Relaxin Therapy Reverses Large Artery Remodeling and Improves Arterial Compliance in Senescent Spontaneously Hypertensive Rats

Qi Xu, Arindam Chakravorty, Ross A.D. Bathgate, Anthony M. Dart, Xiao-Jun Du

Abstract—Hypertension and aging are associated with large artery structural remodeling and stiffening, which are known to increase cardiovascular risk. Relaxin is a peptide hormone with potent antifibrotic action in multiple organs. Although relaxin is able to reduce peripheral vascular resistance and improve arterial compliance in rats, it remains unclear whether the improvement in compliance is indirectly attributed to a vasodilatory action or whether relaxin is able to reverse arterial remodeling and stiffening directly in aged hypertensive animals. Senescent spontaneously hypertensive rats (17 months old) were treated with relaxin for 2 weeks (0.5 mg/kg per day) followed by a 1-week washout period. We determined large artery compliance using in vivo and in vitro techniques and quantified arterial remodeling by morphological and chemical means. Relaxin therapy significantly reversed aortic remodeling (ie, increases in vessel size, wall thickness, and collagen content) and improved arterial compliance, effects independent of its vasodilatory action. In relaxin-treated spontaneously hypertensive rats, arterial collagen content showed a greater reduction (−31%; P<0.05) than that of elastin (−8%), resulting in an increased elastin:collagen ratio (0.63±0.03 versus 0.47±0.02; P<0.05). In conclusion, our results demonstrated that relaxin is potent in mediating reversal of arterial remodeling and improving arterial structural compliance in aged hypertensive rats. (Hypertension. 2010;55:1260-1266.)

Key Words: relaxin  ■  hypertension  ■  SHR  ■  artery compliance  ■  vascular remodeling  ■  aging

Cardiovascular protective actions of the peptide hormone relaxin and its recent use as a new drug have received increasing interest.1–3 Recent clinical trials have shown beneficial effects of relaxin therapy in patients with acute heart failure.4 Several studies have provided strong evidence for relaxin as a potent vasodilator in vitro and in vivo.5–7 The signaling mechanisms involve stimulating nitric oxide synthases or endothelin-β-mediated vasodilatory signaling8 and antagonizing vasoconstriction induced by angiotensin II, endothelin 1, and catecholamines.9 Conrad and colleagues7,10 showed that treatment with relaxin either acutely or for a period of 10 days increased large artery compliance and cardiac output without a change in blood pressure in normotensive or hypertensive rats.

Another unique feature of relaxin is its potent antifibrotic action.11,12 In relaxin knockout mice, fibrosis occurs with aging in multiple organs.11,13–15 Conversely, administration of relaxin for 2 weeks effectively reversed organ fibrosis in relaxin knockout mice or other models of cardiac fibrosis,11 including spontaneously hypertensive rats (SHR) showing reduced cardiac and renal fibrosis by relaxin therapy for 2 weeks.16 Relaxin achieves its antifibrotic action through multiple mechanisms involving suppression of collagen synthesis via inhibiting fibroblast activation/proliferation and promotion of collagen degradation through activating matrix metalloproteinases.11

Previous studies on the cardiovascular properties of relaxin have focused on its antifibrotic actions in the heart or blood pressure–lowering effect,2,7 and it remains unknown whether chronic relaxin treatment in hypertensive models is able to reverse remodeling of large arteries. Progress in this area would advance our understanding of the therapeutic potential of relaxin. In the current study, we have addressed the hypothesis that relaxin therapy in SHRs is able to reverse large artery remodeling with accompanied improvement in arterial compliance. To make our findings more applicable to the clinical setting of primary hypertension, aged SHRs were used.

Materials and Methods

An expanded Materials and Methods section is provided in the online Data Supplement (please see http://hyper.ahajournals.org).

