors found in the aged animals a decrease in endothelium-dependent relaxation responses of vascular smooth muscles, whereas others found an increased response. In addition, there are only a few reports comparing the influences of age on the regulatory action of endothelium in the vascular responses between normotensive and hypertensive rats.

The present study was designed to elucidate whether age could alter endothelium-dependent relaxations in aorta of both spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats.

Materials and Methods

Young (age 4–6 weeks), adult (age 3–6 months), and old (age 12–25 months) male SHR and WKY rats that were used for the study had been maintained in the Institute of Experimental Animals in Kyushu University and had been fed a commercial diet containing approximately 250 mg sodium and 800 mg potassium/100 g. The average age and weight of SHR and WKY rats in each group were:
were fixed in half-Kalnousky fixatives, and the presence or absence of endothelial cells was verified by scanning electron microscopy.

After measurement of the systolic blood pressure with tail-cuff plethysmography in the unanesthetized state, the rats were stunned and bled. The thoracic descending aorta was immediately excised and placed in room temperature, modified Krebs solution as follows: Na⁺ 137.4, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.5, Cl⁻ 134.4, H₂PO₄⁻ 1.2, HCO₃⁻ 15.5, and glucose 11.5. The solution was aerated with 95% O₂ and 5% CO₂, and the pH was maintained at 7.2-7.3. The blood vessels were cleaned of adherent connective tissue and cut into rings (5 mm long) under a dissecting microscope. In some preparations, the endothelium was removed by gently rubbing the intimal surface with a polyethylene tubing. The remaining rings were carefully handled so as not to injure the inner surface of the aorta. The ring was mounted in a 25 ml volume organ bath, and perfusion was carried out at a rate of 3 ml/min (Micro tube pump MP-32, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at a temperature of 35-36°C with a thermo-unit with a pump (H-80, Taiyo Co. Ltd., Tokyo, Japan). Two stainless steel wires were inserted through the lumen of the vessel ring; one was anchored to a stationary support and the other connected to a strain gauge transducer (UL-10GR, Shinkoh Co., Ltd., Nagano, Japan) coupled to a polygraph. A resting stretch optimal tension estimated to be 3×10⁻⁷ M.

In preliminary experiments, the dose-response relations to norepinephrine (10⁻² to 10⁻⁵ M) were constructed, and the doses of norepinephrine causing a 60–70% maximal contractile response were estimated to be 3×10⁻⁷ M.

Acetylcholine chloride, adenosine 5’-triphosphate, norepinephrine hydrochloride, and sodium nitroprusside (all obtained from Sigma Chemical Co., St. Louis, Missouri) were dissolved in distilled water. Indomethacin (Sigma Chemical Co.) was dissolved in ethanol and CV-4151 (Takeda Chemical Industries, Osaka, Japan) in dimethyl sulfoxide. At the concentrations used in these experiments, ethanol or dimethyl sulfoxide alone had no effect on basal tension or contractile responses to norepinephrine (3×10⁻⁷ M) in aortic rings. Drug concentrations are expressed as final molar concentrations in the bath solutions.

Results are given as mean±SEM. Statistical analyses were done by repeated measures of analysis of variance and unpaired Student’s t test. A p value less than 0.05 was considered significant.

Blood Pressure

The systolic blood pressures of SHR and WKY rats at three different ages were as follows: 113±6 and 109±4 mm Hg for the young group; 207±4 and 127±4 mm Hg for the adult group; and 207±5 and 125±5 mm Hg for the old age group, respectively. The systolic pressures of the adult and old SHR were significantly higher than those of the young SHR (p<0.001) and also those of the corresponding WKY rats (p<0.001).

Morphological Findings

Histological examinations after completion of the experiment revealed that all aortic rings with endothelium (n=127), except the regions pressed by wires, had a normal endothelial covering whereas the aortic vessels subjected to mechanical rubbing (n=36) had no endothelial cells.

