Aldosterone plays an important role in the pathogenesis of cardiovascular disease that is independent of angiotensin II (Ang II). For example, patients with primary aldosteronism, in which the Ang II levels are usually very low, have a higher incidence of left ventricular hypertrophy and stroke than do patients with essential hypertension.1 It has been shown that in addition to standard therapy, treatment with eplerenone, a selective mineralocorticoid receptor (MR) antagonist, improves cardiovascular function and survival rates.2 Experimental animal data also support a role for aldosterone in mediating cardiovascular injury.3

Excess sodium intake is intimately involved in the pathogenesis of hypertension. In large populations, significant correlations between the level of salt intake, blood pressure (BP), and the frequency of hypertension have been reported. Several studies have shown that high salt intake reduces not only circulating renin-angiotensin system (RAS) but also tissue RAS in normal rat. However, augmented local RAS by a high sodium diet is seen in...
Dahl salt-sensitive (DS) hypertensive rats, which may contribute to hypertension.4,5

Tissue angiotensinogen is known as an important effector system for the regulation of BP. The overexpression of the angiotensinogen gene in the heart increases BP and cardiac hypertrophy6 and young spontaneously hypertensive rats show an elevation of tissue angiotensinogen expression. Angiotensin-converting enzyme (ACE) 2, through the generation of the vasodilator Ang-(1-7) and by hydrolyzing part of Ang II, counterbalances the vasopressor effect of ACE that is mediated by Ang II. Genetic inactivation of ACE2 in mice showed an elevation of tissue angiotensinogen expression. Ang II, counterbalances the vasopressor effect of ACE that is mediated by Ang II. Genetic inactivation of ACE2 in mice resulted in severe cardiac dysfunction and mild elevation of BP.7 We hypothesized that the beneficial effects of an MR antagonist or a type 1 Ang II receptor (AT1R) blocker in the treatment of salt-sensitive hypertension could be mediated by reduced activation of the local RAAS and changes in the balance between the opposing activities of ACE and ACE2.

Methods
Animal Experiments

All experiments were performed according to the guidelines for the use of experimental animals of the Animal Research Committee of Kanazawa University. Male Dahl salt-sensitive (DS) rats and Dahl salt-resistant (DR) rats (Seac Yoshitomi, Yoshitomi cho, Japan), 4 to 5 weeks old, were initially fed a standard chow purchased from Nippon Charles River (Kanagawa, Japan). Both DS and DR rats were fed low sodium chow (0.45%) or high sodium chow (7%) for 8 weeks (n = 20 in each group) with or without the addition of eplerenone (100 mg/kg/d; Pharmacia/Pfizer Groton, CT). Eplerenone was synthesized at Pharmacia/Pfizer and incorporated into the Teklad 22/5 rodent diet at a concentration of 1.0 mg/g of chow as previously reported.3 The DS rats were treated orally for 8 weeks with candesartan cilexetil alone (10 mg/kg/d; Takeda Chemical Industries, Osaka, Japan) or with the combination of eplerenone and candesartan cilexetil. Candesartan was given orally by a gastric tube. All rats were housed in metabolic cages and daily urinary excretions were collected.

Blood pressure was determined by the tail–cuff method using photoelectric volume oscillometry (BP-98A, Softron, Tokyo, Japan). In several rats, intra-arterial BP was measured as previously reported.8 The BP data measured by the tail–cuff method agreed with the data obtained from direct intra-arterial measurements. Blood was collected from the tail vein as previously reported.8 Plasma aldosterone concentrations (PAC) were estimated with radioimmunoassay (RIA) after extraction with a Sep-Pak C18 cartridge (Waters associates, Milfold, MA) as previously reported.8 Plasma renin activity (PRA) was measured using a commercial RIA kit.

Perfusion Experiments

Eight rats from each group were used for experiments involving mesenteric arterial perfusion.9 After the rat was anesthetized with pentobarbital, the superior mesenteric artery was immediately cannulated and perfused with Krebs–Ringer solution (pH 7.4) at a temperature of 37°C and oxygenated with a 95% O2–5% CO2 gas mixture at a constant flow rate of 3 mL/min. All connections of the mesenteric vascular bed to the small intestine were carefully dissected as previously reported.9 Noradrenalin was added to a scaled reservoir that was continuously gassed with 95% O2–5% CO2 and kept at 37°C, and from which Krebs solution was continuously perfused to the mesenteric vascular bed. Acetylcholine was injected in a volume of 10 µL into the perfusate in the silicone rubber close to the vascular bed. The perfusion pressure was constantly monitored and recorded by means of a pressure transducer connected to a polygraph (RM 600; Nihon-Koden, Tokyo, Japan).