Animals and Relaxin Therapy

Male SHR and normotensive Wistar-Kyoto (WKY; n=10) rats were used at 17 months of age. Experimental procedures were approved.
Table 1. Body Weight and Systemic Hemodynamics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>SHR-V</th>
<th>SHR-R</th>
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</thead>
<tbody>
<tr>
<td>No.</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Body weight, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 2</td>
<td>495±6</td>
<td>427±5*</td>
<td>408±7*</td>
</tr>
<tr>
<td>Week 3</td>
<td>493±6</td>
<td>426±5*</td>
<td>394±6†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 2</td>
<td>266±4</td>
<td>255±13</td>
<td>332±10†</td>
</tr>
<tr>
<td>Week 3</td>
<td>232±2</td>
<td>246±9</td>
<td>221±7</td>
</tr>
<tr>
<td>Systolic arterial pressure, mm Hg</td>
<td>123±3</td>
<td>169±17*</td>
<td>161±9*</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>106±3</td>
<td>139±12*</td>
<td>132±6*</td>
</tr>
<tr>
<td>Cardiac output, mL/min</td>
<td>87±5</td>
<td>62±6*</td>
<td>72±7</td>
</tr>
<tr>
<td>Stroke volume, mL</td>
<td>0.38±0.02</td>
<td>0.26±0.03*</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>Cardiac index, mL/min per kg</td>
<td>177±9</td>
<td>148±13</td>
<td>181±15</td>
</tr>
</tbody>
</table>

Week 2 data were collected before removal of relaxin-containing minipumps.

*P<0.05 vs WKY rats.
†P<0.05 vs SHR-Vs.

Determination of Hemodynamics and Arterial Compliance

As described previously,7,16 SHRs were treated with vehicle (SHR-V; n=7 with 1 lost during the experimental period) or H2 relaxin (SHR-R; 0.5 mg/kg per day; n=7)11,14-17 for 2 weeks followed by 1 week of washout. Heart rate was determined by electrocardiography at the end of weeks 2 and 3.

Measurement of Arterial Compliance Ex Vivo

The left carotid artery was cannulated for infusion via a syringe pump and superfused in calcium-free HEPES-Kreb buffer. Images of the artery were taken at every 20-mm Hg increment from 0 to 240 mm Hg. The external diameter was measured, and dimension/ pressure/distanceability curves were constructed.7,19,20

Histology

Collagen and elastin contents, internal and outer circumferences, media thickness, and the number of vascular smooth muscle cells (VSMC) of the ascending aorta were determined. All of the measures were conducted in blind fashion.

Quantification of Elastin and Collagen

Biochemical analysis was performed on the descending thoracic aorta for contents of elastin and collagen, as described previously.16,19,21,22

Statistical Analysis

Results were expressed as mean±SEM. Between-group comparisons were made by 1-way ANOVA followed by Bonferroni post hoc test. P<0.05 was considered statistically significant.

Figure 1. Systemic arterial properties in WKY rats (n=10), SHR-Vs (n=6), and SHR-Rs (n=7). Systemic vascular resistance (SVR), augmentation pressure, and augmentation index were greater, whereas global arterial compliance (AC), measured as the ratio of stroke volume:pulse pressure (SV/PP) or AC_area was lower in SHR-Vs. SHR-Rs showed an improved arterial compliance. *P<0.05 vs WKY rats, †P<0.05 vs SHR-Vs.

Results

Body Weight, Hemodynamics, and Arterial Properties

At 18 months of age, SHRs showed significant lower body weight compared with WKY rats (Table 1). SHR-Rs had a lower body weight at week 3 than at week 2, likely because of the surgical procedure for minipump removal. Heart rate was higher in SHR-Rs during relaxin treatment but was similar to the 2 control groups after the washout period. Arterial pressures were significantly higher in both vehicle- and relaxin-treated SHRs compared with WKY rats (both P<0.05; Table 1) but were similar between SHR-V and SHR-R groups. Compared with WKY rats, cardiac output and stroke volume were significantly lower in SHR-Vs (both P<0.05; Table 1), and cardiac index also tended to be lower (P=0.09). Relaxin therapy tended to improve cardiac pumping capacity.