Vascular Responses to Acetylcholine

Significant differences in relaxation responses to acetylcholine of vessels with intact endothelium were apparent among the three different age groups in both SHR (young vs. adult p<0.01, young vs. old p<0.001, and adult vs. old p<0.01) and WKY rats (young vs. adult p<0.05, young vs. old p<0.001, and adult vs. old p<0.005) as shown in Figure 1. Acetylcholine produced dose-dependent relaxations in young SHR and young and adult WKY rats as well. In adult and old SHR and in old WKY rats, however, higher doses (10⁻⁶ and 10⁻⁵ M) of acetylcholine increased vascular tone instead of dose-dependent relaxations (Figures 1 and 2). The maximal relaxation responses to acetylcholine significantly decreased in both SHR and WKY rats with increasing age (SHR: young vs. adult p<0.001, adult vs. old p<0.01; WKY: adult vs. old p<0.001). Responses of either relaxation or contraction to acetylcholine completely disappeared among all groups after removal of the endothelial cells (Figure 2).
**FIGURE 1.** Line graphs showing endothelium-dependent responses to acetylcholine in aorta with endothelium of spontaneously hypertensive rats (SHR) (left panel) and Wistar-Kyoto (WKY) rats (right panel) at three different ages that were precontracted with norepinephrine (3x10^{-7} M). Responses were significantly different among three age groups in both SHR (young vs. adult p<0.01, young vs. old p<0.001, and adult vs. old p<0.01) and WKY rats (young vs. adult p<0.05, young vs. old p<0.001, adult vs. old p<0.005).

The endothelium-dependent relaxations to acetylcholine were not altered by treatment with indomethacin in young SHR and in young and adult WKY rats; whereas in old WKY rats as well as adult and old SHR, the increases in tone at the high concentration of acetylcholine were abolished and the relaxations enhanced dose dependently (Figure 3). As a result, acetylcholine-induced relaxations were almost equal in all groups during treatment with indomethacin. In rings with endothelium from the old WKY rats, CV-4151 (KT 5 M) partially but significantly depressed the tension development evoked by high doses of acetylcholine (Figure 4). However, tranylcypromine (10^{-4} M) did not significantly affect these vascular responses in old WKY rats.

Vascular responses to acetylcholine in the absence of preconstriction by norepinephrine were examined in aortic segments from old WKY rats. Cumulative addition of acetylcholine caused a slight but significant contraction in a dose-dependent manner in unrubbed aortic rings. At the highest concentration of acetylcholine (10^{-5} M), the magnitude of force development was approximately 10% of the force developed in response to 3x10^{-7} M norepinephrine. Indomethacin completely abolished these contractions evoked by acetylcholine. After removal of the endothelium, dose-dependent contractile responses to acetylcholine were not recognized.

**Vascular Relaxations to Adenosine 5'-Triphosphate**

In all rats, ATP was effective in evoking dose-dependent relaxation in the presence of an intact endothelium (Figure 5), although after removal of endothelium, ATP-induced relaxation was almost abolished (young: SHR p<0.05 [n=6], WKY rats p<0.05 [n=6]; adult: SHR p<0.001 [n=5], WKY rats p<0.001 [n=7]; old: SHR p<0.001 [n=5], WKY rats p<0.001 [n=6]). ATP-induced relaxations in SHR were almost similar to those in WKY rats at any age (young: SHR [n=6] vs. WKY [n=6] p>0.05 NS; adult: SHR [n=5] vs. WKY rats [n=7] p<0.05; old: SHR [n=5] vs. WKY rats [n=7] p>0.05 NS). In addition, the degree of relaxations by ATP was not altered by aging in both SHR and WKY rats.

**Vascular Relaxations to Sodium Nitroprusside**

Sodium nitroprusside produced dose-dependent relaxation of aortic rings, which was not altered by removal of the endothelium. The number of unrubbed and rubbed (in parentheses) aorta from SHR and WKY rats used was 6 (5) and 7 (6) from the young, 6 (5) and 6 (7) from the adult, and 7 (7) and 7 (6) from the old age group. The degree of relaxation by sodium nitroprusside did not differ significantly either between SHR and WKY rats or among the three different age groups (Figure 6).
Koga et al  Effect of Age and Hypertension on Endothelium 545

Discussion

The present study demonstrates that maximal endothelium-dependent relaxations to acetylcholine were decreased with advancing age in both SHR and WKY rats, and the tensions at higher doses of acetylcholine were increased in adult and old SHR and in old normotensive WKY rats. However, these tension developments were abolished by treatment with indomethacin, and relaxations were enhanced dose dependently resulting in identical acetylcholine-induced relaxations in all groups. These findings suggest that the increases in tension observed at higher concentrations of acetylcholine are mediated by a cyclooxygenase-dependent contracting factor. Moreover, since both vascular relaxant and contractile responses to acetylcholine were abolished by removal of the endothelium in all preparations, acetylcholine-induced contractions in unrubbed rings of old WKY rats as well as those of SHR are dependent on the endothelium. Similarly, Lüscher and Vanhoutte6 observed previously that adult SHR aorta generates endothelium-dependent contraction to acetylcholine and that indomethacin blocked this contraction. The existence of endothelium-dependent contraction in the present study was also supported by the appearance of the small but significant dose-dependent contractile responses to acetylcholine in quiescent rings with endothelium from old WKY rats.

