Abstract—Experimental and clinical studies show that aldosterone/mineralocorticoid receptor (MR) activation has deleterious effects in the cardiovascular system that may cross-talk with those of angiotensin II (Ang II). This study, using a transgenic mouse model with conditional and cardiomyocyte-restricted overexpression of the human MR, was designed to assess the cardiac consequences of Ang II treatment and cardiomyocyte MR activation. Two-month-old MHC-TA/tetO-hMR double transgenic males (DTg) with conditional, cardiomyocyte-specific human MR expression, and their control littermates were infused with Ang II (200 ng/kg per minute) or vehicle via osmotic minipump. Ang II induced similar increases in systolic blood pressure in control and DTg mice but a greater increase in left ventricle mass/body weight in DTg than in control mice. In DTg mice, Ang II–induced left ventricle hypertrophy and diastolic dysfunction without affecting systolic function, as assessed by echography. These effects were associated with an increase in the expression of collagens and fibronectins, matrix metalloproteinase 2 and matrix metalloproteinase 9 activities, and histological fibrosis. Ang II treatment of DTg mice did not affect inflammation markers, but oxidative stress was substantially increased, as indicated by gp91 expression, apocynin-inhibitable NADPH oxidase activity, and protein carbonylation. These molecular and functional alterations were prevented by pharmacological MR antagonism. Our findings indicate that the effects of Ang II and MR activation in the heart are additive. This observation may be relevant to the clinical use of MR or of combined Ang II type 1 receptor-MR antagonists for hypertrophic cardiomyopathies or for heart failure, particularly when diastolic dysfunction is associated with preserved systolic function. (Hypertension. 2008;52:1060-1067.)

Key Words: mineralocorticoid ▪ remodeling ▪ heart ▪ oxidative stress

The deleterious role of aldosterone (aldo) in cardiovascular diseases has been documented by the Randomized Aldactone Evaluation Study (RALES) and Eplerenone Post-AMI Heart Failure Efficacy and Survival Study (EPHESUS). Both trials have shown a major additive benefit of the administration of mineralocorticoid receptor (MR) antagonists on top of blockers of inhibitors of angiotensin-conversion enzyme (ACE) or blockers of the angiotensin II (Ang II) type I receptor (AT1R). Pharmacological MR antagonism has also been shown to provide organ protection in several animal models (spontaneously hypertensive rats and uninephrectomized rats treated with aldo-salt, heart failure after myocardial infarction, or aortic banding). However, we know little about the mechanisms underlying the interplay involving aldo- and Ang II–induced damage in the development of myocardial remodeling and in the progression of cardiovascular diseases.

Ang II appears to be a major contributor to cardiovascular damage. There is increasing evidence that aldo, acting via MR, may mediate or even exacerbate the damaging effects of Ang II. Robert et al reported that administration of the AT1R antagonist losartan fully prevented both the increases in collagen I and III mRNAs and also collagen accumulation in the aldo-salt rat model, indicating cross-talk between AT1R activation and MR activation. Similar results have been reported by Sun et al. Salt loading and uninephrectomy are necessary in most models to generate both cardiac fibrosis and high circulating aldo levels, although Iglarz et al reported cardiac remodeling and fibrosis in rat infused with aldo alone. In all of the cases, cardiac fibrosis was prevented by AT1R antagonism. This AT1R/MR cross-talk is not restricted to the heart, and MR antagonism is also beneficial in renal and vascular remodeling. Indeed, synergistic effects between aldo/MR activation and Ang II have been reported in vascular smooth muscle cells, suggesting that this interaction is a common mechanism and may be an essential component for the effects of the renin-angiotensin-aldosterone system in the cardiovascular system.
The blood was collected by aorta puncture, and plasma was isolated. Appropriate, canrenoate (20 mg/kg per day) was administered in the drinking water for 2 months via osmotic minipumps (Alzet, Charles River Laboratories, Inc; Figure S1, please see the data supplement online at http://hyper.ahajournals.org). When angiotensin II (200 ng/kg per minute) or vehicle was infused for 2 months via osmotic minipumps, littermates were used as controls (Ctls). Ang II (200 ng/kg per minute) was given to the pregnant mothers containing doxycycline (1 g/kg) was given to the pregnant mothers to obtain MHCtTA/tetO-hMR double transgenic (DTg) mice with conditional, cardiomyocyte-specific human MR expression. To preclude the embryonic lethality reported previously in DTg mice,11 food containing doxycycline (1 g/kg) was given to the pregnant mothers until birth of the progeny to avoid human MR expression in the pups. Two-month-old DTg males were used for this study, and the littersmates were used as controls (Ctls). Ang II (200 ng/kg per minute) or vehicle was infused for 2 months via osmotic minipumps (Alzet, Charles River Laboratories, Inc; Figure S1, please see the data supplement online at http://hyper.ahajournals.org). When appropriate, canrenone (20 mg/kg per day) was administered in drinking water. The animals were euthanized at the end of treatment. The blood was collected by aorta puncture, and plasma was isolated by centrifugation for determination of plasma aldosterone levels. Hearts were removed, rinsed in PBS, weighed, cut into several transverse slices, frozen in liquid nitrogen, and kept at −80°C. The use of animals was in accordance with the guidelines of the European Community and approved by our institutional animal care and use committee.

