Respective role of humoral factors and blood pressure in cardiac remodeling of DOCA hypertensive rats

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Abstract

Objectives: Recent studies have shown that beside elevated arterial blood pressure, humoral factors such as angiotensin II, aldosterone, endothelin or bradykinin might play a role in the cardiac hypertrophy and fibrosis secondary to hypertension. In addition, it seems that perivascular fibrosis and interstitial fibrosis are controlled by independent mechanisms. Therefore, the goal of our study was to evaluate the respective role of the increased arterial pressure and of humoral factors on cardiac remodeling in an experimental hypertension model. Methods: Uninephrectomized rats received DOCA, a high salt diet, and when hypertension was installed, they were treated for 6 weeks with either a long-acting calcium antagonist, mibefradil (30 mg/kg day⁻¹), an ACE inhibitor, enalapril (3 mg/kg day⁻¹), or a mixed ETA-ET B endothelin receptor antagonist, bosentan (100 mg/kg day⁻¹). A group of hypertensive rats was left untreated and a sham-operated group of normotensive rats was used for control. At the end of treatment, maximal coronary blood flow was measured in isolated perfused hearts. Cardiac hypertrophy and interstitial as well as perivascular fibrosis were evaluated by quantitative morphometry. Results: DOCA-salt hypertensive rats exhibited a marked cardiac hypertrophy associated with a decrease of maximal coronary blood flow and interstitial and perivascular fibrosis. The calcium antagonist nearly normalized arterial pressure and suppressed all these changes. Enalapril had no effect on arterial pressure and perivascular fibrosis but decreased subendocardial fibrosis. Bosentan had a very small effect on arterial pressure but decreased cardiac hypertrophy and both perivascular and subendocardial fibrosis. Conclusions: We conclude that in DOCA salt hypertension, humoral factors such as endothelin may play a role beside high blood pressure in cardiac remodeling. In addition, the different components of this remodeling (decrease of vascular reserve, cardiac hypertrophy and cardiac fibrosis) are controlled independently.

Keywords: Endothelin receptor antagonists; Hypertension; Calcium antagonists; Hypertrophy; Rat

1. Introduction

Human and experimental hypertension are associated with a complex cardiac remodeling combining cardiac hypertrophy [1], decrease of coronary vascular reserve [2,3], interstitial and perivascular fibrosis [4–6]. Interestingly, the increased arterial pressure does not seem to be the only cause of this cardiac remodeling. Drugs such as hydralazine can normalize arterial pressure without affecting cardiac hypertrophy [7,8]. In addition to blood pressure, several humoral factors, circulating or produced locally, are likely to be involved [9,10]. Among them, angiotensin II (Ang II) [11], aldosterone [11], and endothelin [12] seem to play an important role in inducing cardiac fibrosis and hypertrophy. Several studies have shown that inhibition of the renin–angiotensin system with angiotensin converting enzyme (ACE) inhibitors can prevent cardiac hypertrophy [13], the decrease of coronary vascular reserve [14,15] and cardiac fibrosis [16] in spontaneously
or renovascular hypertensive rats. This effect of ACE inhibitors might be achieved not only by decreasing blood pressure, but also by blocking the cardiac formation of angiotensin II or decreasing the local degradation of bradykinin [17]. In addition to its potent vasoactive effect, endothelin-1 (ET-1) has also been shown to be a growth factor. In vitro, ET-1 can induce hypertrophy of cardiomyocytes associated with the induction of muscle-specific gene transcripts [18]. Cultured rat cardiomyocytes express prepro ET-1 transcripts and release mature ET-1 into the culture medium [19], and endothelial cells modulate both cardiac fibroblast collagen synthesis and degradation via ET-1 [20]. However, these experiments have been performed with exogenous administration of endothelin and in vivo data on the blockade of the effects of endogenous endothelin are lacking.