Quantification of mRNA of Type III Collagen, Angiotensinogen, ACE, and ACE2 in the Heart

Before the animals were killed, they were anesthetized intraperitoneally with pentobarbital (100 mg/kg), intubated, and mechanically ventilated. The chest was opened by a median sternotomy, and the heart, aorta, and mesenteric artery were removed. The right and left ventricles (plus the interventricular septum) were weighed. Total RNA was extracted from the heart by using TRIzol (Invitrogen Japan, Tokyo, Japan) according to the manufacturer’s protocol. Real-time quantitative reverse transcription–polymerase chain reaction was done using the TaqMan One-Step RT-PCR Master Mix Reagent Kit with an ABI Prism 7000 HT Detection System (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer’s protocol. The sequences of sense and antisense primers and probes for angiotensinogen, ACE, and ACE2 were designed as previously reported.10,11 To obtain a calibration curve, serial dilutions of stock standard RNA were used. The relative amount of each mRNA was normalized to the housekeeping gene, 18 ribosome mRNA. Northern blot analysis of mRNA of type III collagen was done as previously reported.12

Western Blot Analysis of Cardiac ACE and ACE2

Membrane proteins from hearts were isolated and subjected to Western blot analysis as previously reported by Crackower et al.7 For detection of ACE or ACE2, nitrocellulose membranes were incubated with mouse ACE monoclonal antibody (Chemicon, Temecula, CA) or an affinity-purified rabbit ACE2 antibody.7 Signals on Western blots were quantified by densitometry and corrected for β-actin.

Data are expressed as the mean ± SEM. Data were compared by a two-way ANOVA or Friedman’s test and Fisher’s protected least significance or Scheffe’s F test was
performed when each ANOVA indicated significance. Statistical significance was accepted for $P < .05$.

**Results**

Table 1 summarizes the data on body weight, PRA, and PAC, and parameters of cardiac hypertrophy and fibrosis. After 4 or 8 weeks of salt-loading, BP increased significantly in DS rats ($183 \pm 4.3$ mm Hg, $240 \pm 5.4$ mm Hg, respectively) (Fig. 1). High sodium diet did not increase BP in DR rats. Treatment with eplerenone or candesartan for 4 or 8 weeks blunted the increase in BP (eplerenone, $146 \pm 3.9$ mm Hg; candesartan, $152 \pm 9.1$ mm Hg at 4 weeks; eplerenone, $164 \pm 4.0$ mm Hg; candesartan, $178 \pm 8.6$ mm Hg at 8 weeks). The DS rats treated with eplerenone and candesartan for 4 or 8 weeks did not show any differences in BP compared with DS rats fed a low sodium diet or DR rats fed a high sodium diet. A high sodium diet significantly decreased PRA and PAC in DS and DR rats. Treatment with eplerenone slightly increased PRA but did not influence PAC. A high sodium diet increased the heart-to-body weight ratio and type III collagen mRNA levels in the heart ($P < .05$). Treatment with eplerenone or candesartan significantly decreased both the heart-to-body weight ratio and type III collagen mRNA levels in the heart ($P < .05$). Treatment with eplerenone and candesartan normalized the heart-to-body weight ratio and cardiac type III collagen mRNA levels in DS rats given a high sodium diet.

Acetylcholine-induced relaxation was blunted in DS rats given a high sodium diet compared with those given a low sodium diet (Fig. 2). Treatment with eplerenone or candesartan for 8 weeks improved EDR and no significant difference was seen between the two groups. The combination of candesartan and eplerenone normalized EDR.

Cardiac angiotensinogen mRNA levels were elevated in DS rats given a high sodium diet compared with those given a low sodium diet (Fig. 3). Treatment with eplerenone or candesartan decreased angiotensinogen mRNA expression in the heart of DS rats. Cardiac angiotensinogen mRNA levels in DR rats on a high sodium diet were not influenced by eplerenone or candesartan (data not shown). The high sodium diet did not affect expression of ACE mRNA in the heart of DS or DR rats. However, treatment with eplerenone or candesartan significantly decreased cardiac ACE mRNA levels in DS rats ($P < .05$) (Fig. 3). The ACE mRNA/18S ribosome mRNA expression ratio ($2.5 \pm 0.6$) in the heart of DR rats was decreased by the treatment with eplerenone ($1.6 \pm 0.2$) or candesartan ($1.5 \pm 0.1$). Although cardiac ACE2 mRNA levels were significantly reduced in DS rats fed a high sodium diet, the mRNA levels in the heart of DR rats were not decreased.