**BP, Urine Collection, and Echocardiographic Analysis**

The BP was measured by the tail-cuff method, as published previously.12 Twenty-four-hour urine samples were collected in individual metabolic cages (Phymep Marty Technology) for 8 days. Urine aldosterone concentration was determined by radioimmunoassay (Diagnostics Products). Echocardiography was performed on lightly anesthetized mice (isoflurane, Abbot, in oxygen), as described previously.13 Briefly, the heart was visualized in the long axis parasternal view for M-mode left ventricle (LV) dimension measurement and posterior wall pulse wave tissue Doppler measurement. An apical 4- to 5-chamber view was obtained from the subcostal view for diastolic function assessment with pulse wave spectral LV inflow and outflow and for pulse wave tissue Doppler measurement of the mitral annulus velocities.13

Real-Time RT-PCR and Western Blots

Detailed methods are given in the data supplement.

**Ventricular Fibrosis**

Ventricular samples were fixed in formalin and mounted in paraffin-embedded blocks. Sections 7-μm thick were cut, and ventricular fibrosis was measured after Sirius red staining. Image analysis was performed in a blinded fashion using IPLab software (IPLab).

**Gelatin Zymography**

Matrix metalloproteinase (MMP) 2 and MMP-9 activities were measured by gelatin zymography. Ventricular extracts containing 40 μg of proteins were analyzed by electrophoresis on 10% SDS-PAGE containing 1 mg/mL of gelatin (Sigma-Aldrich, United Kingdom) under nonreducing conditions, as described previously.14 Bands appeared as clear areas of lysis against a blue background and were quantified using the Multigauge software (Las3000, Fuji). Lung extract was used as a positive control.

**NADPH Oxidase Activity Assay**

NADPH-dependent superoxide production by 100-μg aliquots of ventricular homogenates was assayed in modified HEPES buffer (140 mmol/L of NaCl, 5 mmol/L of KCl, 0.8 mmol/L of MgCl₂, 1.8 mmol/L of CaCl₂, 1 mmol/L of Na₂HPO₄, 25 mmol/L of HEPES, and 1% glucose [pH 7.2]) in a 96-well microplate luminometer (GloMax, Promega) in the presence of NADPH (300 μmol/L, Sigma-Aldrich) and dark-adapted lucigenin (5 μmol/L, Sigma-Aldrich). Measurements were made in duplicate. The NADPH oxidase inhibitor, apocynin (300 μmol/L, Sigma-Aldrich), was used to confirm that the assay was determining reactive oxygen species (ROS) production from NADPH oxidase.

**In Situ Detection of Superoxide**

ROS production in situ was detected with dihydroethidium. Cryosections were air dried at room temperature and stained with dihydroethidium (3.5 μmol/L) in PBS for 30 minutes in the dark. The slides were then rinsed with PBS and coverslipped. Analysis and image recording were performed with a Leica microscope equipped for epifluorescence. All of the images were recorded with the same time, light, and contrast parameters. Two independent observers scored the results in a blind manner for semiquantitative analysis: fluorescence was scored from 0 (no signal) to 6 (most nuclei).4

Oxyblot

The formation of carbonyl groups in the proteins was assessed with the Oxyblot oxidized protein detection kit, following the manufacturer’s instructions (Chemicon International). Aliquots of 10 μg of protein were used for each assay. To quantify protein oxidation, we defined an oxidation index as the ratio of the densitometric value of the Oxyblot bands to that of the corresponding bands stained with Ponczeau Red (Las3000 DarkBox, Fuji Photo Film Europe, GMBH).

**Statistics**

Data are expressed as means±SEMs. Differences between groups were assessed with multifactor ANOVA, using JMP-6 software (SAS Institute, Inc). Values of P<0.05 were considered significant.

**Results**

Ang II infusion for 8 weeks induced similar increases in systolic BP in Ctl and DTg mice (Table 1). Steady-state plasma and urinary aldosterone levels were similar in Ang II–treated Ctl and DTg mice (Table 1). The canrenone dose used had no effect on these variables. Both DTg and Ang II–treated control mice showed cardiac hypertrophy, as assessed from the heart:body weight ratio or heart:tibia length ratio (Table 1); however, the LV end diastolic diameter:body weight ratio.
estimated by echocardiography (Table 2) indicated the absence of LV dilation. The heart/body weight ratio was higher in Ang II–treated DTg mice indicating an additive effect of Ang II and MR signaling (Table 1). Ang II–treated DTg mice displayed LV hypertrophy associated with a significant left atrium (LA) enlargement (Table 2).

We studied the functional consequences of the cardiac hypertrophy associated with cardiac MR expression and Ang II challenge (Table 2). Systolic function was assessed by studying several variables, including ejection fraction and tissue Doppler imaging; there were no significant differences between the experimental groups. Ang II treatment in control mice did not change diastolic echographic variables (Table 2). In contrast, MR-overexpressing mice treated with Ang II showed an increased isovolumic relaxation time and decreased tissue Doppler diastolic wave velocity without any increase in LV filling pressure, as indicated by the unchanged maximal velocity of the LV inflow:maximal diastolic velocity of the mitral annulus ratio (Table 2); thus, they suffered from LV diastolic dysfunction. Pharmacological antagonism of MR by canrenoate prevented cardiac hypertrophy and diastolic dysfunction (Tables 1 and 2).