Thus, the aim of the present study was to evaluate the respective role of the elevated arterial pressure and of humoral factors in the cardiac remodeling occurring in experimental hypertension. These experiments were performed in rats subjected to experimental deoxycorticosterone acetate (DOCA) hypertension. This model is characterized by low circulating renin levels due to suppression of the renin–angiotensin system [21,22]. In this model the role of the increased arterial pressure could be assessed by treating the rats with mibebradil, a new long-acting calcium antagonist [23] which is able to nearly normalize arterial pressure in these rats. The role of endogenous endothelin was assessed by blocking both ET\textsubscript{A} and ET\textsubscript{B} receptors with bosentan, a new endothelin receptor antagonist [24]. Finally, the role of angiotensin II (or of the inhibition in bradykinin catabolism) could be assessed by using enalapril, an ACE inhibitor.

2. Methods

2.1. Animal preparation

Six-week-old male normotensive Wistar rats (Füllinsdorf, Switzerland) were anesthetized with sodium hexobarbital (100 mg/kg i.p.). The right kidney was removed through a flank incision, and one pellet of DOCA (40 mg) was implanted subcutaneously over the nape of the neck every 2 weeks. These rats were offered 1% saline to drink. Sham-operated rats were used as control rats and received tap water to drink. Four weeks after surgery, arterial blood pressure of DOCA rats was measured indirectly by the tail-cuff method, and only rats with systolic arterial blood pressure (ABP) > 190 mmHg were selected for the study.

2.2. Study design

Five groups of rats were compared. One group was sham-operated and received no treatment (\(n = 18\)). The selected DOCA hypertensive rats were randomly allocated to 4 different groups and were treated with oral doses of mibebradil (30 mg/kg day\(^{-1}\) as food admix, \(n = 18\)), enalapril (3 mg/kg day\(^{-1}\) as food admix, \(n = 18\)), and bosentan (100 mg/kg day\(^{-1}\) as food admix, \(n = 18\)). The fourth DOCA group of rats was left untreated (untreated DOCA group, \(n = 18\)). Preliminary dose–response experiments using telemetry for the measurements of ABP have shown that these doses of mibebradil and bosentan give a maximal blood pressure decrease (data not shown). Since enalapril has no effect on arterial pressure in this model, we chose the antihypertensive dose which was previously used and shown to be effective in two-kidney, one-clip rats [25]. All the measurements were performed in 9 to 12 randomly selected surviving rats from each group. Systolic ABP was measured in each rat at the middle and the end of the 5-week treatment period. The rats were sacrificed after 5 weeks of treatment.

In order to assess if left ventricular hypertrophy was already established at the beginning of the treatment period, we compared left and right ventricular weights as well as left ventricular surface area in a group of sham-operated rats (\(n = 11\)) versus another group of DOCA rats (\(n = 11\)) subjected to 4 weeks of hypertension.

2.3. Measurements of maximal coronary blood flow (MCFB) in isolated perfused hearts

Rats previously heparinized were anesthetized with sodium hexobarbital, 100 mg/kg i.p., in order to prevent thrombosis of the coronary circulation, and thereafter killed by cervical dislocation. The hearts were isolated, cannulated from the aorta, and retrogradely perfused in a Langendorff apparatus with a modified Krebs-Henseleit solution of the following composition (mM): NaCl 114.7, KCl 4.7, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.5, NaHCO\(_3\) 25, CaCl\(_2\) 2.5, and glucose 11.1. The solution was gassed with 95% O\(_2\)-5% CO\(_2\), and pH was adjusted to 7.3. Coronary arterial flow was measured at the outflow from the right atrium with an electromagnetic flowmeter (Narcomatic RT 500, Narco BioSystems Inc., Houston, TX). During the preparation, the heart was beating but did not work. Maximal coronary vasodilation was obtained by adding adenosine (10\(^{-7}\) M) to the perfusion solution. In each rat, abolition of reactive hyperemia by adenosine was verified. During maximal coronary vasodilation, coronary blood flow was measured at perfusion pressures of 90, 80, 70, 60, and 50 mmHg. After perfusion, the hearts were blotted and weighed.

