Prevention of Aortic and Cardiac Fibrosis by Spironolactone in Old Normotensive Rats

Patrick Lacolley, MD, PHD, Michel E. Safar, MD, Bernadette Lucet, TECH, Katia Ledudal, TECH, Carlos Labat, TECH, Athanase Benetos, MD, PHD

Paris, France

OBJECTIVES

Because the synthesis of aldosterone is mainly modulated by angiotensin II through type I receptor stimulation and because converting enzyme inhibition (CEI) does not modify aortic extracellular matrix in old normotensive rats, the aim of the present study was to determine whether inhibition of aldosterone formation was able to prevent aortic fibrosis in old Sprague-Dawley normotensive rats.

BACKGROUND

We have previously shown that long-term aldosterone antagonism prevents the age-related increase in aortic collagen accumulation in young spontaneously hypertensive rats, independent of blood pressure changes. In contrast, we reported that the positive effects of CEI in the prevention of aortic collagen accumulation were related to the inhibition of angiotensin II actions on angiotensin II type I receptors.

METHODS

For this purpose, we studied the histomorphometric and stiffness (echo-tracking technique) changes of an eight-week treatment with the aldosterone antagonist spironolactone by comparison with placebo.

RESULTS

At the end of treatment, spironolactone in conscious animals did not change intra-arterial blood pressure, aortic and carotid wall thickness, and cardiac weight. Cardiac collagen density and, to a lesser extent, carotid collagen and elastin densities and contents were significantly decreased in association with an increase of carotid distensibility.

CONCLUSIONS

These results show that in old normotensive rats, spironolactone can markedly prevent cardiac and, to a lesser extent, arterial fibrosis and improve arterial stiffness, despite a lack of hypotensive effect. (J Am Coll Cardiol 2001;37:662–7) © 2001 by the American College of Cardiology

Aging is associated with an increased arterial stiffness (1). Modifications of arterial structure and function are considered to be responsible for this alteration independently of blood pressure changes. With age, aortic medial thickness and cross-sectional area (CSA) of the aorta increase significantly, together with the development of extracellular matrix, principally collagen (1). All these vascular changes, which predominate on central arteries, can be theoretically reversed by appropriate drug treatments. For instance, aminoguanidine prevents the age-related increase of arterial stiffness, without modifying wall thickness or the total amount of extracellular matrix, an effect probably resulting from changes in collagen cross-linking and advanced glycosylation end products (2). In contrast, converting enzyme inhibition (CEI), which prevents aortic collagen accumulation in young spontaneously hypertensive rats (SHRs) (3), has no comparable effect in old normotensive rats. In these animals, CEI reduces significantly blood pressure and aortic wall thickness but does not modify the amount of extracellular matrix (4).

At the early phase of development in SHRs, spironolactone prevents the accumulation of aortic collagen with minimal changes of blood pressure (5). This action may be explained on the basis of aldosterone-mediated mechanisms. First, aldosterone might act physiologically on specific mineralocorticoid receptors of large vessels (5). A similar effect has been previously described in detail for the myocardium, in which spironolactone reduces subendocardial collagen without changing cardiac weight (6–8). Second, aldosterone release is known to be modulated by the renin-angiotensin-bradykinin systems (5–8). Studies of old normotensive rats, in which minimal effects of CEI have been observed on vascular extracellular matrix (4), may be relevant to evaluate better the mechanisms of action of spironolactone on large arterial vessels.

The purpose of the present study was to determine in old Sprague-Dawley normotensive rats the preventive effect of spironolactone on cardiac, aortic and carotid collagen accumulation as judged by histomorphometry. Changes in carotid arterial stiffness were studied in parallel using high-resolution echo-tracking techniques.