Systemic arterial properties are illustrated in Figure 1. Systemic vascular resistance was significantly higher in SHRs than in WKY controls (P<0.05). Relaxin treatment significantly reduced systemic vascular resistance (P<0.05 versus SHR-V). Arterial compliance, as measured by global arterial compliance and AC_area was significantly lower in...
Elasticity in their in vivo working pressure range (80 to 120 mm Hg) than arteries from SHR-Vs in their working range (120 to 180 mm Hg; Figure 2B). Relaxin therapy markedly altered the elasticity of the SHR carotid artery. The increment over WKY rats in the arterial diameter of SHR-Vs at 0 mm Hg was reversed by 54% after relaxin treatment, whereas the diameter at the plateau was unaffected (Figure 2A). The arterial elasticity of SHR-Rs within their working pressure range was greater than that of SHR-Vs (Figure 2B).

Changes in Aorta Structural Remodeling
At 18 months of age, lumen size (outer circumference: +18%; internal circumference: +16%), media width (+25%), and cross-sectional area (+47%) were greater in SHR-Vs than in WKY rats (all P<0.05; Figure 3). Relaxin treatment markedly attenuated the aorta enlargement (−7%), media thickening (−11%), and cross-sectional area (−17%, all P<0.05 versus SHR-V; Figure 3). Although the total number of VSMCs per cross-sectional area was comparable among all 3 of the groups, SHR-Vs had significantly lower cell density (−30%; P<0.05) when compared with WKY rats, whereas relaxin treatment partly restored VSMC density by 15% (P<0.05 versus SHR-V; Figure 3).

Aortic Content of Elastin and Collagen
Weights of dried and delipidated aortic segment were 58% greater in SHR-Vs versus WKY rats, whereas relaxin treatment reduced this increment by 38% (Table 2). The absolute amount of collagen measured biochemically or morphologically was significantly higher in SHR-Vs than WKY rats, which was paralleled by an increased total elastin content (Table 2 and Figure 4). These measures, when expressed as the percentage of media area or dry weight, were similar to those of age-matched WKY rats (Table 2). Relaxin therapy markedly reduced the total collagen content (P<0.05 versus SHR-V) but had little effect on elastin content, leading to a higher elastin:collagen ratio in SHR-Vs (Table 2 and Figure 4; P<0.05 versus SHR-V or WKY). Elastin fibers were curly shaped in aortas of aged WKY rats but were straight in aortas from SHR-Vs (Figure 4B). Relaxin therapy showed no effect on the morphology of elastin fibers.

Discussion
We have made novel findings in senescent SHRs receiving relaxin administration for 2 weeks. Relaxin therapy partially reversed morphological remodeling of the aorta, most notably resulting in a reduction in aorta size, wall thickness, media size, and collagen content. These structural changes in the large arteries are responsible for the reduced arterial stiffness found in vivo and ex vivo. Our findings strongly suggest a therapeutic potential for relaxin in the setting of hypertension with stiffened large arteries.

The protocol and methodologic features adopted in the current study merit discussion. The treatment period with human recombinant relaxin was for 2 weeks because of the concern that a longer period of treatment would be limited...
Vascular actions of relaxin have been well described.1 Conrad and colleagues1,7,10,26,27 have conducted a series of studies documenting vasodilatation and arterial pressure-lowering action of relaxin of exogenous or endogenous sources. Relaxin levels in pregnant rodents increase markedly together with the development of more compliant arteries, leading to the notion that endogenous relaxin plays a key role in the hemodynamic adjustment during pregnancy.28 Vasodilation by relaxin therapy in normotensive and hypertensive models has also been described.1,6,7,10 Several signal mechanisms are believed to contribute to this vasodilation, including activation of nitric oxidase synthase, enhancing endothelin-β signaling, and antagonizing actions of vasoconstrictors.1,9,12 Although improved large artery compliance after relaxin therapy was reported previously, including a study using 3-month–old SHRs,7,10 there has been no study that has examined the therapeutic effect of relaxin on structural remodeling of large arteries in the setting of hypertension.