2.4. Hormonal measurements

Immunoreactive ET-1 was measured by radioimmunoassay as described recently [26]. Plasma renin activities were measured by radioimmunoassay of the angiotensin I generated by the incubation of plasma with an excess of angiotensinogen provided...
in renin-free plasma obtained from rats binephrectomized 24 h previously [27].

2.5. Morphometrical analysis

Quantitative morphometry was performed in order to quantify cardiac hypertrophy (left ventricular wall thickness and area) as well as interstitial and perivascular fibrosis (collagen content and density). The hearts were arrested in diastole using a saturated KCl solution and then fixed under a perfusion pressure of 80 mmHg using 4% paraformaldehyde. The left and right ventricles were dissected, blotted and weighed. Left ventricles were dehydrated with ethanol and xylol and embedded in paraffin. Two 4-µm-thick coronal sections, taken at the equator of the heart, were mounted on glass slide and colored with the collagen-specific stain Sirius red F3BA (Pfaltz and Bauer Inc., Stanford, CT) [28]. Measurements of left ventricular hypertrophy, and collagen volume fraction in the interstitium and in the perivascular areas were determined by quantitative morphometry [29] using a video camera (Sony, Tokyo, Japan) connected to an image analysis processor (Nacpet 1500, Nacpet, Evry, France) and a microcomputer (Macintosh II, Apple, Cupertino, CA). The investigator responsible for the morphometrical analysis was blinded as to each experimental group.

2.6. Measurement of left ventricular hypertrophy

Both myocardial sections were analyzed macroscopically (a complete section per field) using a ×2.5 objective giving a final calibration of \(6.7 \times 10^{-3}\) mm/pixel. Total surface area and left ventricular wall thickness were averaged over both sections per rat.

2.7. Measurement of myocardial collagen content

For measurement of collagen in the interstitium, a study of the running mean and of the running variance allowed to determine that measurement of 20 fields in the subendocardium and 20 fields in the subepicardium was suitable to get a convergent estimation of the variables. Systematic field displacement over subepicardial and subendocardial regions was done with 1 measured field over 3. Therefore, over the 5 groups, a total of 2240 fields was quantified. For each field analyzed, the interstitial collagen density was determined as the ratio of collagen surface area over myocardial surface area. Rare focal areas of necrosis, microscopic scars (thick collagen fibers located in areas previously occupied by myocytes) and all fields containing coronary artery (diameter > 10 µm) were excluded from the analysis. Perivascular collagen (PVC) was measured separately around every coronary artery (diameter > 10 µm) visible in the analyzed field. Only collagen surrounding and connected to the coronary wall was considered as perivascular collagen. The total perivascular collagen sur-

tace area and the perimeter of the coronary lumen were measured for each selected field. The perivascular collagen area per unit of lumen perimeter length was calculated in order to normalize the amount of collagen with respect to the vessel size.

2.8. Statistical analysis

All results are expressed as mean ± s.e.m. All variables were compared by a one-way analysis of variance (ANOVA). Where a significant F value was obtained, the data were further analyzed using Fischer’s PLSD test. A P level of less than 0.05 was considered as significant.

The investigation was performed in accordance with the Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986, published by HMSO, London.