**Table 1.** Body weight, the ratio of heart weight to body weight, plasma renin activity, plasma aldosterone concentration, and relative amount of mRNA levels of type III collagen in each experimental group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BW (g)</th>
<th>HW/BW ($\times 10^{-3}$)</th>
<th>PRA (ng/mL/h)</th>
<th>PAC (pg/mL)</th>
<th>Collagen III/18S mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS ($n = 20$)</td>
<td>455 ± 9</td>
<td>2.9 ± 0.07</td>
<td>3.1 ± 0.52</td>
<td>197 ± 9.8</td>
<td>1.1 ± 0.07</td>
</tr>
<tr>
<td>HS ($n = 20$)</td>
<td>442 ± 10</td>
<td>3.0 ± 0.06</td>
<td>0.52 ± 0.23†</td>
<td>73 ± 6.6†</td>
<td>1.2 ± 0.05</td>
</tr>
<tr>
<td>DS rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LS ($n = 20$)</td>
<td>449 ± 3</td>
<td>3.1 ± 0.03</td>
<td>3.3 ± 0.99</td>
<td>175 ± 7.8</td>
<td>1.3 ± 0.03</td>
</tr>
<tr>
<td>HS ($n = 20$)</td>
<td>431 ± 8</td>
<td>4.1 ± 0.04†</td>
<td>0.48 ± 0.12†</td>
<td>68 ± 5.1†</td>
<td>3.2 ± 0.12†</td>
</tr>
<tr>
<td>MRB ($n = 20$)</td>
<td>405 ± 6</td>
<td>3.5 ± 0.1*</td>
<td>1.4 ± 0.34*</td>
<td>59 ± 5.8</td>
<td>1.9 ± 0.03*</td>
</tr>
<tr>
<td>ARB ($n = 20$)</td>
<td>405 ± 6</td>
<td>3.8 ± 0.1*</td>
<td>2.1 ± 0.67*</td>
<td>48 ± 6.0</td>
<td>2.1 ± 0.06*</td>
</tr>
<tr>
<td>MRB + ARB ($n = 20$)</td>
<td>394 ± 3</td>
<td>3.4 ± 0.09*</td>
<td>0.73 ± 0.42*</td>
<td>51 ± 5.3</td>
<td>1.5 ± 0.03*</td>
</tr>
</tbody>
</table>

LS = low sodium diet; HS = high sodium diet; MRB = DS rats treated with eplerenone in the presence of a high sodium diet; ARB = DS rats treated with candesartan in the presence of a high sodium diet; BW = body weight; HW/BW = the ratio of heart weight to body weight; PRA = plasma renin activity; PAC = plasma aldosterone concentration.

* $P < .05$ v HS; † $P < .05$ v LS.
ACE protein expression in DS rats (0.38 ± 0.02) and in DR rats (0.32 ± 0.07) (Fig. 4). Treatment with eplerenone (0.14 ± 0.01) or candesartan (0.17 ± 0.04) decreased ACE protein levels in DS rats fed a high sodium diet. The decreased ACE2 protein levels (0.052 ± 0.003) was seen in DS rats fed a high sodium diet. Combination therapy with eplerenone and candesartan normalized ACE2 protein levels in DS rats fed a low sodium diet (0.047 ± 0.002) (Fig. 4).

Discussion

In the present study, treatment with eplerenone or candesartan partially decreased BP and combination therapy with the two drugs normalized BP and improved cardiac hypertrophy and fibrosis in DS rats and restored endothelial-dependent relaxation. Nagase et al. reported that eplerenone lowered BP by in DS rats. However, Nakata et al. found that eplerenone did not decrease BP in the same animal model. We and Nagase et al. started eplerenone at the age of 5 weeks (prehypertensive stage) but Nakata et al. treated rats with eplerenone at the age of 12 weeks (hypertensive stage). The antihypertensive effect of eplerenone in several kinds of hypertensive rat models was summarized in Table 2. Both eplerenone and AT1R blocker were reported to be efficacious in reducing BP in hypertensive blacks and whites. Pitt et al. reported that the combination of eplerenone and an ACE inhibitor was more effective in reducing left ventricular mass and systolic BP than eplerenone alone in essential hypertensive patients. Griffin et al. reported that the renal protection by ACE inhibition or aldosterone blockade in spontaneously hypertensive rats was BP dependent. However, several investigators have suggested that the pathogenesis of cardiovascular and renal damage is, at least in part, independent of BP and aldosterone blockade in spontaneously hypertensive rats.
mediated by the direct tissue damage-promoting effects of the RAAS.18,19

A high sodium intake markedly suppressed circulating RAAS in DS rats and treatment with eplerenone did not increase PAC. Treatment with eplerenone in patients with hypertension increases both PRA and PAC. We found that in DS rats fed a low sodium diet, eplerenone increased PRA and PAC (data not shown). A high sodium diet may be more effective in decreasing activation of the circulating RAAS compared with the diuretic effect of eplerenone, which activates the RAAS.

