Cardiac Aldosterone Production in Genetically Hypertensive Rats

Yoshiyu Takeda, Takashi Yoneda, Masashi Demura, Isamu Miyamori, Hiroshi Mabuchi

Abstract—Aldosterone is synthesized in extra-adrenal tissues, both blood vessels and brain. We undertook the present study to determine whether the rat heart produces aldosterone and to investigate the effects of adrenalectomy, ACE inhibition, and angiotensin II on aldosterone synthesis in the heart. To clarify the pathophysiological role of cardiac aldosterone in the hypertensive heart, we compared the synthesis of aldosterone in the hearts of stroke-prone spontaneously hypertensive rats (SHRSP) with that in Wistar-Kyoto rats. The effects of the aldosterone antagonist spironolactone on myocardial hypertrophy in adrenalectomized SHRSP were also studied. Isolated rat hearts were perfused for 2 hours, and the perfusate was analyzed with HPLC and mass spectrometry. The activity of aldosterone synthase was estimated on the basis of the conversion of [14C]deoxycorticosterone to [14C]aldosterone. The levels of aldosterone synthase gene (CYP11B2) mRNA were determined with competitive polymerase chain reaction. Aldosterone production, the activity of aldosterone synthase, and the expression of CYP11B2 mRNA were increased in hearts from adrenalectomized rats and rats treated with angiotensin II. ACE inhibitors decreased cardiac aldosterone synthesis. Cardiac aldosterone, aldosterone synthase activity, and CYP11B2 mRNA levels in hearts from 2- and 4-week-old SHRSP were significantly greater than those of age-matched Wistar-Kyoto rats. Spironolactone prevented cardiac hypertrophy in adrenalectomized SHRSP. These results suggest that the rat heart produces aldosterone and that endogenous cardiac aldosterone may affect cardiac function and hypertrophy in hypertension in rats. (Hypertension. 2000;36:495-500.)

Key Words: heart ■ rats, inbred strains ■ aldosterone ■ hypertrophy ■ cytochrome p450 ■ mRNA

Aldosterone receptors are present in cardiac myocytes, endocardial endothelial cells, and cardiac fibroblasts. The peripheral infusion of aldosterone in rats causes cardiac hypertrophy and cardiac fibrosis without increasing the blood pressure. In cultured neonatal rat fibroblasts or cardiomyocytes, aldosterone increases collagen synthesis and protein synthesis. Physiologically significant amounts of mineralocorticoids are believed to be synthesized only in the adrenal cortex. However, extra-adrenal steroid 21-hydroxylation and 11β-hydroxylation occur in a variety of human tissues. Circulating progesterone is converted to deoxycorticosterone (DOC) and DOC sulfate in adult and fetal human extra-adrenal tissues. The extra-adrenal expression of steroid 21-hydroxylase and 11β-hydroxylase by a benign testicular Leydig cell tumor has also been reported. We reported that aldosterone, synthesized in the vasculature, is in part controlled by angiotensin (Ang) II and participates in the development of vascular hypertrophy that correlates with Ang II concentration.

We performed the present study to determine whether the rat heart produces aldosterone and to investigate the effects of adrenalectomy, ACE inhibitor, and Ang II on aldosterone synthesis, aldosterone synthase activity, and the expression of aldosterone synthase gene (CYP11B2) in the heart. Next, to clarify the pathophysiological roles of cardiac aldosterone in the hypertensive heart, we compared both aldosterone synthesis and the expression of CYP11B2 mRNA in the hearts of stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto (WKY) rats. The effects of the aldosterone antagonist spironolactone on myocardial hypertrophy in adrenalectomized SHRSP were also studied.