### Table 1. Global Parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Ctl</th>
<th>Ctl + A</th>
<th>Ctl + A + C</th>
<th>DTg</th>
<th>DTg + A</th>
<th>DTg + A + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma aldosterone, pg/mL (n = 7 to 8)</td>
<td>104±9</td>
<td>107±12</td>
<td>101±13</td>
<td>103±10</td>
<td>108±10</td>
<td>102±7</td>
</tr>
<tr>
<td>Urine aldosterone, pg/mL (n = 4 to 8)</td>
<td>255±29</td>
<td>240±60</td>
<td>236±11</td>
<td>253±28</td>
<td>257±30</td>
<td>251±16</td>
</tr>
<tr>
<td>BP, mm Hg (n = 7 to 8)</td>
<td>103±3</td>
<td>154±2*</td>
<td>150±2*</td>
<td>108±7</td>
<td>156±3.6†</td>
<td>146±2†</td>
</tr>
<tr>
<td>Body weight, g (n = 7 to 15)</td>
<td>31.4±0.8</td>
<td>31.7±0.6</td>
<td>30.9±0.4</td>
<td>32.3±0.6</td>
<td>32.3±0.6</td>
<td>29.7±0.4</td>
</tr>
<tr>
<td>HW/BW, mg/g (n = 7 to 15)</td>
<td>5.3±0.1</td>
<td>6.9±0.2*</td>
<td>5.6±0.1</td>
<td>6.6±0.2*</td>
<td>7.6±0.1*§</td>
<td>6.3±0.2*</td>
</tr>
</tbody>
</table>

Ctl + A indicates Ctl treated with angiotensin; Ctl + A + C, Ctl treated with angiotensin and canrenoate; DTg + A, DTg treated with angiotensin; DTg + A + C, DTg treated with angiotensin and canrenoate; LV EDD, LV end diastolic diameter; BW, body weight; EF, ejection fraction; Vcfc, velocity shortening of circumferential fibers; Sa and Spw, maximal systolic velocity of the mitral annulus and posterior wall; IVRT, isovolumic relaxation time; Ea and Epw, maximal diastolic velocity of the mitral annulus and the posterior wall; E, maximal velocity of the LV inflow.

### Table 2. Echocardiographic Evaluation of the Effects of Ang II Treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctl</th>
<th>Ctl + A</th>
<th>Ctl + A + C</th>
<th>DTg</th>
<th>DTg + A</th>
<th>DTg + A + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>13</td>
<td>8</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>35±0.9</td>
<td>35±1.5</td>
<td>31±0.6</td>
<td>33.8±0.9</td>
<td>34.6±1.2</td>
<td>28.8±0.9†‡§</td>
</tr>
<tr>
<td>Age, d</td>
<td>122±4</td>
<td>121±5</td>
<td>110±1</td>
<td>118±4</td>
<td>120±3</td>
<td>107±1</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>556±14</td>
<td>531±8</td>
<td>519±10</td>
<td>487±14</td>
<td>479±16</td>
<td>519±13</td>
</tr>
<tr>
<td>Cardiac remodeling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA, mm</td>
<td>2.49±0.09</td>
<td>2.81±0.11</td>
<td>2.76±0.11</td>
<td>2.77±0.1</td>
<td>2.97±0.12*</td>
<td>2.65±0.13</td>
</tr>
<tr>
<td>LV EDD/BW, mm/g</td>
<td>0.12±0.001</td>
<td>0.12±0.01</td>
<td>0.14±0.002</td>
<td>0.14±0.004</td>
<td>0.14±0.01</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>LV mass/BW, mg/g</td>
<td>4.29±0.16</td>
<td>5.14±0.27</td>
<td>5.2±0.35</td>
<td>5.04±0.19</td>
<td>6.28±0.47†§</td>
<td>5.65±0.47</td>
</tr>
<tr>
<td>LV systolic function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF, %</td>
<td>80±4</td>
<td>74±2</td>
<td>83±2§</td>
<td>76±3</td>
<td>78±3</td>
<td>81±3</td>
</tr>
<tr>
<td>Vcfc, circumference per second</td>
<td>3.29±0.28</td>
<td>2.55±0.18</td>
<td>3.27±17§</td>
<td>2.92±0.14</td>
<td>3.15±0.2</td>
<td>3.34±0.2§</td>
</tr>
<tr>
<td>Sa, cm/s</td>
<td>3.14±0.19</td>
<td>2.69±0.13</td>
<td>2.56±0.09</td>
<td>2.87±0.11</td>
<td>2.53±0.17</td>
<td>2.65±0.19</td>
</tr>
<tr>
<td>Spw, cm/s</td>
<td>3.84±0.16</td>
<td>3.01±0.15</td>
<td>2.97±0.15*</td>
<td>3.33±0.12</td>
<td>3.07±0.21</td>
<td>2.94±0.22</td>
</tr>
<tr>
<td>LV diastolic function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>16.5±0.7</td>
<td>18±0.65</td>
<td>17.7±0.42</td>
<td>18.2±0.79</td>
<td>21.8±1.44§</td>
<td>19.1±3.32</td>
</tr>
<tr>
<td>Ea, cm/s</td>
<td>4.7±0.27</td>
<td>4.24±0.23</td>
<td>4.43±0.2</td>
<td>4.25±0.22</td>
<td>3.64±0.29</td>
<td>4.42±0.33</td>
</tr>
<tr>
<td>Epw, cm/s</td>
<td>4.95±0.36</td>
<td>4.39±0.25</td>
<td>4.13±0.32</td>
<td>4.06±0.15</td>
<td>3.56±0.13*§</td>
<td>3.7±0.19</td>
</tr>
<tr>
<td>E/Ea</td>
<td>22±1.7</td>
<td>25.2±1.54</td>
<td>22±0.74</td>
<td>23±0.9</td>
<td>26±2.9</td>
<td>22±1.48</td>
</tr>
</tbody>
</table>