Angiotensinogen is synthesized locally in the heart, and its expression is augmented in pressure overload-induced hypertrophy. Local overexpression of angiotensinogen in the heart of mice induces hypertrophy without hypertension.6 Kobori et al5 reported that a high sodium diet decreased circulating RAS in both DS rats and DR rats. However, intrarenal angiotensinogen was enhanced in DS rats but not in DR rats, and these investigators suggested that paradoxical responses of tissue RAS by a high sodium diet contribute to the development of hypertension. Salt loading reduces tissue ACE2 expression in salt-sensitive hypertensive rats and has no effect on its expression in control salt-resistant normotensive rats. In this study, a high sodium diet increased angiotensinogen mRNA and decreased ACE2 mRNA in the hearts of DS rats. Treatment with eplerenone decreased both angiotensinogen and ACE mRNA levels, but did not affect ACE2 mRNA expression. These data suggest a protective role for eplerenone by possibly decreasing formation of Ang II in the heart. Our data also showed that treatment with candesartan increased ACE2 mRNA and decreased angiotensinogen mRNA in the heart. Several lines of experimental evidence suggest a beneficial role for ACE2 in cardiovascular function. Yamamoto et al23 reported that deletion of ACE2 accelerates pressure overload-induced cardiac dysfunction by increasing local Ang II. In ACE2−/−mice, administration of candesartan improved cardiac hypertrophy. Cardiac ACE2 mRNA expression was increased after administration of the AT1R blocker losartan, and the effect was independent of BP.24 Ishiyama et al25 reported that candesartan upregulated ACE2 mRNA after myocardial infarction. Huentelman et al26 reported that overexpression of the mouse ACE2 gene in the hearts of Sprague-Dawley rats protected against Ang II-induced cardiac hypertrophy and fibrosis. Thus, ACE2 may be responsible for the beneficial actions of treatment with candesartan on cardiac hypertrophy and fibrosis in DS rats.

In conclusion, the blockade of aldosterone or angiotensin II improved vascular endothelial function and decreased cardiac mRNA expression of angiotensinogen and ACE and increased ACE2 mRNA. Dual blockade of aldosterone and Ang II further improved endothelial function and inactivated the cardiac RAAS. These changes were concomitant with improvement of cardiac hypertrophy and fibrosis in salt-sensitive hypertensive rats. These results suggest that the local RAAS is an important contributing factor to the progression of hypertension, cardiac hypertrophy, and fibrosis in salt-sensitive hypertension.27–29

### References


### Table 2. Effect of eplerenone on blood pressure in several models of hypertensive rats

<table>
<thead>
<tr>
<th>Rats</th>
<th>Age (week-old)</th>
<th>Dose (mg/kg)</th>
<th>Duration of the treatment</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS</td>
<td>6</td>
<td>125</td>
<td>5 weeks</td>
<td>Decrease</td>
<td>(13)</td>
</tr>
<tr>
<td>DS</td>
<td>12</td>
<td>100</td>
<td>7 weeks</td>
<td>No change</td>
<td>(14)</td>
</tr>
<tr>
<td>DS</td>
<td>11</td>
<td>100</td>
<td>7 weeks</td>
<td>No change</td>
<td>(27)</td>
</tr>
<tr>
<td>SHRSP</td>
<td>9</td>
<td>100</td>
<td>5 weeks</td>
<td>Decrease</td>
<td>(3)</td>
</tr>
<tr>
<td>Licorice-treated</td>
<td>13</td>
<td>182</td>
<td>2 weeks</td>
<td>Decrease</td>
<td>(28)</td>
</tr>
<tr>
<td>Aldosterone-treated</td>
<td>4</td>
<td>100</td>
<td>4 weeks</td>
<td>Decrease</td>
<td>(29)</td>
</tr>
</tbody>
</table>

DS = Dahl salt-sensitive rat; SHRSP = stroke-prone spontaneously hypertensive rat.