Methods

Animals

SHRSP/Izm (2, 4, and 9 weeks old, n=24; 3 weeks old, n=20) and WKY/Izm rats (2 and 4 weeks old, n=24; 9 weeks old, n=96) donated by the Disease Model Cooperative Research Association (Kyoto, Japan) were housed in metabolic cages with free access to tap water and normal rat chow (0.1 mmol/g Na+, 0.24 mmol/g K+; Nippon Charles River). Animals were maintained in a constant temperature environment, with 12-hour light/dark cycles.

Experimental Protocol

In the first study, male 9-week-old WKY rats were used. The first group of animals (n=24) underwent bilateral adrenalectomy under...
ether anesthesia and were allowed free access to 0.9% NaCl as drinking water for 7 days. The control group (n=24) consisted of sham-operated animals that were supplied 0.9% NaCl solution. The second group of rats (n=24) was administered the ACE inhibitor quinapril (0.3 mg/d PO; Parke-Davis Co, Ltd) for 14 days. The third group of rats (n=24) was administered Ang II (9 μg/h) via an implanted osmotic minipump for 2 weeks as previously described.9 Age- and weight-matched WKY rats that were allowed access to tap water were used as controls. We confirmed no residual adrenal tissues after experiments at necropsy on visual inspection.

In the second study, 2-, 4-, and 9-week-old SHRSP (n=24 in each group) and age-matched WKY rats were used for the heart perfusion studies. Eight rats from each group were used for the quantification of CYP11B2 mRNA. The blood pressure was determined according to the plethysmographic tail-cuff method as previously reported.10 Blood was collected from the tail vein as previously reported. Plasma concentrations of corticosterone and aldosterone were estimated with radioimmunoassay (RIA) after extraction with a Sep-Pak C18 cartridge (Waters).

In the third study, 3-week-old SHRSP (n=36) were used to clarify the relationship between cardiac aldosterone and cardiac hypertrophy in SHRSP. Twenty adrenalectomized SHRSP were fed a high-sodium diet (0.9% drinking solution), and 10 rats were subcutaneously administered spironolactone (20 mg·kg·day-1·d-1) via an implanted osmotic minipump for 8 weeks as previously described.11 Ten adrenalectomized SHRSP treated with vehicle (polyethylene glycol) for 8 weeks were used as controls. Sixteen sham-operated SHRSP with (n=8) or without (n=8) spironolactone were fed a high-sodium diet. The carotid artery was cannulated and blood pressure was recorded with a pressure transducer connected to a polygraph (RM 600; Nihon-Koden). Arterial blood pressure was measured in a conscious and unrestricted state. Before the animals were killed, they were anesthetized with pentobarbital (100 mg/kg IP), intubated, and mechanically ventilated. The chest was opened via median sternotomy, and the heart and lungs were removed. The right and left (plus septum) ventricles were weighed. All procedures were performed according to guidelines from the American Physiological Society and were approved by the Animal Research Committee of Kanazawa University (permit 28078).

Isolated Perfused Heart
Rats were heparinized and anesthetized with pentobarbital (100 mg/kg IP). Once the rat was deeply anesthetized, the heart was removed via sternotomy and placed in ice-cold Krebs-Ringer solution. The heart was cannulated immediately via the aorta, and retrograde perfusion was performed in a Langendorff apparatus under constant pressure (90 mm Hg) with modified Krebs-Henseleit solution.12 The solution was gassed with 95% O2/5% CO2 and adjusted to pH 7.4. Studies were started after a 20-minute equilibration perfusion.