Ctl + A indicates Ctl treated with angiotensin; Ctl + A + C, Ctl treated with angiotensin and canrenoate; DTg + A, DTg treated with angiotensin; DTg + A + C, DTg treated with angiotensin and canrenoate; LV EDD, LV end diastolic diameter; BW, body weight; EF, ejection fraction; Vcfc, velocity shortening of circumferential fibers; Sa and Spw, maximal systolic velocity of the mitral annulus and posterior wall; IVRT, isovolumic relaxation time; Ea and Epw, maximal diastolic velocity of the mitral annulus and the posterior wall; E, maximal velocity of the LV inflow.

*P<0.05 vs Ctl.
†P<0.05 vs DTg.
‡P<0.05 vs DTg + A.
§P<0.05 vs Ctl + A.
Then, we studied alterations of the extracellular matrix possibly associated with cardiac remodeling involved in diastolic dysfunction by analyzing interstitial collagen deposition and the mRNAs for collagen 1a (Col1a) and collagen 3a (Col3a). Histological staining with Sirius red showed that both Ang II infusion and cardiac MR overexpression caused a significant increase in cardiac fibrosis (Figure 1A and 1B). The combination of the 2 factors led to an additive increase in interstitial fibrosis (Figure 1A and 1B). The abundance of Col1a and Col3a mRNAs was greatly increased by either Ang II treatment or cardiac MR overexpression, and the combination of both led to an increase of ≈100-fold (Figure 1C and 1D). The rise in collagen gene expression was associated with a similar increase in fibronectin gene expression (Figure S2).

MR antagonism with canrenoate had no effect on the Ang II–induced fibrosis or Col1a/Col3a/fibronectin expression in control mice, but it strongly reduced fibrosis (Figure 1A and 1B) and the expression of Col1a/Col3a/fibronectin (Figures 1C, 1D, and S2) in mice with combined Ang II treatment and cardiac MR overexpression. CTGF (a profibrotic factor likely involved in cardiac Ang II–induced fibrosis) was upregulated in Ang II–treated mice and not affected by cardiac MR expression or by MR antagonism (Figure 1E). Extracellular matrix turnover, mediated through MMPs, may be involved in the observed fibrosis. Both Ang II administration and cardiac MR overexpression increased MMP-2 and MMP-9 activity assessed by gelatin zymography (Figure 1F through 1H); the effect when Ang II was administered to MR-overexpressing mice was additive, and canrenoate administration prevented the additive effect (Figure 1F through 1H). Neither ACE nor AT1R mRNA expression was modified by cardiomyocyte MR overexpression (Figure S3A and S3B), whereas AT1R expression was increased by Ang II treatment (Figure S3B).

To investigate the underlying mechanisms leading to the Ang II–worsened cardiac remodeling when cardiomyocyte MR is activated, we studied inflammation and myocardial oxidative stress in our model. Macrophage infiltration was studied by cardiac immunostaining with the F4/80 antibody directed against a macrophage epitope or by testing for CD68 gene expression: neither Ang II treatment nor cardiomyocyte MR overexpression induced macrophage infiltration (Figure S4A and S4B). The monocyte chemoattractant protein 1 inflammation marker was not increased in the heart by MR overexpression (Figure S4C). However, Ang II administration increased monocyte chemoattractant protein 1 expression, and this increase was not altered by canrenoate treatment, indicating that MR activation was not involved in this Ang II effect. Cardiac mRNA expression of several markers of inflammation, like intercellular adhesion molecule, vascular cell adhesion molecule, or tumor necrosis factor-α, was not altered by systemic Ang II or cardiomyocyte MR overexpression (Figure S4D through S4F). Importantly, the expression of inflammation markers was not enhanced by the
combination of cardiac MR overexpression and Ang II treatment.