3. Results

3.1. Hemodynamic variables

Before treatment, 5 weeks after nephrectomy, the 4 DOCA-treated groups had equivalent body weights, heart rates and blood pressures (216 ± 5, 215 ± 5, 215 ± 4 and 214 ± 4 mmHg in the untreated DOCA, mibefradil, enalapril and bosentan-treated groups respectively) (Fig. 1). Blood pressure in the sham-operated group was significantly lower (154 ± 2 mmHg). In the untreated DOCA group 2 rats out of 18 died, 1 in the bosentan group and 6 out of 18 in the enalapril-treated group. No animals died in the mibefradil group. The decreased body weight of the surviving hypertensive animals was significantly less marked in the mibefradil group (P < 0.05). As compared to the untreated DOCA group, mibebradil decreased heart rate significantly. Bosentan and enalapril had no effect on heart rate (Table 1).
Table 1
Mortality, body weight and hemodynamic parameters in the five groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Mortality (%)</th>
<th>BW (g)</th>
<th>ABP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>Untreated</td>
<td>0</td>
<td>366±9</td>
<td>155±2</td>
<td>370±5</td>
</tr>
<tr>
<td>DOCA</td>
<td>Untreated</td>
<td>11.1</td>
<td>300±12</td>
<td>240±6</td>
<td>363±9</td>
</tr>
<tr>
<td>DOCA</td>
<td>Mibefradil</td>
<td>0</td>
<td>328±5</td>
<td>175±5</td>
<td>313±8</td>
</tr>
<tr>
<td>DOCA</td>
<td>Enalapril</td>
<td>33.3</td>
<td>322±6</td>
<td>243±5</td>
<td>382±15</td>
</tr>
<tr>
<td>DOCA</td>
<td>Bosentan</td>
<td>5.6</td>
<td>320±11</td>
<td>228±5</td>
<td>352±7</td>
</tr>
</tbody>
</table>

+++ P < 0.001 vs. sham-op.
* P < 0.05, ** P < 0.01, *** P < 0.001 vs untreated DOCA.

BW = body weight, ABP = systolic arterial blood pressure, HR = heart rate.
Mortality is expressed in percent rate of death from the initial group size of 18 rats.

The ABP of the sham-operated rats did not change after the treatment period. In contrast with that of the untreated DOCA group which increased significantly, ABP decreased dramatically with mibefradil although it was still higher than in the sham-operated group (P < 0.01). Blood pressure decrease with bosentan was not statistically significant. Enalapril was totally ineffective on blood pressure (Table 1, Fig. 1).

3.2. Hormonal changes

The endothelin-1 plasma concentration was significantly increased in the untreated DOCA and the enalapril groups (P = 0.04) with respect to the sham-operated group (Fig. 2A). Bosentan increased ET-1 plasma concentrations by two-fold.

All the 4 DOCA groups showed a dramatic drop in plasma renin activities (2.4 ± 0.2, 1.6 ± 0.1, 3.4 ± 0.4, 2.4 ± 0.2 ng Ang I/ml h⁻¹) in the untreated, mibefradil, enalapril and the bosentan groups, respectively) compared to the sham-operated rats (47.0 ± 4.0 ng Ang I/ml h⁻¹) (Fig. 2B).

3.3. Cardiac changes

3.3.1. Coronary reserve

During adenosine infusion, coronary autoregulation was abolished and CBF was linearly related to perfusion pressure (Fig. 3). MCBF measured at a pressure of 90 mmHg was decreased by 45% in untreated DOCA rats, 39% in enalapril-treated rats and 43% in bosentan-treated rats. The mibefradil group was the only one in which MCBF improved significantly (P = 0.005) by 36% with respect to the untreated DOCA group, being 1/4 of that of the sham-operated rats (Table 2).

3.3.2. Left ventricular hypertrophy

After 4 weeks of hypertension and just before the beginning of the treatments, left ventricular hypertrophy was already present as shown by the significant increases in the ratios of left ventricular weight over body weight and the left ventricular area over body weight (Table 3).