Identification of Aldosterone in the Perfusate
The perfusate was extracted with a Sep-Pak C18 cartridge and subjected to reversed-phase HPLC using methanol/water (40:100%) as the mobile phase at a flow rate of 1.5 mL/min for 60 minutes. The retention times of 18-hydroxy corticosterone, aldosterone, corticosterone, DOC, progesterone, and pregnenolone were 35, 32, 43, 49, 55, and 59 minutes, respectively. The fraction corresponding to synthetic aldosterone was collected and analyzed with gas chromatography/ mass spectrometry (GC/MS) as previously reported.7

Measurements of Aldosterone in the Perfusate
After 20 minutes of equilibration, the perfusate was collected for 2 hours. The perfusate with titrated aldosterone (3000 cpm; American Japan) added to determine recovery was extracted with a Sep-Pak C18 cartridge before HPLC separation as described earlier. The aldosterone concentration in the perfusate was measured with RIA after HPLC separation. At the end of the experiment, the heart was homogenized in 10 mL Krebs-Ringer buffer solution in a tissue grinder. Protein was assayed according to the method of Bradford.13

Measurements of Aldosterone Synthase Activity in the Heart
Aldosterone synthase activity was estimated on the basis of the conversion of [14C]DOC to [14C]aldosterone. Diced heart tissues were homogenized in 2 mL Krebs' buffer (without BSA) with three 10-second bursts in a Ystral homogenizer, and the homogenate was assayed for protein colorimetrically. Steroid conversion rates were assayed in triplicate by the addition of homogenate (1 mg protein) to 1 mL buffer containing 0.5 μmol/L (0.001 μCi) [14C]DOC, 250 mmol/L sucrose, 25 μmol/L isotocitrate, and 50 mmol/L Tris-HCl, pH 7.4. After 120 minutes of incubation at 37°C, the incubation medium was extracted on a Sep-Pak C18 cartridge. The steroids were separated with reversed-phase HPLC. The fraction that corresponded to DOC and aldosterone was counted in a scintillation counter.

Quantification of CYP11B2 mRNA in the Heart
Eight rats from each group were used for quantification of CYP11B2 mRNA in the heart. Rats were anesthetized and decapitated, and the heart was immediately removed. The tissue was weighed, frozen in liquid nitrogen, and stored at −80°C before analysis. Total RNA from rat hearts was isolated with guanidinium thiocyanate followed by centrifugation in a cesium chloride solution. Quantification of CYP11B2 mRNA was performed with the competitive polymerase chain reaction (PCR) method as previously reported.14 Sequences for the sense and antisense primers for CYP11B2 have been reported previously.15 The intra-assay and interassay variabilities of the competitive PCR were 12.5% and 13.8%, respectively. To test the yield and the efficiency of the reverse transcriptase reaction, 1 μg total RNA was subjected to reverse transcription (RT) as described, with 5 μmol/L [32P]dCTP (New England Nuclear) added to the reaction as previously reported.16

Southern Blot
The RT-PCR products from 10-μL aliquots were electrophoresed on a 3% agarose gel and transferred to nylon membranes. Hybridization was performed as previously reported,17 with a specific oligoprobe for CYP11B2 (5'-GATGCCTGGGATGTCATCTC-3') that had been end-labeled with [32P]ATP (6000 Ci/mmol, New England Nuclear) with a 5'-end oligonucleotide labeling kit.

Northern Blot Analysis of α1-III Collagen mRNA
Poly(A+) RNAs (5 μg/lane) of rat hearts from each experimental group were separated by formaldehyde/agarose gel electrophoresis, transferred to a nylon membrane (Hybond-N; Amersham Japan), and hybridized with 32P-labeled oligonucleotide probe specific for α1-III collagen cDNA.18 The filters were washed, and radioactive bands were detected with autoradiography. The hybridized signals were analyzed with a BAS 2000 Bioimaging Analyzer (Fuji Photo Film Co Ltd). For quantification of relative levels of expression of α1-III collagen mRNA, the autoradiographic signals were standardized to signals determined from β-actin mRNA in each preparation to control for amounts of RNA loaded per lane.17

Data are expressed as the mean±SEM. Differences were assessed by 1-way ANOVA and multiple comparison tests. Statistical significance was accepted for a value of P<0.05.

