Role of oxidative stress in age-related reduction of NO-cGMP-mediated vascular relaxation in SHR

Jason A. Payne, Jane F. Reckelhoff, and Raouf A. Khalil

1Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, Mississippi 39216-4905; and 2Research and Development, Department of Veterans Affairs Medical Center, West Roxbury 02132 and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

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Payne, Jason A., Jane F. Reckelhoff, and Raouf A. Khalil. Role of oxidative stress in age-related reduction of NO-cGMP-mediated vascular relaxation in SHR. Am J Physiol Regul Integr Comp Physiol 285: R542–R551, 2003; 10.1152/ajpregu.00056.2003.—The incidence of hypertension increases during the late stages of aging; however, the vascular mechanisms involved are unclear. We investigated whether the late stages of aging are associated with impaired nitric oxide (NO)-mediated vascular relaxation and enhanced vascular contraction and whether oxidative stress plays a role in the age-related vascular changes. Aging (16 mo) male spontaneously hypertensive rats (SHR) nontreated or treated for 8 mo with the antioxidant tempol (1 mM in drinking water) or vitamin E (E; 5,000 IU/kg chow) and vitamin C (C; 100 mg·kg⁻¹·day⁻¹ in drinking water) and adult (12 wk) male SHR were used. After the arterial pressure was measured, aortic strips were isolated from the rats for measurement of isometric contraction. The arterial pressure and phenylephrine (Phe)-induced vascular contraction were enhanced, and the ACh-induced vascular relaxation and nitrite/nitrate production were reduced in aging compared with adult rats. In aging rats, the arterial pressure was nontreated (188 ± 4), tempol-treated (161 ± 6), and E + C-treated (187 ± 1 mmHg). Phe (10⁻⁵ M) caused an increase in active stress in nontreated aging rats (14.3 ± 1.0) that was significantly (P < 0.05) reduced in tempol-treated (9.0 ± 0.7) and E + C-treated rats (9.8 ± 0.6 × 10⁻⁴ N/m²). ACh produced a small relaxation of Phe contraction in nontreated aging rats that was enhanced (P < 0.05) in tempol- and E + C-treated rats. L-NAME (10⁻⁴ M), inhibitor of NO synthase, or ODQ (10⁻⁵ M), inhibitor of cGMP production in smooth muscle, inhibited ACh relaxation and enhanced Phe contraction in tempol- and E + C-treated but not the nontreated aging rats. ACh-induced vascular nitrite/nitrate production was not different in nontreated, tempol- and E + C-treated aging rats. Relaxation of Phe contraction with sodium nitroprusside, an exogenous NO donor, was smaller in aging than adult rats but was not different between nontreated, tempol- and E + C-treated aging rats. Thus, during the late stages of aging in SHR rats, an age-related inhibition of a vascular relaxation pathway involving not only NO production by endothelial cells but also the bioavailability of NO and the smooth muscle response to NO is partially reversed during chronic treatment with the antioxidants tempol and vitamins E and C. The data suggest a role for oxidative stress in the reduction of vascular relaxation and thereby the promotion of vascular contraction and hypertension during the late stages of aging.

INCREASES IN ARTERIAL PRESSURE are often observed with aging and have traditionally been considered a normal or physiological component of the aging process. However, the increased arterial pressure in elderly individuals is becoming a major health and economic problem because of the aging of the population. Accumulating evidence suggests that the increased arterial pressure with aging is a pathophysiological disorder that could lead to increased cardiovascular morbidity and mortality in the elderly (24, 29). Epidemiological studies have shown that more than one half of the U.S. population aged 65 or older has hypertension (29). Also, the course of hypertension progresses with aging and increases the risk of stroke and coronary artery disease (2, 21, 24, 29).

Although aging is often associated with increases in arterial pressure, the vascular mechanisms involved are not clearly understood. Structural changes in the vessel wall have been suggested as possible causes of the age-related increase in arterial pressure (29). In support of the age-related vascular structural changes, several studies have shown an increase in the vascular wall stiffness and a decrease in the vascular compliance with aging (18, 19, 24). Additional age-related functional changes in the blood vessels could also increase the vascular resistance and contribute to the increase in arterial pressure. Clinical studies have shown that the forearm blood flow is reduced in elderly individuals and suggested possible impairment of endothelium-dependent vascular relaxation with aging (32). Because mechanistic studies in humans could be difficult and costly, the vascular mechanisms of the age-related increase in arterial pressure can be studied in experimental animals at different stages of the aging process. Although some studies have suggested age-related reduction in endothelial cell function in...
spontaneously hypertensive rats (SHR) (23, 37), other studies have shown no change in endothelial function with age in San Juan hypertensive rats or even up-regulation of endothelial function with age in SHR and heart failure-prone rats (4, 17, 36). The discrepancy in findings of these studies may be related, in part, to the fact that the vascular functions have often been measured in rats during the early stages of the aging process at 12 to 24 wk of age (23, 36), which may not be indicative of the actual vascular changes that occur during the late stages of aging, and therefore made it necessary to study the vascular mechanisms of the age-related increase in arterial pressure in older rats at 12 to 24 mo of age.