METHODS

Twenty-six Sprague-Dawley rats (Iffa Credo, L’Abresle, France) were housed in our animal room (five to seven per cage), which was maintained at a temperature of 20° C to 22° C, a humidity of 55% to 65%, and a 12-h light/dark cycle. The rats were fed a standard diet (0.13 mEq/g Na⁺ and 0.205 mEq/g K⁺) and had free access to tap water. Rats were randomly allocated to two groups (n = 13 per group).
Doppler technique is used to place the probe perpendicularly over the common carotid artery, 1 cm below carotid bifurcation, using gel as transmitting medium. The artery is simply exposed and not dissected. The different treatments were administered once daily by gavage. The study was performed according to the international guidelines recommended for animal experiments.

**Arterial pressure and heart rate evaluation in conscious rats.** Animals were anesthetized with pentobarbital (50 mg/kg body weight intraperitoneally). A catheter (PE-50 fused to PE-10) was placed in the lower abdominal aorta via the femoral artery. The catheter was filled with heparinized saline (50 U/ml), tunneled under the skin of the back, and excised between the scapulas. The animals were then allowed to recover from anesthesia for 24 h in individual cages. Then arterial pressure measurements were performed in conscious, freely moving rats in their own cage after at least a 30-min rest. Arterial pressure and heart rate were evaluated 24 h after the last drug administration after at least a 30-min rest. Arterial pressure and heart rate were recorded by means of a Statham P23 ID pressure transducer (Gould) connected to a Gould Brush recorder (model G 4133) according to previously described method and standard ethical rules on animal experiments.

**Carotid hemodynamic study.** At the end of the eight-week treatment the rats were anesthetized with 50 mg/kg pentobarbital intraperitoneally. The femoral catheter (0.9 mm inner diameter), filled with saline and coupled to a Statham P2S1D pressure transducer (Gould Statham, Oxnar, California) was advanced toward the right common carotid artery and placed in the middle of the lumen. Changes in left carotid arterial diameter were determined noninvasively using a high-precision A-mode ultrasonic device described previously in man and rats (9,10).

Briefly, this device measures internal diameter and its systolic-diastolic variations with a precision close to 50 and 1 μm, respectively. This degree of resolution is made possible by oversampling (5,000 arterial diameter measurements/s) and averaging 16 consecutive values. Because this frequency is established as asynchronous with the instrument clock, the resolution of the measurement increases with the square root of the number of independent time intervals acquired. A 10-MHz focalized transducer is steretactically positioned over the common carotid artery, 1 cm below carotid bifurcation, using gel as transmitting medium. The artery is simply exposed and not dissected. The Doppler technique is used to place the probe perpendicularly to the arterial axis, in its largest cross-sectional dimension. After the transducer is switched to radio-frequency mode, the backscattered echoes from both anterior and posterior walls are visualized on an oscilloscopic screen. The radio-frequency signals of both were exhibiting a high signal-to-noise ratio and were then easily tagged by an electronic tracker so that their movement could be derived. The blood pressure is measured as described above on the right common carotid artery, simultaneously to the determination of the left carotid arterial diameter, using a Statham transducer (P23 Db) and a Gould processor (Cleveland, Ohio).

From the two continuous signals of pulsatile changes in arterial diameter (systolic diameter, diastolic diameter) and blood pressure, the computerized acquisition system fits the diameter-pressure curve within the diastolic-systolic range of PP, and then calculates the arterial lumen CSA-pressure curve, using an arc tangent function as previously described (9,10). Mean diameter is integrated from the diameter-time curve and mean CSA is deduced from this parameter. Because the arterial and the PP signals were not determined on the same side, we checked previously (9,10) that there was no time delay between the diameter and the pressure signals due to the electronic processing. In addition, we verified in groups of anesthetized rats that the pressure-CSA curve did not differ whether the diameter signal was recorded on the right side and the pressure signal on the left side of the common carotid artery, or the opposite design was used.

The reproducibility of the method was studied in nine Sprague-Dawley rats using the coefficient of variation (SD expressed as a percentage of the mean of 10 successive measurements). The reproducibility of carotid diameter measurements and their systolic-diastolic variation was assessed over five measurements, each performed by two observers over a 30-min period. Under these conditions the mean intraobserver coefficients of variation were 3 ± 1 and 6 ± 2%.

