Increased Cardiac Types I and III Collagen mRNAs in Aldosterone-Salt Hypertension

Valérie Robert, Nguyen Van Thiem, Sean Lean Chev, Christian Mousas, Bernard Swynghedauw, Claude Delcayre

Abstract Cardiac fibrosis is one of the deleterious events accompanying hypertension that may be implicated in the progression toward heart failure. To determine the mechanisms involved in fibrosis and the role of hemodynamic versus humoral factors, we studied the expression of genes involved in hypertrophy and fibrosis in the heart of rats treated with aldosterone for 2 months with addition of 1% NaCl and 0.3% KCl in water. This treatment induced arterial hypertension, a moderate left ventricular hypertrophy, and a decrease in plasma thyroxine. Equatorial sections of hearts from treated rats showed numerous foci of proliferating nonmuscular cells and a biventricular fibrosis. Computerized videodensitometry demonstrated an increase of collagen volume fraction by 152% and 146% and of the ratio of the perivascular collagen area and vascular area by 86% and 167% in left and right ventricles, respectively. As measured by slot blot, this cardiac fibrosis was accompanied by an increase in $\alpha_1$ procollagen mRNA by 75% and 160% ($P<.01$) and in $\alpha_1$-III mRNA by 76% and 319% ($P<.01$) in left and right ventricles, respectively. Atrial natriuretic peptide mRNA was induced only in the hypertrophied left ventricle. We conclude that fibrosis is occurring and involves pretranslational regulation of collagen synthesis. Whereas hypertrophy and atrial natriuretic peptide mRNA increase are restricted to the left ventricle, fibrosis is initiated in both ventricles, supporting the hypothesis that this cardiac response is independent of hemodynamic factors. (Hypertension. 1994;24:30-36.)

Key Words • hypertension, experimental • heart hypertrophy • aldosterone • fibrosis • collagen • atrial natriuretic peptide

During hypertension, the accumulation of components of the extracellular matrix (ECM) can be excessive and result in myocardial fibrosis. Cardiac collagen, the major component of ECM, is mainly composed of types I and III collagens (85% and 11% of total cardiac collagen, respectively). Because of the mechanical and electrical properties of collagen, its overdeposition compromises cardiac function by increasing diastolic stiffness, favoring arrhythmias, and so may be a determinant factor of chronic heart failure. Thus, elucidation of the mechanisms that induce and maintain the excessive development of ECM is essential.

It is now well documented that mechanical overload is the main determinant of left ventricular (LV) hypertrophy. Also, both pressure overload and mechanical stretch of isolated fibroblasts result in increased cardiac collagen synthesis. However, several observations suggest that tissue stretch alone may not be an adequate stimulus for fibrosis. For example, exercise training, arteriovenous fistula, and hyperthyroidism are not associated with fibrosis. On the other hand, histochemical studies have evidenced a perivascular and interstitial fibrosis in the LV (hypertrophied) but also in the right ventricle (nonhypertrophied) in various models of hypertension. Recent experimental results have implicated humoral factors in fibrosis induction: (1) An increase in plasma aldosterone concentration induces a biventricular fibrosis in the rat that may be prevented by the aldosterone antagonist spironolactone; (2) angiotensin II and aldosterone can separately induce collagen synthesis in cultured fibroblasts; and (3) inhibitors of angiotensin-converting enzyme even at nonhypotensive doses prevent myocardial fibrosis in pressure-overloaded hearts. Thus, if hormones of the angiotensin-aldosterone system seem determinant, the biochemical pathways that ultimately result in increased collagen and related proteins are far from being understood.

The aim of this study was to gain further insight into the mechanisms of myocardial fibrosis induced by aldosterone-salt treatment. We measured the cardiac mRNA contents of types I and III collagen, the major proteins of cardiac ECM. To appreciate the role of hemodynamic versus humoral factors in the fibrotic response, this molecular approach was used in the LV and RV in parallel with the study of atrial natriuretic peptide (ANP) gene expression as a marker of cardiac load.