The vascular endothelium is known to release endothelium-derived relaxing factors such as nitric oxide (NO) (7, 13). NO diffuses into the smooth muscle, where it stimulates the enzyme guanylate cyclase leading to increased cGMP production and smooth muscle relaxation (7, 10, 14). The bioactivity of NO and the NO-cGMP-mediated vascular relaxation could be significantly reduced in the presence of reactive oxygen species (ROS) such as superoxide (11). These potentially harmful vascular effects of superoxide are normally counterbalanced by the enzyme superoxide dismutase and other antioxidants such as vitamins E and C (23, 26, 30, 34, 35). An imbalance between the production of ROS and the level of protective antioxidants could lead to significant increases in oxidative stress and arterial pressure (11), and restoration of this balance by 2- to 12-wk treatment with antioxidants has been shown to decrease the arterial pressure in Wistar-Kyoto (WKY) rats and SHR during the early stages of aging at 12–24 wk of age (5, 26, 31, 34, 35). However, little information is available on whether oxidative stress plays a role in the vascular changes during the late stages of aging, particularly in aging rats at 12–24 mo of age. Also, whether chronic long-term treatment with antioxidants could be beneficial in preventing the vascular changes during the late stages of aging is unclear.

The purpose of this study was to test the hypothesis that the late stages of aging are associated with impaired vascular relaxation and enhanced vascular contraction and that oxidative stress plays a role in the age-related vascular changes. To test this hypothesis, we compared the vascular responses in nontreated aging (16 mo) and adult (12 wk) SHR. We also compared the vascular responses in aging SHR nontreated or treated for 8 mo with tempol, a superoxide dismutase mimic, or vitamins E and C, to investigate whether chronic treatment of aging rats with antioxidants would reverse any age-related changes in vascular contraction/relaxation. Experiments were designed to determine 1) whether the vascular contraction to the α-adrenergic agonist phenylephrine (Phe) is enhanced and the vascular relaxation to ACh is reduced in aging compared with adult rats; 2) whether the age-related changes in vascular contraction and relaxation involve alterations in the endothelium-dependent NO-cGMP pathway; and 3) whether chronic treatment of aging rats with antioxidants such as tempol and vitamins E and C would reverse the age-related changes in vascular contraction, vascular relaxation, and the NO-cGMP pathway.

METHODS

Animals. Aging male SHR (Harlan Sprague Dawley, Indianapolis, IN) were obtained at 8 mo of age. The baseline mean arterial pressure was measured in the rats at 8 mo of age, and the average value was 181 ± 3 mmHg. The rats were then randomized to three aging treatment groups, 10 rats each: nontreated, tempol treated, and vitamins E and C treated (E + C treated) for 8 mo. The nontreated aging rats were maintained on standard rat chow (1% salt) and tap water ad libitum in an environment with a 12:12-h light-dark cycle. The tempol-treated rats received tempol (4-hydroxy-2,2,6,6-tetramethylpipеридинь, Sigma, St. Louis, MO, 1 mM in drinking water) (6). The E + C-treated rats received vitamin E (5,000 IU/kg chow, Harlan Teklad, Madison, WI) and vitamin C (100 mg·kg⁻¹·day⁻¹ in the drinking water) (6).

We have previously shown in aging Sprague-Dawley rats that vitamin E supplementation results in reduction in oxidative stress as measured by reduction in kidney F2-isoprostanes and thiobarbituric acid-reactive substances and decreased expression of heme oxygenase-1 (27). Data from the aging rats were compared with data obtained in parallel in adult (12 wk) male SHR (n = 10) purchased from Harlan Sprague Dawley and maintained in the animal facility for a 1-wk acclimation period. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee at the University of Mississippi Medical Center and the American Physiological Society.

Urinary F2-isoprostanes. To determine the level of oxidative stress in the various groups, the 24-h urinary excretion of the oxidative stress marker F2-isoprostanes was measured by gas chromatography and mass spectrometry as previously described (27).

Measurement of mean arterial pressure. On the day of the experiment, each rat was anesthetized with the thiobarbiturate Inactin (Research Biomedical International, Natick, MA) (110 mg/kg), placed on a temperature-regulated surgery table, and underwent a surgical procedure for catheter implantation. A PE-50 arterial catheter was placed in the femoral artery and connected to a pressure transducer (Cobe model CDX III, Sema, Birmingham, AL), and the mean arterial pressure was recorded on a Grass polygraph (model 7D, Astro-Med, West Warwick, RI). The mean arterial pressure was measured acutely in anesthetized rats and was averaged over a 40-min period to indicate the mean arterial pressure value for each rat.