The distension of an artery (change in volume) during a cardiac cycle depends on the elastic characteristics of the vessel wall (and the surrounding tissue) and the local PP (5,9,10). Local arterial cross-sectional distensibility, assuming a constant length of a cylindrical vessel, is defined by the percent systolic-diastolic change in luminal CSA for a given change in intravascular pressure (ΔP). In relation to the nonlinearity of the mean blood pressure-CSA curve, arterial distensibility decreases curvilinearly as mean blood pressure increases. Thus, the distensibility-pressure curve over the systolic-diastolic range was established by deriving the equation of the mean blood pressure-CSA curve, allowing the evaluation of distensibility at any given value of arbitrary pressure. In this context, “operational” distensibility was defined as the distensibility corresponding to the steady-state mean arterial pressure of each animal. As for distensibility, it was possible to define from the CSA-mean blood pressure curve an operational value of compliance. Compli-
ance is calculated as $\Delta \text{CSA}/\Delta P$, where $\Delta \text{CSA}$ represents the absolute pulsatile change in carotid lumen CSA, and therefore the product of distensibility by CSA. The carotid incremental elastic modulus is calculated as the ratio between the carotid lumen on wall thickness ratio and distensibility, carotid medial thickness and CSA being measured as detailed in the next section.

**Histomorphometric study.** Histomorphometric parameters of the carotid and thoracic aorta were measured according to the following procedure. At the end of the hemodynamic study, and after median thoracotomy, rats were exsanguinated via a catheter placed in the right auricle while saline was injected into the femoral catheter. When the liquid from the auricle ran clear, the circulatory tract was rinsed with a 4% formaldehyde solution. The animals died very shortly after the formaldehyde infusion was started. After 1 or 2 min, a clamp was positioned on the auricle while saline was injected into the femoral catheter. Where the liquid from the auricle ran clear, the circulatory tract was rinsed with a 4% formaldehyde solution. The thoracic aorta and the carotid artery dissected and preserved in a 4% formaldehyde solution until the histological study was performed.

The different structures of the aortic and carotid media were studied in a vascular segment longitudinally embedded in paraffin. Three successive sagittal sections 5 $\mu$m thick were treated with specific stains to obtain a monochromatic color associated with each structure of the vessel media. Sirius red was used for collagen staining, orcein for elastin, and hematoxylin after periodic acid oxidation for nuclear staining. Histomorphometric methods have been previously published in detail (3,5–8,11–13).

For the evaluation of the left ventricular subendocardial collagen, each heart was cut perpendicularly to the apex to base axis into four parts of the same thickness. These specimens were dehydrated through graded ethanol solution and embedded in paraffin. Two representative sections, $3 \mu$m each, were studied from each block, mounted on glass slide and treated with Sirius red, used for collagen staining of the left ventricle (14). Then the slides stained with the Sirius red were examined at a magnification of $\times 250$. Morphometric analysis was performed as described for aortic segments.

**Statistical analysis.** Results are expressed as mean $\pm$ 1 SD. Data were analyzed by one-way analysis of variance (ANOVA). When $F$ was $< 0.05$, a Bonferroni test was performed for intergroup comparisons. A value of $p \leq 0.05$ was considered significant. Univariate correlations were performed in the overall population using standard techniques.

**RESULTS**

**Blood pressure and heart rate in conscious rats.** Table 1 shows the mean values of intra-arterial mean and pulse blood pressure measured in conscious animals at the end of the eight-week treatment period, 24 h after the last gavage. Spironolactone did not induce any significant change in blood pressure and heart rate when compared to the placebo group. Body weight was not affected by spironolactone. None of the placebo- or spironolactone-treated animals died throughout the treatment period nor during the experimental study.

**Carotid arterial mechanical and histomorphological changes.** For the same mean blood pressure, carotid artery distensibility was significantly higher and carotid elastic modulus significantly lower in spironolactone- than in placebo-treated animals (Table 2), whereas there was no significant change in mean and pulsatile diameter.