Table 2. Elastin and Collagen in the Aorta

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WKY</th>
<th>SHR-V</th>
<th>SHR-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>10</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Aorta dry weight, mg/cm of aorta</td>
<td>3.65±0.10</td>
<td>5.78±0.21*</td>
<td>4.97±0.17†</td>
</tr>
<tr>
<td>Elastin content, % of dry weight</td>
<td>18.0±0.3</td>
<td>17.1±0.3</td>
<td>18.4±0.4†</td>
</tr>
<tr>
<td>Total elastin content, mg/cm of aorta</td>
<td>0.65±0.02</td>
<td>0.99±0.04*</td>
<td>0.92±0.04*</td>
</tr>
<tr>
<td>Collagen content, % of dry weight</td>
<td>42.5±1.0</td>
<td>36.7±1.3*</td>
<td>29.4±1.0†</td>
</tr>
<tr>
<td>Total collagen content, mg/cm of aorta</td>
<td>1.54±0.03</td>
<td>2.12±0.10*</td>
<td>1.46±0.07†</td>
</tr>
<tr>
<td>Elastin:collagen ratio</td>
<td>0.42±0.01</td>
<td>0.47±0.02</td>
<td>0.63±0.03†</td>
</tr>
</tbody>
</table>

*P<0.05 vs WKY rats. †P<0.05 vs SHR-Vs.

by the formation of antirelaxin antibodies. Considering the well-known vasodilatory and tachycardiac actions of relaxin,7,12 1 week was allowed for washout of relaxin before invasive hemodynamic assessment was performed. Previous studies on rats, including SHRs, clearly showed heart rate increment by relaxin treatment.10,16 We found that, although heart rate level was increased by relaxin treatment, it had become comparable to the 2 control groups after the washout period. Thus, the functional impact of relaxin treatment is entirely attributable to its reversal of vascular remodeling in SHR-Vs. Using the SHR model, numerous studies have tested drug or nondrug interventions on structural remodeling of large arteries with significant reversal in certain aspects of vascular remodeling achieved after 6 to 12 months.23–25 This is in contrast to our study where a 2-week period of relaxin therapy effectively, although partially, reversed aorta remodeling and improved compliance. Methodologically, we conducted quantitative morphological analysis on aortic remodeling and correlated changes with that of arterial compliance estimated both in vivo and ex vivo. Importantly, these findings were made on 18-month–old senescent SHRs, a model with severe large artery remodeling.

Figure 3. Aortic remodeling in SHRs and regression after relaxin therapy. A, Hematoxylin/eosin-stained transverse sections of ascending aortas from WKY rats (n=10), SHR-Vs (n=6), or SHR-Rs (n=7). Magnification ×4 (top) and ×20 (bottom). Bar=100 µm. B, Compared with WKY rats, SHR-Vs had greater outer or inner circumferences, tunica media width, cross-sectional area, and cell density in media, but total VSMC number was similar. Treatment with relaxin partially reversed these changes. *P<0.05 vs WKY rats, †P<0.05 vs SHR-Vs.
In the current study, relaxin therapy for 2 weeks reduced inner and outer circumferences of the aorta, media thickness, and cross-sectional area. Additional histological analysis revealed a reduction in collagen content, whereas elastin content was largely maintained, all pointing to a beneficial regression of large artery structure in the senescent SHRs.

Earlier studies showed that SHRs could maintain cardiac output until the late stage of hypertension. In the present study, cardiac output was 30% lower in senescent SHRs versus age-matched WKY rats. This was attributed entirely to a reduced stroke volume, because heart rate remained unchanged. Relaxin treatment partially improved cardiac output until the late stage of hypertension. In the present study, hemodynamic determination was performed after a 7-day washout period to determine changes in arterial compliance as a consequence of vascular remodeling by relaxin therapy. In this setting, treated SHRs had comparable blood pressure versus vehicle-treated controls, although systemic vascular resistance was lowered by 20%. Thus, the changes in the parameters of arterial compliance seen in SHR-Rs are because of regression of arterial remodeling independent of the vasodilation or heart rate change of relaxin.

Compared with WKY rats, senescent SHRs studied here showed clear sign of a stiffer arterial tree. Likewise, the morphological changes in the aorta induced by relaxin would lead to improved arterial compliance. We carefully assessed large artery compliance by hemodynamic determination in vivo, similar to that adopted by Conrad et al and, further, tested distensibility of the carotid artery ex vivo. All of the parameters clearly point to a more compliant arterial system in SHR-Rs, including global arterial compliance and augmentation index. By determining the pressure/dimension relationship of the carotid artery, the artery of SHR-Rs had a smaller diameter, a finding similar to that of the ascending aorta, and improved distensibility at the working range of arterial pressures.