Tissue preparation. After measuring the arterial pressure, the rats were euthanized by inhalation of isoflurane. The thoracic aorta was rapidly excised, placed in oxygenated Krebs solution, and cleaned of connective tissue. The aorta was cut transversely into 3-mm-wide rings. Aortic rings were cut open into strips. For endothelium-intact aortic strips, extreme care was taken throughout the procedure to avoid injury of the endothelium. For endothelium-denuded aortic strips, the endothelium was removed by gently rubbing the vessel interior with wet filter paper.

Isometric contraction. One end of the aortic strip was attached to a glass hook using a thread loop, and the other end was connected to a Grass force transducer (FP103). Aortic strips were stretched to Lmax (1.5 the unloaded initial length, L). The strips were allowed to equilibrate for 1 h in a water-jacketed, temperature-controlled tissue bath filled with 50 ml
Krebs solution continuously bubbled with 95% O₂-5% CO₂ at 37°C. The changes in isometric contraction were recorded on a Grass polygraph (model 7D).

A control contraction was elicited by applying phenylephrine (Phe, 10⁻⁵ M) to the tissue bath solution. Once the Phe contraction reached a plateau, the tissue was rinsed with Krebs solution three times, 10 min each. The whole procedure of contraction and washing was repeated twice. Increasing concentrations of Phe were applied, the contractile responses were recorded, and concentration-response curves were constructed. The maximal Phe response was measured at the plateau of each individual Phe concentration-response curve, and the data from all curves were used to calculate the average maximal Phe response. Similarly, the ED₅₀ was determined from each individual Phe concentration-response curve, and the data from all curves were used to calculate the average Phe ED₅₀.

Nitrite/nitrate production. Endothelium-intact aortic strips were placed in test tubes containing 2 ml Krebs solution aerated with 95% O₂-5% CO₂ at 37°C, and the solution was changed every 30 min for 1 h. Samples for basal accumulation of nitrite formed from released NO were first taken. The Krebs solution was replaced, and the strips were stimulated with different concentrations of ACh for 5 min. The strips were rapidly removed, dried, and weighed. The incubation solutions were assayed for the stable end product of NO, NO₃⁻. Briefly, samples of incubation solution (50 μl, in triplicate) were mixed in a 96-well microtiter plate with 100 μl Griess reagent. The colorimetric reaction was measured at 550 nm spectrophotometrically using a microtiter plate reader (Bio-Tek, Winooski, VT). The concentration of nitrite was calculated using a calibration curve with known concentrations of NaNO₃ (8).

**Results**

On the day of the experiment, the mean arterial pressure in adult SHR was 162 ± 1 mmHg. The arterial pressure was significantly greater (P < 0.01) in nontreated aging rats (188 ± 4 mmHg) compared with adult rats. The arterial pressure was significantly reduced (P < 0.01) in tempol-treated (167 ± 1 mmHg) compared with nontreated aging rats. However, the arterial pressure was not significantly different between E+C-treated (187 ± 6 mmHg) and nontreated aging rats. In nontreated aging rats, the 24-h urinary F₂-isoprostanes excretion was 1.84 ± 0.12 ng/mg creatinine (Cr). The urinary F₂-isoprostanes level was significantly reduced (P < 0.05) in tempol-treated (1.28 ± 0.05 ng/mg Cr) and E+C-treated (1.50 ± 0.13 ng/mg Cr) compared with nontreated aging rats. We did not observe any significant differences in body weight between nontreated (404.6 ± 16.0 g), tempol-treated (406.4 ± 26.2 g), and E+C-treated aging rats (397.1 ± 16.7 g). Also, no significant differences in kidney weight could be detected between nontreated (3.74 ± 0.05 g), tempol-treated (3.82 ± 0.08 g), and E+C-treated aging rats (3.82 ± 0.13 g).

In endothelium-intact aortic strips of all groups of rats, Phe caused concentration-dependent increases in active stress (Fig. 1). The Phe-induced vascular contraction was enhanced in nontreated aging rats compared with adult rats (Fig. 1A, Table 1). When the Phe response was presented as percentage of maximum Phe contraction and the ED₅₀ was calculated, Phe was significantly more potent (P = 0.006) in aging rats than adult rats (Fig. 1B, Table 1). Removal of the endothelium significantly enhanced the Phe-induced active stress in adult rats but not in nontreated aging rats (Fig. 1A, Table 1). Phe was significantly more potent (P = 0.039) in causing contraction in endothelium-denuded than endothelium-intact strips of adult rats (Fig. 1B, Table 1). The potency of Phe was not significantly different between endothelium-denuded and endothelium-intact strips of nontreated aging rats (Fig. 1B, Table 1).