Results
Table 1 summarizes the data for plasma renin activity (PRA) and plasma aldosterone and corticosterone concentrations for each experimental group. PRA was significantly higher in adrenalectomized rats than in sham-operated rats. In adrenalectomized rats, plasma aldosterone was not detected in 1 mL plasma on RIA. Rats treated with an ACE inhibitor had significantly lower plasma aldosterone concentrations than control rats (P<0.05). The plasma aldosterone concentration was significantly higher in rats that received Ang II (P<0.05). There were no significant differences in the
plasma corticosterone concentration between rats treated with ACE inhibitor, rats that received Ang II, and control rats.

The HPLC elution profile for immunoreactive aldosterone in the perfusate showed 1 large peak with a retention time of 32 minutes that corresponded to synthetic aldosterone (Figure 1). The aliquot of the fraction with a retention time of 32 minutes was injected into a GC/MS system, and the mass spectra pattern was identical to that for synthetic aldosterone (Figure 1). The sensitivity of the assay of aldosterone was 30 fmol. The overall recovery was 70%, interassay variation was 13.5%, and intra-assay variation was 9.5%.

**Figure 1.** HPLC elution profiles of immunoreactive aldosterone (aldosterone LI) in the perfusate of rat hearts (top). Bottom, Mass spectrum of synthetic aldosterone and the sample of aldosterone from perfusate of rat hearts. The mass spectra of synthetic aldosterone and samples isolated from the perfusate of rat hearts were identical.

**Figure 2.** Analysis of CYP11B2 mRNA levels by competitive PCR. Increasing the concentration of competitive template for CYP11B2 from 0 to 160×10⁻³ amol/μL increasingly inhibited the amplification of endogenous CYP11B2 cDNA in the heart or in the adrenal gland. In comparison to competitive PCR of adrenal CYP11B2, 25-fold higher concentrations of RT-products were used in competitive PCR of heart CYP11B2.

**Figure 3.** Aldosterone production (top), aldosterone synthase activity (middle), and concentrations of CYP11B2 mRNA (bottom) in the hearts for each experimental group. ATII indicates Ang II; ADX, adrenalectomized rats. Values are mean±SEM. *P<0.05 vs control.

<table>
<thead>
<tr>
<th>Group</th>
<th>PRA, ng·L⁻¹·s⁻¹</th>
<th>P-Aldo, nmol/L</th>
<th>P-B, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEI (n=24)</td>
<td>1.2±0.3*</td>
<td>0.23±0.02*</td>
<td>39±9</td>
</tr>
<tr>
<td>Ang II (n=24)</td>
<td>0.21±0.09*</td>
<td>1.8±0.3*</td>
<td>43±10</td>
</tr>
<tr>
<td>ADX (n=24)</td>
<td>1.3±0.2*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control (n=24)</td>
<td>0.46±0.08</td>
<td>0.55±0.09</td>
<td>41±11</td>
</tr>
</tbody>
</table>

PRA indicates plasma renin activity; P-Aldo, plasma aldosterone concentration; P-B, plasma corticosterone concentration; ADX, adrenalectomized rats; ND, not detectable. Values are mean±SEM.

*P<0.05 vs control.
trations of CYP11B2 mRNA in the heart for each experimental group. Aldosterone production in rats treated with ACE inhibitor was significantly reduced compared with that of controls (10±2.5 versus 18±2.2 pmol·mg⁻¹·h⁻¹, P<0.05). Aldosterone production was significantly increased in adrenalectomized rats (30±2.8 pmol·mg⁻¹·h⁻¹) and rats treated with Ang II (38±3.1 pmol·mg⁻¹·h⁻¹; P<0.05). Aldosterone synthase activity in hearts was significantly reduced with ACE inhibitor by 44% (P<0.05) and significantly increased with adrenalectomy (1.7-fold) and Ang II (2.1-fold) (P<0.05). The expression of CYP11B2 mRNA in hearts was significantly increased with ACE inhibitor by 51% (P<0.05) and significantly increased with adrenalectomy (2.1-fold) and Ang II (2.9-fold) (P<0.05).