Methods

Animal Models and Tissues

Male Wistar rats weighing 200 g were used for all experiments. Before surgery, animals were anesthetized with pentobarbital (60 mg/kg). All animals were uninephrectomized and randomly assigned into two groups: (1) For aldosterone-salt hypertensive rats, an osmotic minipump (Alzet, Charles River) was implanted subcutaneously to deliver 0.75 $\mu$g/h d-aldosterone (Sigma Chemical Co) for 8 weeks. Minipumps were changed every 15 days. Animals received 1% NaCl and 0.3% KCl in the drinking water. (2) Sham-operated rats were implanted with an osmotic minipump containing only physio-
logical serum and received no salt in the drinking water. Nonoperated rats of the same age were used as controls. Sham-operated rats were not different from controls for all the parameters studied. All animals were fed ad libitum with M25 pellets (Extralabo). Systolic blood pressure was determined weekly by the tail-cuff method. Control of the urinary sodium-potassium ratio was performed on 24-hour urine collected in metabolic cages. Plasma as well as urinary sodium and potassium concentrations were assayed by flame photometry. At the time of death, the heart was removed, rinsed in ice-cold saline solution, and blotted dry. LVs with septum and RVs were separated, weighed, frozen in liquid nitrogen, and stored at −70°C until use.

**Plasma Thyroxine and Aldosterone Levels**

Blood samples were collected in tubes containing 100 mmol/L EDTA. After centrifugation, the plasma samples were removed and stored at −70°C. Plasma concentrations of aldosterone and total and free thyroxine were measured by radioimmunoassay.

**Total RNA Extraction**

Total RNA from RVs and LVs (300 mg) was prepared according to Chomczynski and Sacchi.22 RNA concentration was calculated by measuring absorbance at 260 nm, assuming 40 μg/mL for 1 absorbance unit. The RNAs were resuspended in 0.1% sodium dodecyl sulfate (SDS), and aliquots were stored at −70°C until use.

**Northern Blots and Slot Blots**

For Northern blots, samples of 20 μg RNA were denatured in 50% formamide, 2.2 mol/L formamide, and 1 x MOPS buffer (pH 8.0) and electrophoresed in a 1% agarose gel. Total RNA was then transferred to a Hybond-N membrane (Amersham). For slot blot analysis, 1, 2, 4, and 8 μg RNA of each sample were spotted on the membranes.

**Hybridization Conditions**

Blots were subsequently hybridized with the following oligomers or cDNA probes: a 40-mer oligonucleotide complementary to a segment in the 3′ translated region of the rat ANP mRNA (Appligene), a 24-mer oligonucleotide specific to the rat ribosomal 18S RNA (Institut Pasteur, Paris, France), a 25- to 30-mer poly-oligo(dT) (Pharmacia), a rat α1-I collagen cDNA of 1600 bp complementary to the carboxy-terminal propeptide,23 and rat α1-III collagen cDNA containing 1300 bp of the 3′ noncoding and coding regions (kindly provided by Dr. Vutrio24). Poly-oligo(dT) hybridization was performed in 5 x Denhardt’s solution, 200 μg/mL herring sperm DNA, 5 x SSC, 0.1% SDS, and 0.1 mol/L phosphate buffer (pH 7.0) at 42°C. Blots were then washed two times in 2 x SSC at room temperature. Hybridizations with ANP and 18S oligomers were performed as previously described.23 Oligonucleotides were 5′ end labeled using [32P]dATP (New England Nuclear) and T4 polynucleotide-kinase (Promega). Hybridizations with collagen cDNAs were carried out in 50% formamide, 5 x Denhardt’s solution, 200 μg/mL herring sperm DNA, 5 x SSC, 0.1% SDS, and 0.1 mol/L phosphate buffer (pH 7.0) at 42°C. Blots were baked in this solution for 12 hours for prehybridization and for 24 hours with cDNA radiolabeled by random primer extension using an Amersham Megaprime DNA labeling system. [32P]dCTP (3000 Ci/mmol) was incorporated to obtain a specific activity of 2 to 8x106 counts per milligram. Washes were done twice in 0.1 x SSC and 0.1% SDS at room temperature and two more times at 50°C to avoid cross-hybridization with ribosomal RNA. Membranes were subsequently exposed to Amersham Hyperfilm at −70°C by using intensifying screens. Different exposures of all autoradiograms were done to ensure that blackening was performed within the linear range of densitometry. The relative amounts of mRNAs were quantified on slot blots by dividing the optical densities measured using the different probes by the optical density measured using the 18S probe. Because slot blots of the LVs and RVs were not hybridized at the same time, results of LV and RV gene expression reported in Table 5 are not directly comparable and can be used only to compare sham-operated versus treated rats.