Table 3 shows that the carotid and aortic wall thickness were unchanged by treatment, whereas carotid elastin and collagen densities and contents were significantly decreased in the spironolactone-treated rats. The elastin-to-collagen ratio and the number and size of nuclei were unchanged in the spironolactone group. Thoracic aorta was poorly modified, with the exception of a slight increase in elastin density but not content.

Univariate correlations in the overall population showed that carotid collagen density (%) was negatively correlated with carotid distensibility ($r^2 = 0.14; p = 0.02$) and positively correlated with carotid artery elastic modulus ($r^2 = 0.20; p = 0.032$).

**Cardiac structural parameters.** Subendocardial collagen of the left ventricular wall was markedly reduced (80%) by spironolactone. Compared to placebo rats, the value was: 2.94 $\pm$ 0.96 vs. 7.55 $\pm$ 2.00 ($p < 0.0001$). There was also a significant decrease in left ventricular thickness (2.96 $\pm$ 0.38 vs. 2.30 $\pm$ 0.25 mm; $p < 0.0001$), whereas total heart

**Table 1.** Body Weight, Intra-arterial Blood Pressure, and Heart Rate (HR) Data in Conscious Animals

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Spironolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>$689 \pm 61$</td>
<td>$638 \pm 93$</td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>$113 \pm 16$</td>
<td>$111 \pm 11$</td>
</tr>
<tr>
<td>Pulse pressure (mm Hg)</td>
<td>$48 \pm 7$</td>
<td>$43 \pm 7$</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>$388 \pm 37$</td>
<td>$381 \pm 35$</td>
</tr>
</tbody>
</table>

Values are mean $\pm$ SD.

**Table 2.** Measurement of Carotid Hemodynamic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Spironolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>$111 \pm 17$</td>
<td>$107 \pm 10$</td>
</tr>
<tr>
<td>Pulse</td>
<td>$33 \pm 12$</td>
<td>$31 \pm 11$</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>$317 \pm 31$</td>
<td>$325 \pm 26$</td>
</tr>
<tr>
<td>Carotid diameter ($\mu$m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>$1.532 \pm 0.142$</td>
<td>$1.506 \pm 0.087$</td>
</tr>
<tr>
<td>Pulse</td>
<td>$0.071 \pm 0.033$</td>
<td>$0.080 \pm 0.009$</td>
</tr>
<tr>
<td>Carotid compliance ($\text{mm}^2, \text{mm Hg}^{-1 \cdot 10^{-2}}$)</td>
<td>$4.05 \pm 1.79$</td>
<td>$5.31 \pm 1.05$</td>
</tr>
<tr>
<td>Distensibility ($\text{mm Hg}^{-1 \cdot 10^{-3}}$)</td>
<td>$2.17 \pm 0.79$</td>
<td>$2.96 \pm 0.51^*$</td>
</tr>
<tr>
<td>Elastic modulus (kPa)</td>
<td>$0.40 \pm 0.15$</td>
<td>$0.31 \pm 0.07^*$</td>
</tr>
</tbody>
</table>

Values are expressed as mean $\pm$ SD.

$^*p < 0.05$. 
and left ventricular weight were not modified by spironolactone (Table 4). A strong positive correlation between carotid and left ventricular collagen (%) was observed in the totality of the animals ($r^2 = 0.23$; $p = 0.007$). Cardiac collagen was also strongly correlated with left ventricular thickness ($r^2 = 0.40$; $p = 0.0001$). Both carotid and cardiac collagen were not correlated with mean blood pressure.

**DISCUSSION**

In the present study we showed that in old Sprague-Dawley rats, long-term spironolactone did not change blood pressure, heart weight, or carotid and aortic medial thickness significantly. In contrast, significant changes occurred in the arterial extracellular matrix, involving a decrease in carotid elastic and collagen densities and contents. Although these vascular changes were less pronounced than the observed decrease in subendocardial collagen density, they were associated with a significant pressure-independent increase of carotid distensibility and decrease of carotid incremental elastic modulus.