Table 2 summarizes the body weight, ratio of heart weight to body weight, hemodynamic parameters, and plasma aldosterone and corticosterone concentrations in SHRSP and WKY rats. Table 3 summarizes the mean blood pressure, body weight, ratio of left or right ventricular weight to body weight, and α1-III collagen gene expression in each experimental rat. Spironolactone decreased blood pressure, although not significantly, in both adrenalectomized and sham-operated SHRSP. Treatment with spironolactone significantly decreased left ventricular weight in both adrenalectomized and sham-operated SHRSP (P<0.05) but did not affect right ventricular weight. The effect of spironolactone on left ventricular weight in adrenalectomized SHRSP was smaller.
TABLE 3. Effects of Spironolactone on Body Weight, Mean Blood Pressure, the Ratio of Left or Right Ventricular Weight to Body Weight, and α1-III Collagen Gene Expression in Each Experimental Group

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>MBP, mm Hg</th>
<th>LV/BW, mg/g</th>
<th>RV/BW, mg/g</th>
<th>α1-III Collagen mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenalectomized SHRSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spironolactone (n=10)</td>
<td>282±10</td>
<td>88±5</td>
<td>2.9±0.07*</td>
<td>0.52±0.02</td>
<td>0.44±0.01* 0.44±0.02*</td>
</tr>
<tr>
<td>Vehicle (n=10)</td>
<td>274±12</td>
<td>94±4</td>
<td>3.1±0.08</td>
<td>0.56±0.02</td>
<td>0.52±0.02  0.49±0.02</td>
</tr>
<tr>
<td>Sham-operated SHRSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spironolactone (n=8)</td>
<td>314±16</td>
<td>175±3</td>
<td>3.4±0.08*</td>
<td>0.73±0.01</td>
<td>0.62±0.02* 0.51±0.01*</td>
</tr>
<tr>
<td>Vehicle (n=8)</td>
<td>320±11</td>
<td>178±3</td>
<td>4.3±0.09</td>
<td>0.74±0.01</td>
<td>0.75±0.02  0.70±0.02</td>
</tr>
</tbody>
</table>

MBP indicates mean blood pressure; BW, body weight; LV/BW, left ventricular weight/body weight ratio; RV/BW, right ventricular weight/body weight ratio. Values are mean±SEM.

*P<0.05 vs vehicle.

Discussion

Cardiac hypertrophy is regulated by ventricular pressure and volume loading. Perivascular fibrous tissue synthesis is associated with chronic elevations in circulating effector hormones of the renin-angiotensin-aldosterone system, not with arterial hypertension per se. The fibrosis that follows myocyte necrosis is associated with increased plasma Ang II concentration and myocardial K⁺ depletion that accompanies chronic mineralocorticoid excess.19

More than 30 years ago, Ilett and Lockett20 described an aldosterone-like substance produced by heart muscle. The detection of 3β-hydroxysteroid dehydrogenase activity in rat heart also indicates the potential for steroid metabolism in cardiac tissue.21 We confirmed the existence of aldosterone in the perfusate of rat heart with GC/MS. In adrenalectomized rats, plasma aldosterone was not detected; however, the aldosterone concentration in the perfusate was increased compared with sham-operated controls. These results support the hypothesis that the appearance of aldosterone in the perfusate does not reflect uptake from plasma. The findings of the present study, together with the observation of CYP11B2 expression in the heart, confirm that heart can synthesize aldosterone de novo by expressing CYP11B2 and other enzymes essential for the final product aldosterone.