**Collagen Morphometry**

Heart tissue was included in mounting solution (Ytosystem) and frozen at −155°C in liquid nitrogen-cooled isopentane. Equatorial sections (5 μm) containing both LV and RV were cut in a cryostat at −20°C. Two sections were stained with hemalun-eosin. Two other sections were stained with the collagen-specific Sirius red stain (0.5% in saturated picric acid) and were studied blindly by a single examiner. Each field was digitized on a Macintosh IIfx by a gray level camera (Hamamatsu) mounted on a light microscope (Leitz) at a magnification of ×100, and collagen was quantified using image-analysis software (OPTILAB, Grafiek). Interstitial collagen volume fraction (ie, the ratio of interstitial collagen surface area to total ventricular surface area, as a percentage) and perivascular collagen of the stained tissue were determined separately in both LV and RV. Microscopic scarring, defined as replacement fibrosis, was included in the interstitial collagen so that results are referred to as total interstitial collagen volume fraction. Perivascular collagen area was divided by the area of the corresponding artery lumen because a correlation exists between perivascular collagen and vessel luminal area in a given animal.17

**Statistical Analysis**

The results are expressed as mean±SEM. Comparisons between groups were performed using the unpaired Student's t test after variance analysis. All probability values were two-tailed; a value of P<.05 was considered statistically significant.

**Results**

**Blood Pressure and Anatomic Data**

Table 1 shows that at 8 weeks of aldosterone-salt treatment systolic blood pressure in treated rats was increased by 47% (P<.001) compared with sham-operated rats. Body weight of treated rats was significantly lower than in sham-operated rats, although all animals continued to gain weight throughout the study and did not present any sign of cardiac failure. Aldosterone-salt treated rats developed a moderate LV hypertrophy. The ratios of LV weight to body weight and LV weight to RV

<table>
<thead>
<tr>
<th>Table 1. Physiological and Anatomic Data</th>
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<tbody>
<tr>
<td>Rat Group</td>
</tr>
<tr>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>Sham-operated (n=16)</td>
</tr>
<tr>
<td>Aldosterone (n=19)</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; BW, body weight; LVW, left ventricular weight; and RVW, right ventricular weight. Values are mean±SEM.

*P<.001, aldosterone vs sham-operated rats.
TABLE 2. Urinary Data

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Urine Volume, mL for 24 Hours</th>
<th>Na⁺, mg/h</th>
<th>K⁺, mg/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>20±3</td>
<td>1.5±0.1</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td>(n=11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone</td>
<td>85±8*</td>
<td>16.2±1.2*</td>
<td>10.1±0.5*</td>
</tr>
<tr>
<td>(n=17)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM.
*P<.0001, aldosterone vs sham-operated rats.

weight were increased by 45% (P<.001) and 22% (P<.001), respectively. There was no evidence of RV hypertrophy in aldosterone-salt treated rats.

Urinary Data

As shown in Table 2, aldosterone-salt treatment resulted in a marked increase of diuresis, natriuresis, and kaliuresis. Urine volume was augmented fourfold (P<.0001), sodium excretion 10-fold (P<.0001), and potassium excretion threefold (P<.0001). The ratio of urinary sodium to potassium concentrations was increased fourfold essentially because of the more important excretion of sodium compared with potassium. Aldosterone-salt treatment did not modify either Na⁺ plasma concentration (143±1 versus 137±3 mmol/L) or K⁺ plasma concentration (4.36±0.6 versus 5.4±0.5 mmol/L).