Fig. 2. Plasma ET-1 concentrations measured at the end of the treatment period in the five experimental groups (A), and plasma renin activity (PRA) measured by radioimmunoassay of Ang I generated during incubation of plasma samples in vitro (B). * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig. 3. Relationship between perfusion pressure and coronary blood flow measured in the five experimental groups. ** P < 0.01 vs. untreated DOCA rats.
Table 2
Coronary reserve and left ventricular hypertrophy in the five groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>MCBF (ml/min g(^{-1}))</th>
<th>LVW/BW (×1000)</th>
<th>LVA/BW (mm(^2)/g)</th>
<th>LVT/BW (×100) (mm/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>Untreated</td>
<td>23.1 ± 1.2</td>
<td>2.5 ± 0.1</td>
<td>0.15 ± 0.01</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>DOCA</td>
<td>Untreated</td>
<td>12.6 ± 0.7</td>
<td>4.4 ± 0.4 ***</td>
<td>0.26 ± 0.01 ***</td>
<td>0.67 ± 0.06 ***</td>
</tr>
<tr>
<td>DOCA</td>
<td>Mibefradil</td>
<td>17.2 ± 1.0 *</td>
<td>3.3 ± 0.2 **</td>
<td>0.17 ± 0.01 **</td>
<td>0.20 ± 0.04 **</td>
</tr>
<tr>
<td>DOCA</td>
<td>Enalapril</td>
<td>14.0 ± 0.9</td>
<td>3.8 ± 0.2</td>
<td>0.24 ± 0.01</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>DOCA</td>
<td>Bosentan</td>
<td>13.2 ± 0.5</td>
<td>3.9 ± 0.1</td>
<td>0.23 ± 0.01</td>
<td>0.44 ± 0.06 **</td>
</tr>
</tbody>
</table>

+++ \( p < 0.001 \) vs. sham-op.
* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) vs. untreated DOCA.

MCBF = maximal coronary blood flow, LVW = left ventricular weight, LVA = left ventricular area, BW = body weight, LVT = left ventricular wall thickness.

The ratio of right ventricular weight over body weight was slightly but not significantly increased in the DOCA group (Table 3).

Untreated DOCA rats showed a marked left ventricular hypertrophy with significant increases of 73% in the ratio of left ventricular area over body weight (LVA/BW) and of 123% in left ventricular wall thickness over body weight (LVT/BW). Mibefradil and bosentan improved significantly these variables, whereas there was no significant decrease with enalapril (Table 2). The morphometric measures confirmed the increase of 84% in left ventricular weight over body weight in the untreated DOCA group compared to the sham-operated group. The right ventricle absolute weights remained unchanged in the sham-operated and the 4 DOCA groups (0.23 ± 0.01, 0.23 ± 0.02, 0.26 ± 0.02, 0.23 ± 0.02 and 0.23 ± 0.01 g in the sham-operated, untreated DOCA, mibefradil, enalapril and bosentan groups respectively. However, the ratio of right ventricular weight over body weight (×1000) was significantly increased in the DOCA-untreated group compared to the sham-operated group (0.86 ± 0.1 vs. 0.63 ± 0.03, \( p < 0.05 \)). Neither drug had a significant effect on this increase (0.79 ± 0.05, 0.72 ± 0.06, 0.71 ± 0.04 in the mibefradil, enalapril and bosentan groups respectively).

3.3.3. Cardiac fibrosis

Subepicardial interstitial collagen density was similar in all 5 groups (Table 4). In contrast, in the subendocardial region the interstitial collagen density increased by 59% in the untreated DOCA group compared to the sham-operated group. Mibefradil, enalapril and bosentan were beneficial, decreasing significantly the subendocardial interstitial collagen density by 36%, 32% and 25%, respectively, compared to the DOCA-untreated group (Table 4).

Table 3
Arterial blood pressure and left ventricular hypertrophy in DOCA and sham-operated rats before the start of the treatment period

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>BW (g)</th>
<th>ABP (mmHg)</th>
<th>RVW/BW (×1000)</th>
<th>LVW/BW (×1000)</th>
<th>LVA/BW (×1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-op</td>
<td>Untreated</td>
<td>316±7</td>
<td>111±6</td>
<td>0.51±0.02</td>
<td>2.16±0.06</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>DOCA</td>
<td>Untreated</td>
<td>308±4</td>
<td>225±10</td>
<td>0.76±0.17</td>
<td>3.24±0.23 ***</td>
<td>0.21±0.01</td>
</tr>
</tbody>
</table>

* \( p < 0.05 \), +++ \( p < 0.001 \) vs. sham-op.

BW = body weight, ABP = arterial blood pressure, RVW = right ventricular weight, LVW = left ventricular weight, LVA = left ventricular area.