We next analyzed the expression of the NADPH oxidase subunits involved in oxidative stress. There were no differences for the expression of p47 and p67 subunits between DTg and Ctl mice (Figure S5A through S5D); p47 and p67 subunit mRNA expressions, but not protein levels, were more abundant after Ang II treatment, and this effect was not reduced by MR inhibition with canrenoate (Figure S5A through S5D). The absence of difference between Ang II–treated control and Ang II–treated MR transgenic mice (Figure S5A through S5D) indicated that there was no interaction between Ang II and MR pathways concerning these subunits. By contrast, the abundance of the gp91 (NADPH oxidase 2 [NOX2]) subunit mRNA was increased both by Ang II (20-fold increase) and by cardiac MR overexpression (12-fold increase; Figure 2A), the combination of both resulting in a 90-fold increase in gp91 mRNA (Figure 2A). Western-blot analysis showed increases, albeit of lower amplitude, in the amount of gp91 protein (Figure 2B): the combination of Ang II treatment and MR overexpression induced the largest increase in gp91 protein level, and this was partly prevented by canrenoate treatment (note that the effect of Ang II alone was not influenced by MR antagonism). The functional consequences of increased NOX2 expression were investigated through analyses of NADPH activity, superoxide production, and protein carbonylation. Maximal apocynin-inhibitable NADPH oxidase activity was similarly induced by each Ang II treatment and cardiac MR overexpression; the combination of the 2 had a greater effect than either individually (Figure 3A). Canrenoate treatment, which had only a minor effect in control mice treated with Ang II, prevented the increase in NADPH oxidase activity in Ang II–treated DTg mice, suggesting the involvement of MR. Production of superoxide, estimated using a semiquantitative approach based on dihydroethidium...
staining of cardiac tissue (Figure 3B), was significantly higher in Ang II–treated DTg than in Ctrl mice, the effect being prevented by canrenoate administration (Figure 3B). Interestingly increased oxidative stress was associated with a 30% increase in the oxidized protein amount, as estimated by protein carbonylation (Figure 3C).

**Discussion**

We report evidence that interactions between systemic Ang II and cardiac MR-mediated pathways lead to increased fibrosis and cardiac remodeling, possibly as a consequence of a combined action on oxidative stress in the absence of inflammation. Combined increases of systemic Ang II and cardiac MR signaling were associated with LV hypertrophy and diastolic dysfunction, and this was prevented by pharmacological MR antagonism, identifying MR as a potential therapeutic target in diastolic heart failure.

The renin-angiotensin-aldosterone system is involved in the development of cardiac fibrosis,16,17 and there is evidence implicating Ang II as a profibrotic factor.18 MR activation also favors cardiac fibrosis and remodeling in both human and animal models. A biomarker of collagen turnover, the circulating procollagen type III amino terminal peptide, is decreased by pharmacological MR antagonism in patients with severe heart failure.19,20 Pharmacological activation of MR in the aldo-salt model results in extensive miogran fibrosis and remodeling,17 and AT1R activation has been proposed to contribute to the aldo-induced fibrosis.3–5 Conversely, MR antagonism or pharmacological inhibition of aldosterone synthase with FAD 286 decreases organ damage associated with Ang II in the double transgenic rat overexpressing human renin and angiotensinogen.21,22 Spironolactone treatment prevents increased cardiac interstitial fibrosis induced by Ang II in rats23 or mice.24 These various findings are consistent with there being a functional interaction between the activation of the MR and AT1R in the heart.25 However, the multiple systemic changes present in the aldo-salt or aldo-infused animals make it difficult to dissect the local mechanisms responsible for this cross-talk. It is not known whether these interactions are related to direct effects of aldosterone on cardiomyocytes, to hemodynamic changes, to sodium load, or to the consequences of renal MR activation on homeostasis. Moreover, the heart is a complex organ composed of different cell types, including cardiomyocytes, fibroblasts, inflammatory cells, and vascular cells. All of them have been implicated in the cardiac effect of aldosterone, but the precise role of MR signaling within cardiomyocytes in the action of cardiac mineralocorticoids has not been elucidated. The combination of genetic and pharmacological approaches that we used in this study precluded confounding effects of systemic activation of MR signaling pathways, and, consequently, we were able to study the interaction between systemic Ang II and local increase in MR activation. We cannot, however, discriminate between AT1R and Ang II type 2 receptor implications. This would require further experiments using specific pharmacological antagonists.

We demonstrate that cardiomyocyte MR overexpression, at variance from previously reported effects of the aldo/salt/uninephrectomy challenge, which may stimulate MR in other cell types than the cardiomyocyte. Increased local renin-angiotensin-aldosterone system activity has been suggested to be the mechanism by which aldosterone induces cardiac fibrosis. Both losartan and spironolactone treatment prevent the increases in AT1R density and AT1R mRNA level, as reported by Robert et al.3 Increased ACE in the perivascular space and fibrotic scars, but not in cardiomyocytes, was observed by Sun et al4 in aldo-salt rats. The expression of ACE and AT1R in DTg mice was the same as in controls, suggesting that downstream events secondary to chronic activation of cardiac MR and Ang II receptors are involved in this cross-talk. Jaffe and Mendelsohn5 showed that Ang II may activate the MR in vascular smooth muscle cells via AT1R, but the pathways are unknown. Our results suggest that this mechanism may also operate in cardiomyocytes.