**Histomorphometric changes and blood pressure.** Although the role of mechanical factors as a determinant of arterial hypertrophy has been well documented in hypertensive animals and humans (14–16), there are still discrepancies on the components of the arterial wall (vascular smooth muscle, elastin, collagen) that are the most sensitive to pressure load. There is little doubt that the degree of hypertrophy of the arterial wall is strongly influenced by the level of mean blood pressure according to the Laplace law: 1) several studies in the past have indicated a strong positive relation between arteriolar smooth muscle mass and mean blood pressure levels in untreated rats (14), and 2) after drug treatment of hypertension, there is a parallelism between blood pressure reduction and the decrease in size of arterial smooth muscle (15,16). These observations agree with the changes in medial thickness that we observed previously in studies of SHRs treated by AT1 blockade and CEI (3).

In contrast, aortic collagen accumulation is poorly sensitive to the changes of mean blood pressure. Studies in SHRs in vivo (3) have shown that i) aortic collagen is reduced with CEI but not with dihydralazine for the same decrease in medial thickness and the same mean blood pressure reduction, and ii) aortic collagen accumulation is diminished even with nonantihypertensive doses of the CEI. It seems likely that in SHRs, collagen accumulation, which reflects the presence of a stiff wall material, is rather related to the level of pulsatile pressure than to the level of mean arterial pressure. Studies in verapamil-treated SHRs have shown that aortic structural changes were associated with a substantial decrease of PP but not of mean arterial pressure (17).

In the present study, we showed that the decrease in blood pressure produced by spironolactone in old Sprague-Dawley rats was not significant. It might be argued that, in this investigation, intra-arterial mean blood pressure was measured 24 h after the last drug administration, and that, with spironolactone, mean blood pressure at the drug’s peak effect should be lower. However, in a previous pilot experiment, we showed that the mean blood pressure reduction 3 h after drug administration did not exceed $15 \pm 3 \text{ mm Hg}$ in SHRs (5). Thus, in agreement with several previous studies (8,18,19), it is safe to conclude that only a minimal or a lack of mean blood pressure reduction was obtained in our normotensive animals under spironolactone. This finding contrasts with the reduction in cardiac and carotid collagen observed during the present investigation and concords with several results previously reported for cardiac fibrosis (6–8,18,19). Two specific arguments suggest that the mechanism of reduction of cardiovascular fibrosis under spironolactone is poorly influenced by mechanical factors. First, a triterpene acid derived from *Centella asiatica*, a licorice root derivative that is chemically similar to aldosterone, has been found to enhance collagen synthesis in human skin fibroblasts (20). Second, another mineralocorticoid hormone, deoxycorticosterone, has been shown to increase collagen synthesis in minced rat heart tissue (21).

**Histomorphometric changes and aldosterone antagonism.** In the literature, several in vitro investigations indicate that aldosterone acts directly on large arterial vessels. First, immunohistochemical methods have shown that the intensity of staining of mineralocorticoid receptors within the vascular wall predominates in the aorta and decreases markedly with the size of the arteries (22). Second, endogenous vascular synthesis of aldosterone occurs in the rat mesenteric artery, even after adrenalectomy (23–26). Interestingly, the vascular endothelium should be particularly involved in this synthesis (25). Finally, a direct and rapid