The Phe concentration-response curves have shown that the maximal Phe (10⁻⁵ M)-induced active stress was significantly reduced (P < 0.01) in tempol-treated and E+C-treated rats compared with nontreated aging rats (Fig. 2A, Table 1). To determine whether the observed differences between the tempol-treated and E+C-treated rats and the nontreated aging rats are related to possible desensitization with repeated exposure to Phe, additional experiments were performed to test the effects of a single application of the maximal Phe concentration (10⁻⁵ M) on vascular contraction. In these experiments, the Phe (10⁻⁵ M)-induced active stress was 13.9 ± 0.8 × 10⁻⁴ N/m² in nontreated aging rats and was significantly reduced (P < 0.05) in tempol-treated (9.2 ± 0.6 × 10⁻⁴ N/m²) and E+C-treated rats (9.6 ± 0.8 × 10⁻⁴ N/m²). These data suggest that the differences in the Phe-induced concentration-active stress relation between tempol-treated and E+C-treated rats and the nontreated aging rats are not...
related to desensitization with repeated exposure to Phe. Further analysis of the Phe concentration-response curves and calculation of the Phe ED$_{50}$ showed that Phe was more potent ($P = 0.031$) in producing contraction in nontreated compared with tempol-treated or E + C-treated aging rats (Fig. 2B, Table 1). Removal of the endothelium did not significantly affect the Phe-induced active stress in nontreated aging rats but significantly enhanced ($P < 0.01$) the Phe-induced contraction in tempol-treated and E + C-treated aging rats (Fig. 2A, Table 1). Although the potency of Phe was not significantly different between endothelium-denuded and endothelium-intact vascular strips of nontreated aging rats, Phe was more potent ($P = 0.04$) in causing contraction in endothelium-denuded than endothelium-intact strips of tempol-treated aging rats and in endothelium-denuded than endothelium-intact strips of E + C-treated aging rats (Fig. 2B, Table 1).

In endothelium-intact vascular strips of adult rats, pretreatment with l-NAME ($10^{-4}$ M) for 30 min, to inhibit NO synthase, significantly enhanced ($P = 0.013$) the maximal Phe-induced active stress (Table 1). Analysis of the Phe ED$_{50}$ in adult rats showed that Phe was more potent ($P = 0.022$) in causing contraction in the presence than in the absence of l-NAME (Table 1). In contrast, in vascular strips of nontreated aging rats, the maximal Phe-induced stress and the Phe ED$_{50}$ in the presence of l-NAME were not significantly different from that in the absence of l-NAME (Fig. 3, A and D; Table 1). On the other hand, in endothelium-intact vascular strips of tempol-treated and E + C-treated aging rats, pretreatment with l-NAME ($10^{-4}$ M) significantly enhanced ($P < 0.05$) the maximal Phe-induced stress (Fig. 3, B and C; Table 1). Also, analysis of the Phe ED$_{50}$ showed that in tempol-treated and E + C-treated aging rats, Phe was more potent ($P < 0.05$) in causing contraction in the presence than in the absence of l-NAME (Fig. 3, E and F; Table 1).

Similarly, in endothelium-intact vascular strips of adult rats, pretreatment with ODQ ($10^{-5}$ M) for 30 min, to inhibit cGMP production in smooth muscle (12, 25), significantly enhanced ($P = 0.044$) the maximal Phe-induced stress (Table 1). Also, analysis of the Phe ED$_{50}$ in adult rats showed that Phe was more potent ($P < 0.01$) in causing contraction in the presence than in the absence of ODQ (Table 1). In contrast, in vascular strips of nontreated aging rats, the maximal Phe-induced stress and the Phe ED$_{50}$ in the presence of ODQ were not significantly different from that in the absence of ODQ (Fig. 3, A and D, Table 1). On the other hand, in endothelium-intact vascular strips of tempol- and E + C-treated aging rats, pretreatment with ODQ ($10^{-5}$ M) significantly enhanced ($P < 0.05$) the maximal Phe-induced stress (Fig. 3, B and C, Table 1). Also, analysis of the Phe ED$_{50}$ in tempol-treated and E + C-treated aging rats showed that Phe was more potent ($P < 0.05$) in causing contraction in the presence than in the absence of ODQ (Fig. 3, E and F, Table 1).

In endothelium-intact aortic strips of adult rats, ACh caused concentration-dependent relaxation of Phe ($3 \times 10^{-7}$ M) contraction (Fig. 4). The ACh-induced relaxation was significantly reduced ($P < 0.05$) in aging compared with adult rats (Fig. 4). The ACh relaxation was significantly enhanced ($P < 0.05$) in tempol-treated and E + C-treated compared with nontreated aging rats (Fig. 4).