The mechanism responsible for the action of relaxin on vascular remodeling was tested, focusing on changes in the extracellular matrix collagen and elastin. Certain conditions, such as hypertension, atherosclerosis, and intimal injury, stimulate VSMCs to produce extra collagen in the vessel wall. Our study supports this view by showing a 38% increase in total collagen content per centimeter of aortic segment in SHRs over WKY rats. Previous studies revealed that arterial collagen content increased progressively with aging and became more insoluble and cross-linked. In sharp contrast, vascular elastin synthesis is active in newborn and young rats, and becomes quiescent in aged animals resulting a reduced elastin:collagen ratio. Elastin is more extensible, but collagen fibers are very rigid. When transmural pressure rises, large arteries recruiting both elastin and collagen respond with a curvilinear pressure-diameter curve. It is believed that lower elastin:collagen ratio is a key factor responsible for altered vascular stiffness in aged subjects. High blood pressure exacerbates the situation by forcing the artery to work in a less compliant range. We found that, unlike the curly shaped elastin fibers in aortas from aged WKY rats, elastin...
fibers in the aorta of SHRs were straight and remained unchanged after relaxin therapy. Relaxin therapy significantly reduced collagen content, estimated either as total content per centimeter segment or percentage of dry weight but without change in elastin level, resulting in a significant increase in the elastin:collagen ratio and arterial compliance. Thus, a stiffer large artery in senescent SHRs is largely attributable to increased collagen content in the vascular matrix, and relaxin improves arterial compliance largely through its collagen-lowering action. Reduction in vascular collagen by relaxin treatment is most likely attributable to increased breakdown of collagen after increment in matrix metalloproteinase levels, as shown by studies from Jayebal et al. We did not determine matrix metalloproteinase levels, considering the fact that aorta samples were collected 7 days after termination of relaxin treatment.

In conclusion, our study has convincingly shown that relaxin is a potent peptide mediating regression of large artery remodeling in aged hypertensive rats, an action associated with significant improvement in arterial compliance. Thus, our findings imply hypertension and arterial stiffness as possible clinical conditions in which relaxin therapy is likely to be effective.

Perspectives
Our study using an aged SHR model provides proof-of-concept evidence that relaxin is a potent agent in the reversal of large artery remodeling, including a reduction in collagen content, leading to improved compliance. This efficacy was achieved in senescent SHRs within a period of 2 weeks. Considering that reduced large artery compliance is an independent risk factor for cardiovascular events and the recent positive outcomes from the Relaxin for the Treatment of Patients With Acute Heart Failure Trial (Pre-RELAX-AHF), a clinical trial is warranted testing relaxin either as monotherapy or in combination with other drugs in elderly hypertensives.

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Disclosures
None.

References


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Relaxin therapy reverses stiffened large arteries and improves arterial compliance in senescent spontaneously hypertensive rats

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Expanded Materials and Methods

Animals and relaxin therapy.

Male SHR (n=14) and normotensive Wistar-Kyoto (WKY, n=10) rats were used at 17-months of age. Animals were housed and maintained under standard conditions. Experimental procedures were approved by a local Animal Ethics Committee and were in accordance with the NIH guidelines.

The recombinant human gene-2 (H2) relaxin was provided by Cothera Inc. (San Mateo, Calif) and bioactive in rodents. SHRs were randomly allocated into vehicle- (SHR-V, n=7 with 1 lost during the experimental period) or H2 relaxin-treated group (SHR-R, n=7). Briefly, SHRs were anesthetized and ALZET osmotic mini-pumps (model 2ML2, Durect Corporation), loaded either with vehicle (20 mmol/L sodium acetate buffer, pH 5.0) or H2 relaxin at 0.5 mg/kg per day were implanted subcutaneously. Fourteen days later, relaxin-treated animals were anesthetized and minipumps removed. It is well known that relaxin treatment at this dose increases heart rate (HR) in rodents. We measured HR by recording electrocardiogram before minipump removal. One-week was allowed for relaxin washout before invasive functional assessment was conducted. This protocol was designed to exclude vasodilatory action of relaxin allowing determination of therapeutic efficacy on arterial morphology and stiffness.

Determination of hemodynamics and arterial compliance.