---

**Table 3. Histomorphometric Data of the Thoracic Aorta and of the Carotid Artery**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Spironolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoracic aorta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial thickness (μm)</td>
<td>139.4 ± 12.2</td>
<td>132.0 ± 9.5</td>
</tr>
<tr>
<td>Elastin density (%)</td>
<td>23.98 ± 1.56</td>
<td>25.81 ± 3.00*</td>
</tr>
<tr>
<td>Collagen density (%)</td>
<td>16.78 ± 1.51</td>
<td>16.17 ± 1.04</td>
</tr>
<tr>
<td>Elastin content (μm²/mm)</td>
<td>33.299 ± 1.983</td>
<td>33.758 ± 2.275</td>
</tr>
<tr>
<td>Collagen content (μm²/mm)</td>
<td>23.486 ± 3.739</td>
<td>21.379 ± 2.392</td>
</tr>
<tr>
<td>Carotid artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial thickness (μm)</td>
<td>55.3 ± 5.9</td>
<td>57.7 ± 8.1</td>
</tr>
<tr>
<td>Elastin density (%)</td>
<td>22.25 ± 1.88</td>
<td>20.21 ± 2.02**</td>
</tr>
<tr>
<td>Collagen density (%)</td>
<td>18.41 ± 2.22</td>
<td>16.40 ± 1.31**</td>
</tr>
<tr>
<td>Elastin content (μm²/mm)</td>
<td>12.235 ± 888</td>
<td>11.533 ± 820*</td>
</tr>
<tr>
<td>Collagen content (μm²/mm)</td>
<td>10.239 ± 1,938</td>
<td>9.502 ± 1.874*</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*p < 0.05. **p < 0.001.

---

**Table 4. Spironolactone: Cardiac Structural Data**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Spironolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subendocardial collagen density (%)</td>
<td>7.55 ± 2.00</td>
<td>2.94 ± 0.96**</td>
</tr>
<tr>
<td>Heart weight/body weight ratio</td>
<td>2.5 ± 0.4</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>Left ventricular weight/body weight ratio</td>
<td>2.0 ± 0.3</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

± 1 SD.

**p < 0.001.
effect of aldosterone on sodium transport has also been described in vascular smooth muscle cells (27–30), involving in particular the Na\(^+\)/H\(^+\) antiport and the Na\(^+\), K\(^+\)-ATPase pump (31).

Numerous studies have shown that myocardial fibrosis in response to chronic mineralocorticoid excess and salt loading is independent of the degree of hypokalemia, hypertension, and cardiac hypertrophy (8,18,19). Furthermore, low-dose spironolactone administration has been shown to offset the effects of aldosterone on cardiac fibrosis with minimal changes in blood pressure and cardiac mass (5,8). In the present study, spironolactone caused little change in blood pressure and cardiac hypertrophy but a striking reduction in cardiac collagen accumulation was observed. This reduction was much more pronounced than that observed on the carotid artery. One possibility is that collagen content (but not density) is simply higher in the heart than in the arteries, resulting in a more substantial lowering under spironolactone, according to the low of initial value. Another possibility is that the number and/or sensitivity of mineralocorticoid receptors differ markedly in the heart and in the vessels. An indirect argument in favor of this possibility is that these receptors are in higher number in larger than in smaller arteries (22), resulting in a significant increase in distensibility under spironolactone without significant change in mean arterial pressure and hence arteriolar resistance.

In conclusion, the goal of the present study was not to perform an age-dependent/dose-dependent set of experiments with respect to arterial stiffness and spironolactone treatment. It was only to assess that even in old normotensive rats, spironolactone is able to prevent an age-induced increase in extracellular matrix accumulation together with an improvement of arterial elasticity and that this finding was independent of blood pressure changes. Because in old normotensive rats, a similar effect has not been observed under chronic converting enzyme inhibition, the action of spironolactone might be considered as specific. Thus, spironolactone treatment may be proposed in situations involving a development of extracellular matrix as during aging and hypertension. In the latter situation, particularly in essential hypertension in which a positive statistical association has been observed between increased arterial stiffness and increased aldosterone (32), the clinical relevance of this finding should be investigated.

Acknowledgment
We thank Maryse Deboute for her excellent assistance.

Reprint requests and correspondence: Dr. Michel Safar, Médecine Interne 1, Hôpital Broussais, 96 rue Didot, 75674 Paris Cedex 14, France. E-mail: michel.safar@brs.ap-hop-paris.fr.

REFERENCES