Because the Phe contraction was greater in vascular strips of aging rats compared with adult rats, control experiments were performed on strips of nontreated aging rats in which the Phe concentration was lowered to $1 \times 10^{-7}$ M to produce a submaximal contraction that is roughly equal in magnitude to the contraction observed in strips of adult rats precontracted with $3 \times 10^{-7}$ M Phe. These experiments showed that the ED$_{50}$ of ACh in vascular strips of aging rats precontracted with $1 \times 10^{-7}$ M Phe ($2.4 \pm 0.1 \times 10^{-8}$ M) was not significantly different from that in strips precontracted with $3 \times 10^{-7}$ M Phe ($2.3 \pm 0.1 \times 10^{-8}$ M).
Pretreatment of endothelium-intact vascular strips of adult rats for 30 min with L-NAME (10^{-5} M) to inhibit NO synthase, or ODQ (10^{-5} M), to inhibit cGMP production in smooth muscle, significantly reduced \((P < 0.05)\) ACh (10^{-5} M)-induced relaxation to 16.7 \pm 3.0 and 20.6 \pm 3.2\%, respectively, compared with 78.9 \pm 1.8\% relaxation in the absence of L-NAME and ODQ. In contrast, pretreatment of endothelium-intact vascular strips of nontreated aging rats with L-NAME (10^{-4} M) or ODQ (10^{-5} M) did not significantly affect ACh-induced relaxation (Fig. 5A). On the other hand, L-NAME or ODQ significantly inhibited \((P < 0.05)\) ACh-induced relaxation in tempol-treated (Fig. 5B) and E + C-treated aging rats (Fig. 5C). Removal of the endothelium completely inhibited the ACh-induced relaxation of Phe contraction in all groups of rats.

In endothelium-intact vascular strips of adult rats, the basal nitrite/nitrate (NOx) production was 25.2 \pm 6.5 pmol/mg tissue wt. ACh significantly increased NOx production in vascular strips of adult rats (Fig. 6). The basal and ACh-induced NOx production was significantly reduced in aging compared with adult rats. However, the basal and ACh-induced NOx production was not significantly different in tempol-treated and E + C-treated compared with nontreated aging rats (Fig. 6).

In endothelium-denuded vascular strips, SNP, an exogenous NO donor and a standard guanylate cyclase activator, caused concentration-dependent relaxation of Phe contraction. The SNP-induced relaxation was significantly reduced \((P < 0.05)\) in aging compared with adult rats (Fig. 7). The SNP-induced relaxation was not significantly different in tempol-treated and E + C-treated compared with nontreated aging rats (Fig. 7).

**DISCUSSION**

The main findings of the present study are 1) the mean arterial pressure and the Phe-induced vascular

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**Table 1. Maximal Phe-induced active stress and Phe \(\text{pED}_{50}\) in vascular strips of adult SHR and aging SHR nontreated or treated with tempol or vitamins E and C**

<table>
<thead>
<tr>
<th></th>
<th>Adult SHR</th>
<th>Non-treated</th>
<th>Tempol</th>
<th>E + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe (10^{-5} M)-induced active stress, (\times 10^4\text{N/m}^2)</td>
<td>(+) Endo</td>
<td>9.5 \pm 0.6</td>
<td>14.3 \pm 1.0*</td>
<td>9.0 \pm 0.7</td>
</tr>
<tr>
<td></td>
<td>- Endo</td>
<td>11.5 \pm 0.7\†</td>
<td>15.1 \pm 1.1*</td>
<td>11.8 \pm 0.7\†</td>
</tr>
<tr>
<td>L-NAME</td>
<td>11.6 \pm 0.5\†</td>
<td>14.8 \pm 1.1*</td>
<td>11.4 \pm 0.7\†</td>
<td>11.7 \pm 0.6\†</td>
</tr>
<tr>
<td>ODQ</td>
<td>11.4 \pm 0.7\†</td>
<td>15.3 \pm 0.8*</td>
<td>11.8 \pm 0.8\†</td>
<td>11.4 \pm 0.5\†</td>
</tr>
<tr>
<td>Phe (\text{pED}_{50}), -log [M]</td>
<td>(+) Endo</td>
<td>7.3 \pm 0.1</td>
<td>7.9 \pm 0.2*</td>
<td>7.4 \pm 0.1</td>
</tr>
<tr>
<td></td>
<td>- Endo</td>
<td>7.8 \pm 0.1\†</td>
<td>8.0 \pm 0.1*</td>
<td>7.7 \pm 0.1\†</td>
</tr>
<tr>
<td>L-NAME</td>
<td>7.8 \pm 0.2\†</td>
<td>8.2 \pm 0.1</td>
<td>7.8 \pm 0.1\†</td>
<td>7.7 \pm 0.1\†</td>
</tr>
<tr>
<td>ODQ</td>
<td>7.7 \pm 0.1\†</td>
<td>8.1 \pm 0.2</td>
<td>7.7 \pm 0.1\†</td>
<td>7.8 \pm 0.1\†</td>
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Data represent means \(\pm\) SE of measurements in 20–30 vascular strips from 10 adult or aging spontaneously hypertensive rats (SHR). \(\text{pED}_{50}\) (−log [M]) is the concentration required to produce half-maximal phenylephrine (Phe) contraction. \(-\) Endo, endothelium denuded; \(+\) Endo, endothelium intact; L-NAME, N\(^\text{\textsuperscript{-}}\)-nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazole[4,3]-quinoxalin-1-one. *Measurements in nontreated aging rats are significantly different \((P < 0.05)\) from corresponding measurements in adult rats, or aging rats treated with tempol or vitamins E and C (E + C). \†Significantly different \((P < 0.05)\) from corresponding measurement in \(+\) Endo vascular strips.
contraction are enhanced and the endothelium-dependent relaxation is reduced in aging compared with adult SHR, 2) the age-related changes in vascular relaxation and contraction involve alterations in the endothelium-dependent NO-cGMP pathway, and 3) chronic treatment of aging rats with antioxidants such as tempol and vitamins E and C reverses the age-related changes in vascular contraction, vascular relaxation, and the NO-cGMP pathway.