Table 4
Interstitial and perivascular collagen content in the myocardium of the five groups of rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Subepicardial density (%)</th>
<th>Subendocardial density (%)</th>
<th>Perivascular collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total area (mm(^2))</td>
</tr>
<tr>
<td>Sham-op</td>
<td>Placebo</td>
<td>12</td>
<td>2.97±0.25</td>
<td>3.55±0.26</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>DOCA</td>
<td>Untreated</td>
<td>12</td>
<td>2.83±0.32</td>
<td>5.66±0.78 +</td>
<td>0.64±0.11 +</td>
</tr>
<tr>
<td>DOCA</td>
<td>Mibefradil</td>
<td>11</td>
<td>2.94±0.25</td>
<td>3.65±0.30 **</td>
<td>0.30±0.03 **</td>
</tr>
<tr>
<td>DOCA</td>
<td>Enalapril</td>
<td>9</td>
<td>2.27±0.16</td>
<td>3.84±0.54 *</td>
<td>0.48±0.08</td>
</tr>
<tr>
<td>DOCA</td>
<td>Bosentan</td>
<td>11</td>
<td>3.04±0.24</td>
<td>4.27±0.37 *</td>
<td>0.42±0.06 *</td>
</tr>
</tbody>
</table>

++ \( p < 0.01 \), +++ \( p < 0.001 \) vs. sham-op.
* \( p < 0.05 \), ++ \( p < 0.01 \), +++ \( p < 0.001 \) vs. untreated DOCA.

PCL = perimeter of the coronary lumen.
The perivascular collagen also was markedly increased in the untreated DOCA group (Table 4). Mibefradil and bosentan, but not enalapril, decreased significantly this variable (by 53% and 34%, respectively). No treatment was able to completely normalize the perivascular area per unit of perimeter length, but two of them improved it, mibefradil being more effective than bosentan (Table 4).

4. Discussion

The present results show that in DOCA hypertensive rats, blood pressure level is a major effector of cardiac hypertrophy (myocyte growth), but that humoral factors like endothelin may play an important independent role in regulating this hypertrophy as well as collagen accumulation within the left ventricle.

As described previously, DOCA suppressed the renin–angiotensin system. This was shown by the marked decrease of plasma renin activity and by the fact that enalapril had no effect on arterial pressure. Interestingly, despite the very low basal level of plasma renin activity (PRA), enalapril was able to induce a significant reactive rise in PRA. This reactive rise due to the decrease of Ang II [30], shows that in this low-renin model the renin–angiotensin system maintains some functionality of the feedback loop Ang-II–renin. Despite the marked blood pressure reduction, mibefradil did not increase PRA but significantly decreased it. This decrease may be due to a direct effect of mibefradil on renin secretion by juxtaglomerular cells.

DOCA administration increased significantly ET-1 plasma concentrations. Neither mibefradil nor enalapril affected ET-1 plasma levels. Bosentan, however, increased markedly plasma ET-1. As previously reported [31], this increase is due to displacement of ET-1 from ET\textsubscript{A} receptors. Indeed, it has been shown that ET\textsubscript{A} receptors play an important role in the clearance of ET-1 [32]. The fact that bosentan induces a complete blockade of all ET receptors explains a total absence of functional consequences of this increase.

This DOCA model is a severe model of hypertension as shown by the very high blood pressure and by the spontaneous mortality. Enalapril seemed to have increased mortality in this experiment and mibefradil, the only totally effective treatment, seemed to prevent it. However, our study was not designed as a mortality study and the number of rats in each group was not sufficient to draw a firm conclusion. Because of the absence of death in the mibefradil group and the single death in the bosentan group, a selection bias (evaluation of the survivors only) could not have influenced the results in these two groups. This was further confirmed by performing a statistical analysis using a “worst case” scenario, i.e. the dead animal was attributed the worst values of the untreated group. Such an analysis did not change the results for bosentan. However, for enalapril, it is not possible to exclude that the limited effect on interstitial fibrosis was due to the fact that only the survivors were evaluated.

In this experimental model, left ventricular hypertrophy was already present after 4 weeks of hypertension, i.e. before the initiation of the treatments. Therefore, in this case, we cannot dissociate regression and prevention with respect to the effects of the different drugs. Most likely both processes occurred.