It has been reported that adult SHR aorta6 or renal artery27 stimulated by acetylcholine, canine femoral artery5-28 or porcine pulmonary artery29...
were partially inhibited by CV-4151 but not by [55x154]aorta with [55x404]rat and without [55x308]endothelium at various ages.

EDCFs such as 1) vasoconstrictor prostanoids like thromboxane A2,7,31-34 2) angiotensin II,35 3) endothelin,36 4) oxygen-derived free radicals,37 and 5) unidentified substances.5,6,27-30 Because acetylcholine-induced contractions in our model were partially inhibited by CV-4151 but not by tranylcypromine, the findings in the present study suggest, like the previously reported findings, that thromboxane A2 contributes but cannot fully explain endothelium-dependent contraction. In other words, the cyclooxygenase pathway may produce EDCF only in some branches of the pathway (i.e., thromboxane formation, formation of prostanooids other than prostaglandin I2). In addition, it has been demonstrated that oxygen-derived free radicals, which are generated by activation of endothelial cyclooxygenase,38 may mediate endothelium-dependent contraction in the basilar artery of the dog.37 We cannot exclude the participation of oxygen-derived free radicals in endothelium-dependent contractions in our experiments. Yanagisawa et al36 reported the existence of endothelin, a potent vasoconstrictor peptide produced by vascular endothelial cells.36 However, EDCF in our experiments may be different from endothelin because endothelin-induced vasoconstrictions are not blocked by indomethacin.36

Lüscher and Vanhoutte6 suggest that the reduction of acetylcholine-induced relaxation in adult SHR aorta may be due to the release of EDCF in vascular endothelium of hypertensive animals at higher concentrations of acetylcholine. Also, in the present study, reduction of the vasorelaxation effect of acetylcholine with increasing age may be attributed to the appearance of endothelium-dependent contraction. Our findings that, in the presence of indomethacin, the acetylcholine-induced relaxations were identical in both strains and among three ages suggest that the releases of or vascular responsiveness to EDRF stimulated by acetylcholine in rat aorta are well maintained regardless of senescence or high level of blood pressure. In addition, the similarity of ATP-induced endothelium-dependent relaxations in all groups indicates that the release of or vascular response to EDRF by purinergic stimulation might not differ in either strains or age groups. Hongo et al39 demonstrated that ATP-induced relaxations in the carotid artery from WKY rats decreased with advancing age.39 This difference from our observation may be because of the different region of origin of the blood vessels. Thus, it is possible that the generation of EDRF or its response in rat aorta is generally maintained independent of effects of age or hypertension.

The degree of relaxation by sodium nitroprusside was similar at all ages of both SHR and WKY rats, whether with or without endothelium. Shirasaki et al40 demonstrated that relaxation responses to sodium nitroprusside in the aorta of the adult and old rats were increased by removal of the endothelium. This report is in contrast with our experiments. The dissimilarity may be because of the differences in the doses of sodium nitroprusside or the age of rats. In addition, as in our experiments, there are some reports showing that the relaxation evoked by sodium nitroprusside in the aorta of adult rats was similar, whether with or without endothelium.41-43 Because both EDRF and sodium nitroprusside act on smooth muscle by stimulating the accumulation of cyclic guanosine monophosphate,44-47 it seems likely that relaxation responses dependent on the guanylate cyclase system tend to be preserved regardless of increasing age or elevation of blood pressure.

**Figure 6.** Line graphs showing dose-dependent relaxations to sodium nitroprusside in spontaneously hypertensive rat (SHR) (upper panels) and Wistar-Kyoto (WKY) rat (lower panels) aorta with (left panels) and without (right panels) endothelium at various ages.
We conclude that EDCF is released with increasing age independently of the level of blood pressure, resulting in attenuation of endothelium-dependent relaxation. In hypertensive rats, EDCF is synthesized at a younger age as compared against findings in normotensive rats. These contracting factors are thromboxane A<sub>2</sub> and other cyclooxygenase products other than prostaglandin I<sub>2</sub>.

Acknowledgment

We thank the Takeda Chemical Industries for the gift of CV-4151.

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KEY WORDS • age • endothelium-dependent contraction • acetylcholine • endothelium-dependent relaxation • spontaneously hypertensive rats
Age and hypertension promote endothelium-dependent contractions to acetylcholine in the aorta of the rat.
T Koga, Y Takata, K Kobayashi, S Takishita, Y Yamashita and M Fujishima

Hypertension. 1989;14:542-548
doi: 10.1161/01.HYP.14.5.542

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/14/5/542

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