Previous studies have been performed on rats ranging in age between 12 and 24 wk to investigate the possible hemodynamics and vascular changes associated with aging (23, 36). However, studying the hemodynamics and vascular changes during the early stages of aging in rats 12–24 wk of age may not be ideal to understand the vascular mechanisms of the increased arterial pressure in elderly individuals. It would probably be more relevant to study the age-related hemodynamics and vascular changes during the late stages of aging in older rats 12–24 mo of age.

In the present study we have used aging (16 mo) SHR and found that the arterial pressure is elevated in the aging rats compared with adult (12 wk) SHR. We have also found that the vascular contraction to Phe is enhanced in aging compared with adult SHR. These results are in accordance with previous reports that aging is associated with increased arterial pressure and enhanced vascular contraction to norepinephrine (2, 3, 24, 29).

In a search for the possible mechanisms involved in the enhanced vascular contraction in the aging rats, we found that removal of the endothelium enhanced the Phe contraction in adult rats but had minimal effects in aging rats. Also, the ACh-induced
relaxation was reduced in aging rats compared with adult rats. These results provide evidence that an endothelium-dependent relaxation pathway is active in the adult and inhibited during the late stages of aging in SHR (11, 37). The present results are different from reports that endothelial cell function could be upregulated with age in SHR and heart failure-prone rats (17, 36). The difference in the results could be related to the age of the rats: 12–15 wk in other studies compared with 16 mo in the present study. Interestingly, studies in aging (24 mo) Fischer-144 rats have shown significant reduction in endothelium-dependent vasodilation (22), a finding consistent with our present observations in the aging (16 mo) SHR.

The vascular endothelium is known to release relaxing factors such as NO (7, 13). The reduced ACh-induced relaxation in aging rats could be due to a decrease in the synthesis/release of NO from endothelial cells or decreased bioavailability of NO or may reflect a change in the sensitivity of vascular smooth muscle to relaxation by NO. The sensitivity of vascular smooth muscle to relaxation by NO could be evaluated by its sensitivity to relaxation by exogenous NO donors such as SNP. The observation that the relaxation of endothelium-denuded vascular strips by SNP was reduced in aging compared with adult rats suggests that the decreased relaxation in the aging rats is due, in part, to decreased vascular smooth muscle sensitivity to NO. Interestingly, the decrease in SNP-induced re-

![Graph A](image1.png)

**Fig. 5.** Effect of L-NAME and ODQ on ACh-induced relaxation in endothelium-intact aortic strips of nontreated (A), tempol-treated (B), and E + C-treated aging rats (C). Aortic strips were incubated in the presence or absence of L-NAME (10^{-4} M) or ODQ (10^{-5} M) for 30 min. Submaximal Phe contraction was elicited and then ACh was added and the % relaxation of Phe contraction was measured. Data points represent means ± SE of measurements in 12–20 aortic strips from 6–10 rats of each group. *Measurements in the presence of L-NAME or ODQ are significantly different (P < 0.05) from that in the absence of L-NAME or ODQ.

![Graph B](image2.png)

**Fig. 6.** Basal and ACh-induced nitrite/nitrate production in endothelium-intact aortic strips of adult rats, and aging rats nontreated or treated with tempol or vitamins E and C. Data points represent means ± SE of measurements in 12 aortic strips from 6 rats of each group. *Measurements are significantly different (P < 0.05) between aging and adult rats.