The three drugs had very different effects on arterial pressure. Mibefradil was the most efficient and decreased blood pressure to nearly normal levels. Enalapril had no effect. Bosentan decreased arterial pressure by around 15 mmHg but this effect did not reach statistical significance. Therefore, the effects of bosentan on cardiac remodeling are unlikely to be due to a drop of arterial pressure. In the present study we have used maximal effective dose (based on the hemodynamic effects) for each drug, but we cannot exclude that in each therapeutic class lower doses might have had a different effect.

In the present study, we evaluated four different features of cardiac remodeling: (1) the decrease of coronary vascular reserve, (2) the cardiac hypertrophy, (3) the interstitial fibrosis and (4) the perivascular fibrosis. Coronary vascular reserve was assessed by measuring maximal coronary blood flow during maximal coronary vasodilation in isolated perfused hearts. Under these conditions, coronary blood flow depends only on the coronary perfusion pressure and the cross-sectional surface area of the coronary arterioles and capillaries. Thus, maximal coronary blood flow gives a good functional indication of the vascular remodeling including medial vascular wall hypertrophy, decrease of external artery diameter, and ventricular hypertrophy [33].

Cardiac hypertrophy was evaluated not only by weighing the left ventricle but also by measuring the left ventricular wall thickness and surface area which are key determinants of the geometry of the left ventricular wall. Cardiac fibrosis was evaluated by measuring by computer-assisted morphometry the myocardial collagen content using the collagen-specific stain Sirius red [28]. Compared with the hydroxyproline biochemical assay, this technique previously validated [5] has the main advantage of allowing separate estimation of interstitial and perivascular fibrosis and to distinguish them from microscopic scars. In experimental hypertension associated with unilateral renal ischemia, perivascular fibrosis generally appears first and is followed later by interstitial fibrosis [34]. Interestingly deoxycorticosterone has been shown to increase mainly perivascular fibrosis while aldosterone for a similar blood pressure increase has been shown to increase both interstitial and perivascular fibrosis [35].

4.1. Coronary vascular reserve

Coronary vascular reserve, estimated by measuring maximal coronary blood flow, was decreased by 45% in
the DOCA rats. This decrease was in part prevented by mibebradil. Neither enalapril nor bosentan could prevent this decrease despite some effect on cardiac hypertrophy. This suggests that neither endothelin nor angiotensin II or the bradykinin system play an important direct role in the alteration of coronary vascular reserve in DOCA rats. Since only mibebradil is efficacious, it is likely that in this model elevated arterial pressure and consequently left ventricular hypertrophy are the major determinants of the decrease of coronary vascular reserve. This conclusion is also suggested by the inverse correlation existing between maximal coronary blood flow and arterial pressure (Table 2).

4.2. Cardiac hypertrophy

DOCA-salt rats had a marked left ventricular hypertrophy as shown by the increase of left ventricular weight and thickness. Mibebradil almost completely suppressed this hypertrophy. This effect might be due to the marked decrease of arterial pressure or to a direct effect of this calcium antagonist on cardiac growth. Bosentan, despite a very limited effect on arterial pressure, had a beneficial effect on left ventricular thickness which decreased by 34% (P < 0.01). This result confirms in this model several in vitro and in vivo studies which have shown an effect of endothelin on cardiac growth. In vitro, ET-1 induces hypertrophy of cultured rat cardiomyocytes with concomitant induction of several cardiac muscle specific genes such as myosin light chain 2, α actin and troponin I through the possible involvement of protein kinase C activation or intracellular Ca²⁺ mobilization [18]. In vivo, BQ 123, an ET-1 selective receptor antagonist, has been shown to block the increase in left ventricular weight and in the size of cardiomyocytes provoked by aortic banding without affecting arterial pressure [36]. In the present study, we have not determined if endothelin originated from plasma (where it was increased) or was locally synthesized. Cardiomyocytes are able to synthesize and secrete ET-1 [37]. Cardiomyocytes express also ET₄ and ET₅ receptors coupled to phospholipase C via G proteins [38]. Activation of protein kinase C by diacylglycerol formed by phospholipase C activated by endothelin might be the signaling pathway underlying ET-1-induced hypertrophy of cardiomyocytes [39].