There is increasing evidence of the presence of a cardiac renin-angiotensin system with local angiotensin formation.22 Local angiotensin formation in the heart may contribute to the pathogenesis of cardiac hypertrophy, congestive heart failure, and tissue remodeling.23 We have reported that rat and human blood vessels produce aldosterone.7,14 Vascular aldosterone production is controlled by the renin-angiotensin system at the transcriptional level16 and participates in the development of hypertension in rats.17 In this study, treatment with an ACE inhibitor decreased both the synthesis of aldosterone and the activity of aldosterone synthase in the heart. Decreased expression of CYP11B2 mRNA in the heart with ACE inhibitor therapy also was observed. Ang II is an important regulator of adrenal mineralocorticoid biosynthesis and secretion. Chronic treatment with Ang II increased cardiac aldosterone synthesis, aldosterone synthase activity, and the expression of CYP11B2 mRNA. These results suggest that cardiac aldosterone synthesis is controlled in part by the renin-angiotensin system at the transcriptional level. Cardiac aldosterone synthesis may be regulated by both the systemic and local renin-angiotensin system. Silvestre et al24 also reported that Ang II and a low-sodium/high-potassium diet increased the myocardial production of aldosterone in rat.

The SHRSP is a useful animal model of hypertension-induced cardiac hypertrophy.25 The systemic and local renin-angiotensin systems are major pathophysiological factors in the development of hypertrophy in this model.26 Early, long-term treatment of SHRSP with ACE inhibitors increases cardiac contractility and coronary flow and prevents the development of cardiac hypertrophy.27 Long-term treatment with Ang II type 1 receptor (AT₁) antagonists also prevents cardiac hypertrophy in SHRSP.28 In this study, cardiac aldosterone levels were increased in the young prehypertensive SHRSP. Increased levels of CYP11B2 mRNA and aldosterone synthase activity in hearts of SHRSP suggest that local synthesis of aldosterone is increased in the cardiovascular system.

Gohlke et al27 reported that doses of ACE inhibitors that do not decrease blood pressure inhibit the cardiac hypertrophy in SHRSP and suggested that the effects of ACE inhibitors on cardiac function are independent of their antihypertensive and antihypertrophic actions. Increased expression of renin, angiotensinogen, ACE, and AT₁ mRNA in the hearts of SHRSP has been reported.29 The amount of aldosterone produced by the heart is very small compared with the amount of plasma or adrenal aldosterone with CYP11B2 mRNA levels in the heart 100-fold lower than in adrenal gland. Such a ratio is comparable to that of ACE mRNA, of which the total quantity is ~150-fold lower in the heart than in the lung, one of the main sources of this enzyme.30

Robert et al31 reported that aldosterone-induced cardiac fibrosis is independent of blood pressure. In the present study, spironolactone prevented cardiac hypertrophy and fibrosis in both SHRSP and adrenalectomized SHRSP. However, the effect of spironolactone on cardiac fibrosis was greater than than that in sham-operated SHRSP. Levels of α1-III collagen mRNA were significantly decreased in both ventricles of adrenalectomized or sham-operated SHRSP treated with spironolactone (P<0.05).
that on left ventricular weight in adrenalectomized SHRSP. These results indicate that the effect of circulating aldosterone on cardiac hypertrophy is more important than the effect of cardiac aldosterone, but the latter may contribute more for fibrosis. Although CYP11B2 expression, aldosterone synthase activity, and aldosterone production by heart were increased in SHRSP compared with WKY rats, this was only the case in early life. However, the blood pressure of the 2 groups was the same during this time frame, and it was only after 9 weeks that there was a significant difference, at which time the 3 parameters of aldosterone production were equivalent. This would suggest that cardiac aldosterone influences cardiac hypertrophy during the first few weeks of life. Recently, Silvestre et al.\(^{32}\) reported that cardiac aldosterone production is increased in rats after cardiac myocardial infarction and that such increase in myocardial aldosterone level may be involved in post–myocardial infarction and that such increase in myocardial aldosterone level may be involved in post–myocardial infarction ventricular fibrosis. Further study is necessary to clarify the pathophysiological role of the cardiac renin-angiotensin-aldosterone system in cardiovascular disease.

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**References**

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