It has been proposed that the mechanisms by which mineralocorticoids induce cardiac fibrosis involve the induction of oxidative stress,26 whereas the profibrotic effects of Ang II involve the redox-sensitive signaling pathway.24,27 MR blockers decrease Ang II–induced inflammation, fibrosis, and oxidative stress.24 MR blockade, independent of systolic BP reduction, also improves oxidative stress–induced structural and functional changes associated with an increase renin production (ren2 transgenic rat model).28 Therefore, this pathway may also be involved in cardiac MR/Ang II cross-talk. We report a cumulative effect of Ang II and MR overexpression on the expression of the main NADPH oxidase subunit, gp91/NOX2, without increasing the expression of the p67 and p47 subunits. The gp91/NOX2 includes flavin-adenine-dinucleotide– and NADPH-binding domains in its transmembrane regions and is essential for the activity of the NOX2-containing NADPH oxidase. NOX2 is responsible for the Ang II–induced cardiac hypertrophy and aldosterone-induced cardiac fibrosis, as elegantly demonstrated using NOX2 knockout mice.24 NOX2 is also expressed in cardiomyocytes,29 so the increase in NOX2 subunit expression that we report may be the link between cardiac MR activation and fibrosis. It has been suggested that the aldosterone-induced oxidative stress originates from inflammatory cells.30 One of the original findings of our work is that the cardiomyocyte MR can transduce a signal that led to increased oxidative stress, without detectable inflammatory processes. This finding in vivo confirms and extends previous data showing that MR activation stimulated the generation of ROS in isolated adult rat ventricular cardiomyocytes.31

Another original finding of our in vivo study is that the molecular alterations induced by the combination of Ang II and cardiomyocyte MR overexpression have important functional consequences mostly affecting LV diastolic function and LA remodeling with LA enlargement. This is reminiscent of clinical data indicating that pharmacological MR antagonism has beneficial effects on diastolic heart failure. The Resource Utilization Among Congestive Heart Failure clinical trial showed recently that more than half of heart failure patients have diastolic heart failure (DHF).32 The major modification in patients with DHF is diastolic dysfunction, including alterations of relaxation and filling, whereas sys-
tolic LV function is normal. It has been proposed that the development of cardiac fibrosis may account for the development of diastolic dysfunction in DHF patients, with the functional alteration typically described in infiltrative cardiomyopathies, such as sarcoidosis or Fabry disease.15 Our study showed that MR might be important in LA remodeling, because LA enlargement was observed in MR mice treated with Ang II and was prevented by MR blockade. Our study, however, cannot differentiate between the functional consequences of the greater LV hypertrophy induced with Ang II and cardiac MR overexpression and a direct effect related to the molecular consequences of the combination of Ang II treatment and cardiac MR overexpression. Nevertheless, blockade of MR, Ang II, or of both could be of clinical benefit in a situation in which LV hypertrophy is associated with diastolic dysfunction. The role of MR activation in DHF has been assessed in 2 clinical studies, but both included only small samples. Mottram et al16 demonstrated that MR antagonism by spironolactone, in the absence of hemodynamic effects, improved LV function in hypertensive patients with DHF. Similar findings were reported by Orea-Tejeda et al17 in DHF patients: they report favorable effects of spironolactone treatment on top of standard therapy, including ACE inhibitors and AT1 antagonists. Mineralocorticoid signaling has been implicated recently in the transition to heart failure in elderly dogs with hypertensive heart disease associated with diastolic dysfunction.36 Most interestingly, increased expression of MR in cardiac tissue was reported in a hypertensive DHF experimental model (Dahl-sensitive rats fed an 8% NaCl diet); this increased cardiac MR level may account for the beneficial effect of eplerenone on diastolic function reported.37 Our findings clearly support the involvement of MR in diastolic dysfunction.

Clinical Perspectives

Our study provides new insights into the involvement of MR in early cardiac alterations and particularly into the functional consequences of LV fibrosis and hypertrophy with diastolic dysfunction and LA remodeling. Therapeutic MR blockade may decrease both LV diastolic dysfunction and LA remodeling, possibly preventing the occurrence of severe complications.

Acknowledgments

We thank Martine Muffat-Joly for assistance with blood pressure analysis and the Centre d’Explorations Fonctionnelles Intégrées at Bichat Federative Research Institute 02 for help with phenotypic characterization. Marie-Paule Jacob (INSERM U698, Paris, France) was a great help for setting up the zymography assay.

Sources of Funding

A.D.Z. is supported by a Société Française d’Hypertension Arterielle/MSD research grant and a fellowship from the French Medical Academy. A.N.D.C. held a PhD fellowship from the Region Ile de France. This work was supported by grants from the AVENIR INSERM program, the Agence Nationale de la Recherche (ANR08-PC090005), the Fondation de France, and the Centre de Recherche Industrielle et Technique.

Disclosures

None.