![Graph C](image3.png)

**Fig. 7.** Sodium nitroprusside (SNP)-induced relaxation of Phe contraction in endothelium-denuded aortic strips of adult rats, and aging rats nontreated or treated with tempol or vitamins E and C. Submaximal Phe contraction was elicited and then increasing concentrations of SNP were added and the % relaxation of Phe contraction was measured. Data points represent means ± SE of measurements in 12 aortic strips from 6 rats of each group. *Measurements in aging rats are significantly different (P < 0.05) from adult rats.
laxation in nontreated aging rats was not corrected in tempol-treated or E + C-treated rats. These data may imply that a failure of cGMP response in the nontreated aging rats is uncorrected by tempol or vitamins E + C. However, this will be inconsistent with the finding that ODQ, an inhibitor of guanylate cyclase, enhanced the Phe contraction in tempol-treated and E + C-treated but not nontreated aging rats, a finding that indicates that the failure of cGMP response in the nontreated aging rats is corrected by tempol and vitamins E plus C. Alternatively, the decreased vascular sensitivity to NO may be related to possible changes in the vascular wall stiffness and structural limitations on relaxation with aging. This is supported by reports that aging may be associated with structural changes that lead to increased stiffness of the vascular wall and decreased compliance of large vessels such as the aorta of humans and SHR (18, 19, 21). Measurement of vascular relaxation in response to a cGMP/NO-independent relaxing agonist such as isoproterenol would further explore the interesting possibility of structural changes in the vascular wall with aging. However, additional changes in the synthesis/release or bioavailability of NO could still contribute to the impaired ACh-induced relaxation in the aging rats.

We have observed that pretreatment of the vascular strips of adult SHR with L-NAME, which blocks NO synthesis, inhibited ACh-induced vascular relaxation and enhanced Phe-induced contraction (Table 1). However, in vascular strips of the nontreated aging rats, L-NAME did not significantly affect ACh-induced vascular relaxation or Phe-induced contraction (Figs. 3 and 5). These results suggest that NO synthesis by endothelial cells is impaired in the aging rats. This is supported by the observation that both the basal and the ACh-induced nitrite/nitrate production were reduced in vascular strips of aging rats compared with adult rats. This is also supported by reports that the expression and activity of endothelial NOS and the production of NO are reduced in cultured senescent human endothelial cells (20).

The NO produced by endothelial cells is known to promote vascular relaxation by activating guanylate cyclase and increasing the production of cGMP in smooth muscle (10, 14). ODQ, which inhibits guanylate cyclase and decreases cGMP production in smooth muscle (12, 25), inhibited ACh-induced vascular relaxation and enhanced Phe-induced contraction in vascular strips of adult rats (Table 1). However, ODQ did not significantly affect ACh-induced vascular relaxation or Phe-induced contraction in the nontreated aging rats (Figs. 3 and 5). These results further support the hypothesis that NO synthesis/release by endothelial cells and thereby the activity of the NO-cGMP pathway in smooth muscle is reduced in aging rats.

Previous studies have shown that 2-wk administration of tempol, a superoxide dismutase mimetic, attenuates both the hypertension and the renal excretion of 8-isoprostaglandin F_2α, a marker of oxidative stress, in adult SHR (30). Also, tempol decreases the arterial pressure and attenuates ANG II-induced vasoconstriction in vascular strips and perfused mesenteric vascular beds of adult male WKY rats and SHR (31) and ameliorates ANG II-induced reduction in renal medullary blood flow in SHR (5). Furthermore, tempol abolishes ANG II-induced increase in superoxide production in vascular strips, suggesting that the tempol-induced decrease in arterial pressure and inhibition of ANG II-induced vasoconstriction are likely due to reduction in the amount of superoxide (31). Vitamin E supplementation for 3 wk has been shown to lower the arterial pressure and to decrease lipid peroxides in blood vessels of WKY rats and SHR (23, 26). Also, dietary vitamin E and vitamin C supplementation for 9 wk lowers the blood pressure and attenuates renal vascular smooth muscle hyperplasia in SHR (34, 35). Thus relatively short-term (3–12 wk) treatment of adult rats with the antioxidants vitamins E and C appears to reduce the arterial pressure by mechanisms that most likely involve reduction in oxidative stress and superoxide production.

A purpose of the present study was to investigate whether oxidative stress plays a role in the vascular changes observed during the late stages of aging in 16-mo-old rats and whether chronic long-term treatment with antioxidants would prevent the vascular changes in the aging rats. Chronic (8 mo) treatment of the aging rats with the antioxidants tempol and vitamins E and C decreased the oxidative stress marker F2-isoprostanes and reversed the age-related enhancement of vascular contraction and the inhibition of the NO-cGMP vascular relaxation pathway in the aging rats. The reduction in the oxidative stress marker F2-isoprostanes as well as the reversal of the age-related changes in the NO-cGMP vascular relaxation pathway with antioxidants suggests that an increase in ROS in the aging rats may cause a decrease in the bioactivity/bioavailability of NO during the late stages of aging. The lack of difference in basal and ACh-induced nitrite/nitrate production between vascular strips of tempol-treated or E + C-treated rats and nontreated aging rats supports the contention that the reduction in the NO-cGMP activity in the aging rats may be not only due to changes in NO synthesis/release but also to changes in NO bioactivity. This is consistent with reports that superoxide generation increases and NO bioavailability decreases with age in female WKY rats and SHR stroke-prone rats (11).