Hormonal Profile

As shown in Table 3, plasma aldosterone was significantly raised by 174% in the treated group. As thyroid hormones are potent regulators of collagen gene expression, we also evaluated the thyroid status of rats. Aldosterone-salt treatment was associated with a significant decrease in both total (−24%) and free (−26%) plasma thyroxine concentrations.

Histological Studies

Fig 1 presents microscopic examinations of LVs and RVs. Hemalun-eosin-stained sections showed that the amount of nonmuscular cells present in the interstitium was increased and that the interstitial space was focally enlarged in several parts of ventricular equatorial sections of aldosterone-salt–treated rats. Areas of proliferating cells were also evident and were never seen in either sham-operated or control rats (Fig 1a). This was particularly striking in subendocardial areas and around blood vessels of the LV (Fig 1b). These foci could be very large in some hearts and were poor or devoid of myocytes (Fig 1c). After 8 weeks of aldosterone-salt treatment, Sirius red staining showed a fibrotic process in both ventricles (Fig 1e). Collagen deposition was found in the interstitium and around blood vessels, and as shown in Fig 1f, scattered foci of fibrosis could be very important in some hearts.

Ventricular collagen was measured by computerized videodensitometry as described in “Methods.” Table 4 shows the results. Values of interstitial collagen for the LVs and RVs of control rats were in agreement with those obtained by other authors and confirm that the RV contains more collagen than the LV.

Aldosterone treatment resulted in an increase of interstitial collagen area by 152% and 146% in the LV and RV, respectively. In the same hearts, perivascular collagen area normalized to lumen area was increased by 86% and 167% in the LV and RV, respectively.

Ventricular Gene Expression

To gain insight into the mechanisms involved in the accumulation of collagen, we examined types I and III collagen gene expression in both ventricles where fibrotic areas were observed. Type I and type III collagen mRNAs were quantified by slot blot analysis after hybridization with specific rat probes. As shown in Table 5, an increase of both transcripts was found in the LV but also in the nonhypertrophied RV. Interestingly, the increase in collagen gene expression in the RV was more pronounced than in the LV because type I collagen mRNA was increased threefold (P<.01) and type III collagen mRNA fivefold (P<.02). This gene expression pattern was different from that of the LV, in which both types I and III collagen mRNA increases were comparable.

To ascertain that the accumulation of both collagen transcripts in the RV is not dependent on mechanical stimuli, ANP gene expression was also examined. As shown in Fig 2, Northern blot analysis revealed a strong signal and a single band for atrial RNA that corresponded in length to that of the ANP transcript. No signal was detected in liver RNA. Comparison between aldosterone-salt–treated rats and corresponding controls revealed that ANP mRNA was induced in the hypertrophied LV of treated rats but not in the RV.

To make sure that collagen and ANP gene expression in this model of hypertension was specific and not due to an overall increase of RNAs, slot blots were hybridized with a poly(dT) oligonucleotide. The amount of poly(A+) mRNA relative to 18S rRNA was not significantly different in either the LV (0.49±0.05 versus 0.58±0.08) or RV (0.85±0.08 versus 0.79±0.08) of aldosterone-salt–treated rats compared with controls.

Discussion

The main findings of this work are as follows: At 8 weeks of aldosterone-salt treatment, microscopic examination shows cardiac alterations including (1) a perivascular and interstitial fibrosis, (2) a biventricular increase of types I and III collagen mRNAs, and (3) an accumulation of ANP mRNA occurring only in the hypertrophied LV.

Aldosterone excess with high salt intake has seldom been used as an experimental model of hypertension. In this model, Brilla et al were the first to report that such a treatment induced a fibrosis in the rat heart, and our results lead to the same observation. To quantify this cardiac fibrosis, we used a videodensitometric method that allows us to study independently the repartition of...
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Fig 1. Photomicrographs show examination of ventricular sections after hemalun-eosin (panels a through c) and Sirius red (panels d through f) staining. Panels a and d, Sham-operated rats; panels b, d, e, and f, aldosterone-salt-treated rats. After 2 months of aldosterone-salt treatment, the interstitial space is increased (panel b compared with sham-operated rats in panel a). Note the presence of large foci of proliferating cells devoid of cardiac myocytes (panel c). Perivascular and interstitial fibrosis of both ventricles is also evident in both ventricles (panel e) and could be very large (panel f). (Original magnification ×24 [panels a, b, d, e] and ×64 [panels c, f].)