Enalapril in this model had no effect on blood pressure and its absence of effect on cardiac hypertrophy is not surprising, even if a markedly depressed but still functional renin–angiotensin system persists in the kidneys.

4.3. Interstitial fibrosis

In the present study, interstitial fibrosis was present mainly in the subendocardium. This preferential location in the subendocardium has been previously described [40,41] and is likely to be due to the higher intramyocardial pressure in this part of the myocardium. Mibebradil dramatically decreased this interstitial fibrosis. Once again this could be secondary to the arterial pressure decrease. However, it is also possible that the effect of mibebradil was due to the blockade of intracellular entry of calcium in cardiac fibroblasts. Intracellular calcium is an important intracellular signal in mediating cardiac fibroblast collagen synthesis and fibroblast proliferation [42]. Such inhibition of calcium intracellular increase might also explain the inhibition of the direct cardiac effects of mineralocorticoids which have been shown to influence fibrosis independently of arterial pressure [35].

Bosentan had also an effect on subendocardial fibrosis showing that in DOCA rats endogenous endothelin may play a role in the regulation of collagen synthesis. In vitro, ET-1 can induce the proliferation of fibroblasts and the production of fibrous tissue [43]. Indeed, endothelial cells modulate both cardiac fibroblast collagen synthesis and degradation [20]. Collagen synthesis can be induced by ET-1 and ET-3 via ET₄ and ET₅ receptors, while collagen degradation seems to be mostly due to ET₅ receptors [12]. Both ET₄ and ET₅ receptors are present in rat cardiac fibroblasts with a predominance of ET₅ receptors [44].

Since enalapril was able to decrease interstitial fibrosis, angiotensin II might be still functionally important locally in the myocardium despite the systemic and renal inhibition of the renin–angiotensin system. Indeed, several in vitro [45,46] and in vivo [6] studies have shown the potential role of angiotensin II to induce fibrosis. However, the absence of effect of enalapril on cardiac hypertrophy suggests that, even within the myocardium, Ang II is markedly down-regulated. Bradykinin degradation might have been decreased locally because of the inhibition of angiotensin-converting enzyme [47]. Bradykinin receptors have been shown to be present in the myocardial fibrosis tissue and in some conditions could be co-localized with angiotensin-converting enzyme [48,49]. Thus, bradykinin might have played a role in the regulation of subendocardial fibrosis.

It is also possible that the endothelin system interacts with the renin–angiotensin system. ET-1 stimulates endothelial ACE and the conversion of Ang I to Ang II [50]. On the other hand, angiotensin II increases ET-1 secretion from vascular endothelial cells in vivo [51], induces prepro ET-1 mRNA in endothelial cells [52], and up-regulates cardiac ET₅ receptors [53]. However, enalapril had no effect on ET-1 concentrations; it is therefore unlikely that inhibition of ET-1 mediates its effect on interstitial fibrosis.

4.4. Perivascular fibrosis

Perivascular fibrosis was completely suppressed by mibebradil and was partially decreased by bosentan. Enalapril had no significant effect. This suggests that in this model, as for interstitial fibrosis, perivascular fibrosis
may depend on locally produced humoral factors such as endothelin besides arterial pressure. Our results concerning the role of humoral factors in cardiac remodeling confirm previous results by Brilla et al. [6] where perivascular fibrosis has been observed in the non-overloaded and non-hypertrophied right ventricles of rats infused with angiotensin II and aldosterone.

4.5. Conclusion

Our results show that blood pressure plays a predominant role in cardiac remodeling of DOCA hypertension and that mibefradil, a new calcium channel blocker, is able to prevent it. The effects of bosentan and enalapril in this model suggest a role for humoral factors such as endothelin and angiotensin.

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