Rats were anesthetized with intraperitoneal administration of ketamine/xylazine/atropine (KXA) at 60/12/0.6 mg/kg, respectively and placed on a heated pad. A 2-F Millar microtipped transducer catheter was inserted into the carotid artery and then advanced into the ascending aorta. Using the Power-Lab chart 5.1.4 software (ADInstruments Pty Ltd), pressure signals were acquired and systolic, diastolic, mean arterial pressures and pulse pressure were measured. Heart rate was derived from pulse signals. As described previously, cardiac output was determined by using thermodilution techniques. Briefly, a thermocouple probe (MLT1402, ADInstruments Pty Ltd) was inserted into the ascending aorta via the right carotid artery and 200 µL saline (0.9%, 21°C) was injected into the superior vena cava using the Micro Injector (PB600-1, ADInstruments Pty Ltd). The change in blood temperature was recorded and CO determined by the Cardiac Output Module (ADInstruments Pty Ltd). Systemic vascular resistance was calculated as mean arterial pressure-to-cardiac output ratio. Large arterial compliance was evaluated by augmentation pressure, augmentation index, global arterial compliance (gAC) and AC area. As described previously, the augmentation point of arterial waveform was identified as the first zero crossing from positive to negative of the fourth derivative after the foot of the waveform. Augmentation pressure was the difference between the pressure at the augmentation point and systolic arterial pressure, with augmentation index as the ratio of augmentation pressure/pulse pressure in the same cardiac cycle. gAC was calculated as the stroke volume-to-pulse pressure ratio. AC area was calculated from the diastolic decay of the aortic pressure waveform using the area method: AC area = Ad/[systemic vascular resistance (P1-P2)]. Ad was defined as the area under the region of diastolic decay curve, and P1 and P2 were the pressure at the beginning and end of the waveform, respectively.

Measurement of arterial compliance ex vivo.

For the ex vivo determination of arterial compliance, the left carotid artery was isolated and residual blood flushed out. The artery was superfused in a beaker containing calcium-free HEPES-Kreb's buffer with 10^{-4} M EGTA (37°C). After a 30-min equilibration period, a 1.4Fr
A microtipped transducer catheter (Millar) was inserted into the artery and the distal was cannulated with a polyethylene tubing attached to a syringe pump (Harvard Apparatus Co.). After a pre-stretch of the carotid artery by infusion with a luminal pressure reaching to 240 mmHg, intravascular infusion was conducted leading to a stepwise increase in intra-luminal pressure from 0 to 240 mmHg. Images of the carotid artery were taken at every 20-mmHg increment with a digital camera coupled to a dissecting microscope. The external diameter of the carotid artery was measured using Image pro (Version 5.0, Media Cybernetics, Inc., Bethesda, MD) and dimension/pressure and pressure/distensibility curves were constructed.6,8,9

**Histology.**
The ascending and thoracic descending aorta was isolated and residual blood flushed out. Ascending aorta was fixed in 4% paraformaldehyde and embedded in paraffin. Transversally cut sections were stained with Massons-trichromic method for determination of collagen area, or with Orcein for determination of elastin. In sections stained with H.E., we determined internal and outer circumferences, media thickness and the number of vascular smooth muscle cells (VSMC). All measures were conducted in blind fashion.

**Quantification of aortic elastin and collagen.**
Biochemical analysis was performed on the descending thoracic aorta to determine elastin and collagen content as described previously.8,10,11 In brief, a segment was cut from the aorta and thawed. Under a dissecting microscope the length of the segment was recorded and the media separated from the adventitia on ice. After delipidation and drying, the dry weight was recorded. Extracellular proteins other than elastin were solubilised by 0.1 N NaOH at 95°C. The residual, elastin content, was quantified by dry weight and expressed as mg per centimetre of aortic media. The soluble fractions were then hydrolysed in 6M HCl and total collagen content was determined by hydroxyproline analysis as described previously with results converted to collagen content and expressed as mg per centimeter of aortic media.5,12

**Statistical Analysis.**
Results were expressed as mean±SEM. Between-group comparisons were made by one- or two-way ANOVA followed by Bonferroni post hoc test. *P*<0.05 was considered statistically significant.

**References**