interstitial and perivascular fibrosis. Measurements were done on equatorial sections of the hearts. Such an analysis, based on the assumption that an equatorial section is representative of the entire myocardium, has been validated by other laboratories in different models of fibrosis. The quantification of collagen after Sirius red staining showed a dramatic rise of interstitial and perivascular collagen areas in both ventricles (see Table 4). The analysis of the sections showed us that the fibrosis was homogeneously distributed in the ventricles; i.e., no statistical difference could be found between endocardial, median, and epicardial regions. Dense foci of collagen (as in Fig 1f) were also found in all aldosterone-treated hearts. The repartition of these collagen foci was random in the myocardial section. It is widely admitted that such zones with a high density of collagen represent scars and replace myocytes that have undergone necrosis. Furthermore, as shown after hemalun-eosin staining, these collagen foci correspond to large areas of nonmuscular proliferative cells. Related alterations have been reported by Hinglais et al in the heart of 12-month-old spontaneously hypertensive rats. Using both immunohistochemistry and in situ hybridization, these authors show a colocalization of inflammatory cells and a predominant signal for type I collagen mRNA at the periphery of these fibrotic areas. Such a colocalization strongly suggests a possible control of collagen synthesis by inflammatory cells.

The development of ECM as shown histologically was associated with a general increase in the steady-state level of collagen mRNAs in the treated rat heart, indicating that in aldosterone-induced hypertension increased transcription of collagen genes may be responsible for the cardiac collagen accumulation. That the

<table>
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<tr>
<th>Rat Group</th>
<th>Left Ventricle</th>
<th>Right Ventricle</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Interstitial, %</td>
<td>Perivascular/VA</td>
</tr>
<tr>
<td>Sham-operated (n=7)</td>
<td>2.7±0.3</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Aldosterone (n=7)</td>
<td>6.8±0.6*</td>
<td>3.9±0.2*</td>
</tr>
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VA indicates vessel lumen area. Values are mean±SEM. Interstitial collagen data represent the area occupied by total collagen without perivascular collagen and are expressed as a percentage of total tissue area. Perivascular collagen area was evaluated separately and normalized to the vessel lumen area.

*P<.001, aldosterone vs sham-operated rats.
content of collagen mRNAs was still elevated after 8 weeks of aldosterone-salt treatment indicates that fibrosis is still occurring at that time. It also suggests a sustained stimulus for collagen mRNA accumulation. This is contrary to aortic stenosis in which types I and III collagen mRNAs peak at 3 days and return to basal levels by day 7 in the rat LV, this early and transient upregulation accounting for the increased accumulation of type I collagen found 8 weeks later.13,14 Already, the findings of Lund et al30 suggested that the connective tissue response was different in essential hypertension and aortic constriction. Interestingly, we found that the increase in collagen expression was more pronounced in the RV than the LV. This may be related to the fact that the RV collagen concentration is higher than the LV concentration,27 and it can be postulated that its collagen synthesis capacity is higher. In our study, both types of collagen were augmented in a similar fashion in the LV of treated animals, whereas we observed a higher accumulation of type III collagen transcript compared with type I in the RV. This indicates, as already reported by Liau et al,31 that levels of types I and III collagen are not necessarily coordinately regulated.

Our data indicate that pretranslational control is a mechanism that may be involved in the enhanced synthesis of collagen, but they cannot exclude the possibility of a posttranslational regulation. It is interesting to note that the increase of collagen mRNA content is approximately two times higher in the RV than the LV, whereas the increase of interstitial collagen deposition was of the same order of magnitude in both ventricles (approximately 150%). This discrepancy may be due to differences in collagen metabolism between ventricles, at either a posttranscriptional or posttranslational level. For example, Eleftheriades et al32 provide evidence that interstitial fibrosis after aortic stenosis is due to a decrease rate of degradation followed by an increased rate of collagen synthesis. Also, it is known that a significant proportion of the neosynthesized procollagen is immediately degraded instead of being incorporated into the matrix.33 Thus, the collagen deposition is closely dependent on the level of collagenolytic activity.