It is important to emphasize the following cautionary remarks regarding the above interpretations. First, although the decrease in endothelial cell function and the increase in vascular contraction with aging and the reversal of these vascular changes with antioxidants could contribute to the observed alterations in arterial pressure, the vascular changes may also be secondary to arterial pressure alterations. Analysis of the time course of the changes in vascular reactivity and the increase in arterial pressure with aging should help determine whether the relationship between these two parameters is causal or associative in nature. Second, although the age-related reduction in vascular relaxation and increase in vascular contrac-
tion could explain, in part, the increase in arterial pressure, other factors such as the renal, neural, and hormonal control mechanisms of the arterial pressure could also be involved. The parallel decrease in vascular contraction and arterial pressure in tempol-treated aging rats suggests significant contribution of the age-related vascular changes to the increase in arterial pressure. The cause of the lack of change in arterial pressure despite the significant decrease in vascular contraction in the E + C-treated rats is unclear but could be related to possible effects of vitamins E and C on the other neural, hormonal, and/or renal control mechanisms of arterial pressure. Alternatively, the discrepancy between the effects of vitamins E and C on the arterial pressure and vascular contraction could be related to the method used for measuring the arterial pressure and/or the tissues used for measurement of vascular contraction. It could be argued that measurements of the arterial pressure in rats under anesthesia may not detect finite differences in the arterial pressure between the E + C-treated and nontreated rats. This is unlikely because our recent measurements of the arterial pressure in aging female SHR under anesthesia have shown significant reduction in the arterial pressure in E + C-treated compared with the nontreated rats (6). In future studies, measurements of the arterial pressure in chronically instrumented conscious rats should help to further analyze quantitatively any possible differences in the arterial pressure between the E + C-treated and nontreated aging male rats. We should also note that the present vascular changes were observed in aortic vascular strips. Whether similar changes also occur in the physiologically more relevant resistance microvessels of E + C-treated compared with nontreated aging rats should be examined in future experiments. Third, the vascular endothelium releases other vasodilator substances in addition to NO, such as prostacyclin and endothelium-derived hyperpolarizing factor (1, 33). This may explain why in the vascular strips of the aging rats some relaxation to ACh was still observed and was not completely inhibited by L-NAME or ODQ. On the other hand, the complete absence of ACh-induced relaxation in endothelium-denuded strips of aging rats still supports the contention that the ACh-induced relaxation is endothelium dependent. Fourth, although the present results provide evidence that the enhanced vascular contraction in aging rats may involve increases in ROS and consequent inhibition of the endothelium-dependent NO-cGMP vascular relaxation pathway, we cannot rule out the possibility that the increase in ROS with aging may stimulate the release of endothelium-derived contracting factors and/or increase the sensitivity of vascular smooth muscle to contracting factors. The observation that incubation of endothelium-intact vascular strips of adult rats in the presence of L-NAME or ODQ caused an enhancement of Phe-induced contraction to levels that were still significantly less \( (P < 0.05) \) than that observed in nontreated aging rats (Table 1) lends support to the possibility of increased release of endothelium-derived contracting factor with aging. This is also supported by reports that aging is associated with increased endothelin-1 production and that ROS stimulate the production of endothelin-1 in the vasculature (9, 15, 16, 28). The possible increases in ROS and endothelin-1 production with aging could then lead to additional alterations in the cellular mechanisms of vascular smooth muscle contraction. In relation to this point, we should note that the small, yet significant, effect of tempol or E + C treatment on the Phe ED\(_{50}\) in aging rats (Fig. 2B, Table 1) suggests small effect of the antioxidants on the sensitivity to Phe and the apparent affinity of \( \alpha \)-adrenergic receptors to Phe. On the other hand, the dramatic reduction of active stress in tempol-treated and E + C-treated aging rats (Fig. 2A) suggests significant reduction in the signaling mechanisms of smooth muscle contraction downstream from \( \alpha \)-adrenergic receptor activation by antioxidants and should be further investigated in future studies.

In conclusion, an age-related inhibition of a vascular relaxation pathway involving not only NO production by endothelial cells but also the bioavailability of NO and the smooth muscle response to NO in systemic vessels of SHR is partially reversed by the antioxidants tempol and vitamins E and C. The data suggest a role for oxidative stress in the reduction of the mechanisms of vascular relaxation and thereby the promotion of vascular contraction and hypertension during the late stages of aging. The data also highlight the importance of investigating the age-related vascular changes in animal models at an advanced stage in the aging process proportionately relevant to that in elderly individuals.

R. A. Khalil is an Established Investigator of the American Heart Association.

DISCLOSURES

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REFERENCES