References


Cross-Talk Between Mineralocorticoid and Angiotensin II Signaling for Cardiac Remodeling

An Di Zhang, Aurelie Nguyen Dinh Cat, Christelle Soukaseum, Brigitte Escoubet, Aïcha Cherfa, Smail Messaoudi, Claude Delcayre, Jane-Lise Samuel and Frederic Jaisser

Hypertension. 2008;52:1060-1067; originally published online November 3, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.117531

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/52/6/1060

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2008/11/03/HYPERTENSIONAHA.108.117531.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT TO

Cross-talk between mineralocorticoid and angiotensin II signaling for cardiac remodeling

An Di ZHANG 1,2,3, Aurelie NGUYEN DINH CAT* 1,2,3, Christelle SOUKASEUM * 1,2,3, Brigitte ESCOUBET 1,4,5, Aïcha CHERFA 5,6, Smail MESSAOUDI 3,7, Claude DELCAYRE 3,7, Jane-Lise SAMUEL 5,7, Frederic JAISSER 1,2,3

1 Inserm, U772, Paris, F-75005, France;
2 Collège de France, Paris F-75005, France;
3 Univ Paris Descartes, Paris, F-75005, France,
4 Assistance Publique-Hôpitaux de Paris, Hôpital Bichat, Paris, F-75018, France;
5 University Denis Diderot, Paris 7, Paris, F-75018, France;
6 EA 3508, paris, F-75006, France;
7 INSERM U689 ; Paris, F-75010, France.

* These two authors contributed equally to the study

Corresponding AUTHOR:

Frederic JAISSER

Tel 33 1 4427138 / FAX: 33 1 44271591

Email: frederic.jaisser@college-de-france.fr
Supplementary method

Real-time-polymerase chain reaction (RT-PCR)
Total RNA was extracted from cardiac ventricle tissue using the TRIZOL reagent (Invitrogen, Carlsabad, CA). Briefly, 2 µg of total RNA was reverse-transcribed with 200 units of reverse transcriptase using the Superscript™ II kit (Invitrogen, Carlsabad, CA) according to the manufacturer’s recommendations. The RT products were then amplified on a MJ Research PTC-200 PCR apparatus, using Taqman Gene Expression Assays for collagen-I-a, collagen-III-a and Hypoxanthine Phospho Ribosyl Transferase (HPRT) as reference genes (Col1a: Mm00801666, Col3a: Mm00802331, HPRT: Mm00446968). For gp91(NOX2), p47, p67, TNFa, ICAM, VCAM, iCycle iQ apparatus (Biorad Laboratories, Paris, France) was used for RT-PCR with SYBER Green using primers described in Supplementary Table S1. All samples were tested in duplicate and quantitative relative values were calculated by Delta-Delta-Ct method (Analysis of relative gene expression data using real-time quantitative PCR and the 2^[(-delta delta C(T))]_m`ethod)\textsuperscript{14}.

Western blotting for p91, p47 and p67 proteins
Heart samples were homogenized in sodium dodecylsulfate (SDS) lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics, Meylan, France). Proteins (10 µg) were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Biosciences Europe GMBH, Orsay, France). The membranes were incubated with anti-gp91 (1/1000, BD Transduction laboratories, Canada), anti-p47 or anti-p67 (1/1000, Santa Cruz Biotechnologie, U.S.A). Bound antibody was visualized by using horseradish peroxidase-conjugated secondary antibody (Amersham) and ECL Plus reagents
(Amersham) and signals were acquired in a Las3000 DarkBox (Fuji photo Film Europe GMBH, Düsseldorf, Germany). GAPDH was used as internal control for protein loading.

**Immunostaining with F4/80**

Cryostat sections were dried in air, fixed in acetone, and incubated sequentially with a rat anti-mouse F4/80 antigen antibody (Abcam, UK) (1:500), a biotinylated anti-rat IgG(H+L) antibody (Vector, Burlingame, CA) an avidin/peroxidase complex (ABC kit; Vector); bound antibody was visualized with Histogreen (AbCys, France).
## Supplementary Table

### Table S1 Primers’ sequences

<table>
<thead>
<tr>
<th>gene</th>
<th>forward</th>
<th>reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTGF</td>
<td>5' - TGA CCC CTG CGA CCC</td>
<td>5' - TAC ACC GAC CCA CCG</td>
</tr>
<tr>
<td></td>
<td>ACA -3'</td>
<td>AAG ACA CAG -3'</td>
</tr>
<tr>
<td>fibronectin</td>
<td>5' - CCG GTG GCT GTC AGT</td>
<td>5' - CCG TTC CCA CTG CTG ATT</td>
</tr>
<tr>
<td></td>
<td>CAG A -3'</td>
<td>TAT C -3'</td>
</tr>
<tr>
<td>P47-phox</td>
<td>5' - AGC TCC CAG GTG GTA TGA TG -3'</td>
<td>5' - ATC TTT GGC CGT CAG GTA TG -3'</td>
</tr>
<tr>
<td>P67-phox</td>
<td>5' - GCC GGA GAC GCC AGA</td>
<td>5' - GGG GCT GCG ACT GAG</td>
</tr>
<tr>
<td></td>
<td>AGA GCT A -3'</td>
<td>GGT GAA -3'</td>
</tr>
<tr>
<td>Gp91</td>
<td>5' - CGC CCT TTG CCT CCA TTC TC -3'</td>
<td>5' - CCT TTC CTG CAT CTG GGT CTC C -3'</td>
</tr>
<tr>
<td>MCP1</td>
<td>5' - CAT GCT TCT GGG CCT GCT GTT C -3'</td>
<td>5' - CCT GCT GCT GGT GAT CCT CTT G -3'</td>
</tr>
<tr>
<td>CD68</td>
<td>5' - TTC TGC TGT GGA AAT GCA AG -3'</td>
<td>5' - AGA GGG GCT GGT AGG TTG AT -3'</td>
</tr>
<tr>
<td>ICAM</td>
<td>5' - TTC ACA CTG AAT GCC AGC TC -3'</td>
<td>5' - GTC TGC TGA GAC CCC TCT GCT G -3'</td>
</tr>
<tr>
<td>VCAM</td>
<td>5' - ATT TTC TGG GGC AGG AAG TT -3'</td>
<td>5' - ACG TCA GAA CAA CCG AAT CC -3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5' - CAT CTT CTC AAA ACT CGA GTG ACA A -3'</td>
<td>5' - TGG GAG TAG ATA AGG TAC AGC CC -3'</td>
</tr>
<tr>
<td>AT1</td>
<td>5' - ATT CAA CGC TCC CCA TAG GA -3'</td>
<td>5' - TGA ATT TCA TAA GCC TTC TTT AGA GCT -3'</td>
</tr>
<tr>
<td>ACE</td>
<td>5' - TGA GGT GAC CCT GCT GGC AAG GCC -3'</td>
<td>5' - GTG CTG GGA CTG TCA AGG AGA CAG GC -3'</td>
</tr>
<tr>
<td>HPRT</td>
<td>5' - CTC AAC TTT AAC TGG AAA GAA TGT C -3'</td>
<td>5' - TCC TTT TCA CCA GCA AGC T -3'</td>
</tr>
</tbody>
</table>