The most striking result of our study is that collagen mRNA content and collagen deposition were increased in both ventricles, whereas no hypertrophy was seen in the RV. To further analyze the possible dissociation between the development of fibrosis and hypertrophy, we used a molecular marker that has been described to be indicative of an overload at the cellular level. It is largely documented that ventricular overexpression of ANP reflects tissue stretch secondary to volume or pressure overload.25-34 Using the deoxycorticosterone acetate-salt model, Lattion et al35 observed a dramatic increase of ANP mRNA both in atria and in the LV but not in the RV, suggesting that ventricular ANP recruitment increases in response to passive stretch of the cardiac chamber. In agreement with anatomic data, we show that ANP mRNA is strongly expressed in the LV of hypertensive rats but is absent from the RV of these hearts. This result strengthens the idea that systemic hypertension is the main determinant for LV hypertrophy but not for cardiac fibrosis. Such a conclusion is in agreement with the existence of models in which hypertension is not associated with fibrosis (see above) and with previous results that suggest a dissociation between the level of blood pressure and the induction of alterations of ECM.17,30

Several lines of evidence suggest that the hormones of the renin-angiotensin-aldosterone system could play an important role in the pathogenesis of fibrosis. Namely, recent observations are in favor of a direct action of aldosterone in heart. Specific aldosterone receptors have been found in the rabbit heart, with a predominant localization on myocytes and endothelial cells.36 However, aldosterone receptors probably are also present on fibroblasts because aldosterone has been found to increase collagen synthesis in cardiac cultured fibroblasts.19 On the other hand, specific blockade of aldosterone receptors by spironolactone prevents fibrosis in the rat heart.18 Taken together, these results do not provide clear information on the biochemical mechanism by which the binding of aldosterone on myocyte receptors induces the fibroblast activation, but they do support the idea of a cardiac action of this hormone. By analogy with the known action of aldosterone in kidney, one can assume that it is able to induce the synthesis of proteins involved in cellular ionic homeostasis. Na⁺,K⁺-
ATPase could be one plausible target. Indeed, aldosterone increases the expression of both the α₁-subunit and β₁-subunit Na⁺,K⁺-ATPase mRNAs in neonatal rat cardiocytes and in cultured rat aortic smooth muscle cells. Preliminary results have also shown that it increased the number of ouabain binding sites in cardiac myocytes. Keeping with the idea that aldosterone would have a similar action in the heart as in the kidney, other transporters such as the Na⁺-Ca²⁺ exchanger and the amiloride-sensitive Na⁺ channel are potential targets of aldosterone action.

Even if it can be demonstrated that aldosterone activates the synthesis of membrane ionic transporters in cardiac cells in the short term, this does not explain the subsequent fibrosis. An important observation is that amiloride, a potassium-sparing diuretic, prevents the appearance of scars but not the interstitial fibrosis in the heart of aldosterone-treated rats. This points out that amiloride-sensitive Na⁺ channel are potential targets of aldosterone action. 

Harmful effects of aldosterone have been suggested, both directly by the results of Weber's group (Brilla et al.) and indirectly by the results of the CONSENSUS study, which established that elevated plasma aldosterone is associated with the pathogenesis and progression of heart failure. Our results support the idea that aldosterone might be implicated in the genesis of cardiac fibrosis independently of its secondary hemodynamic effects. However, the determination of mechanisms that result in collagen accumulation remains a challenge in the hope of curing pathological fibrosis.

Acknowledgments

This article is dedicated to the memory of our friend and colleague Jean Louis Peyret. This study was supported by grants from INSERM and C.E.E. (Biomed). We thank Drs J. Gerfaut (Montpellier, France) and E. Vuorio (Turku, Finland) for kindly providing plasmids for type I and type III collagen, respectively. We also thank Pr J. Gueris, Drs D. Alijahi, and E. Laprunie for hormonal assays; T. Dakhli for animal handling; and F. Dowell for kind help in preparing the manuscript.

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Hypertension. 1994;24:30-36
doi: 10.1161/01.HYP.24.1.30

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

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