CTGF: connective tissue growth factor; MCP1: Monocyte chemotactic protein-1; ICAM: intercellular adhesion molecule; VCAM: vascular cell adhesion molecule; TNF-α: tumor necrosis factor-alpha; AT1: Angiotensin II receptor type I; ACE: angiotensin-converting enzyme; HPRT: Hypoxanthine Phospho Ribosyl Transferase;
Figure S1. Experimental protocol.
Two month-old double-transgenic MHCreTA/tetO-hMR (DTg) male mice and their control littermates were divided in 6 groups: control (Ctl); control treated with AngII (Ctl+A); control treated with AngII and canrenoate (Ctl+A+C); DTg mice (DTg); DTg mice treated with AngII (DTg+A); and DTg mice treated with AngII and canrenoate (DTg+A+C). Angiotensin II was infused s.c. through an osmotic minipump for 2 months and canrenoate given in the drinking water. The untreated mice were mock treated (the same procedure) with vehicle instead of AngII. Functional assessments were done immediately before sacrifice.
**Figure S2.** Effects of AngII infusion, cardiac MR overexpression and the combination of both on fibronectin mRNA expression. Open bars: Ctl mice, Filled bars: DTg mice. AngII: AngII treatment; Canr: Canrenoate treatment. * p<0.05 versus Ctl, † p<0.05 versus DTg, ‡ p<0.05 versus DTg + Ang, n=7-11.
Figure S3. Effects of AngII infusion, cardiac MR overexpression and the combination of both on mRNA expression of ACE (A) and AT1 (B). Open bars: Ctl mice, Filled bars: DTg mice. AngII: AngII treatment. * p<0.05 versus Ctl, n=7-11
**Macrophage Immunostaining**

Without anti-F4/80  With anti-F4/80

spleen

heart

DTg+A

**Relative value**

0  1  2  3  4

**AngII**

-  +  +  -  +  +

Canr  -  -  +  -  -  +

**Relative value**

0  1  2  3

**AngII**

-  +  +  -  +  +

Canr  -  -  +  -  -  +

**Relative value**

0, 0.4, 0.8, 1.2, 1.6, 2

**AngII**

-  +  +  -  +  +

Canr  -  -  +  -  -  +

**ICAM**

**VCAM**

**CD68**

**MCP1**

**TNF-α**

**Figure S4**
**Figure S4.** Effects of AngII, cardiac MR overexpression and the combination of both on mRNA of inflammation markers. (A): Macrophage immunostaining with anti-F4/80 antibody indicating the absence of macrophage infiltration in heart from controls (Ctl) and DTg+AngII (DTg+A) mice. Spleen was used as a positive control and incubation without anti-F4/80 antibody was used to assess non-specific staining in tissue sections; (B): CD68 mRNA abundance is not altered in the various experimental conditions; (C) MCP1 mRNA is induced by AngII only, independently of MR overexpression or MR antagonism. Expression of ICAM (D), VCAM (E) and TNFα (F) are not modified. Open bars: Ctl mice, gray bars: DTg mice. AngII: AngII treatment; Can: Canrenoate treatment. * p<0.05 versus Ctl, † p<0.05 versus DTg. n=7-11.
Figure S5
**Figure S5**. Effects of AngII, cardiac MR overexpression and the combination of both on mRNA of p47 and p67 subunits. Expression of p47 and p67 mRNAs (AC) are increased by AngII but not cardiac MR overexpression, while p47 and p67 proteins (B-D) are not altered either by AngII or cardiac MR overexpression. Open bars: Ctl mice, gray bars: DTg mice. AngII: AngII treatment; Canr: Canrenoate treatment. * p<0.05 versus Ctl, † p<0.05 versus DTg. n=7-11.