Role of increased circulating and renal adrenomedullin in rats with malignant hypertension

TOSHIKO NISHIKIMI,1,2 FUMIKI YOSHIHARA,1 AKIO KANAZAWA,3
ICHIRO OKANO,1 TAKESHI HORIZO,3 NORTOISHI NAGAYA,1 CHIKAO YUTANI,4
HISAYUKI MATSUO,1 HIROAKI MATSUOKA,2 AND KENJI KANGAWA1

Departments of 3Medicine and 4Pathology, National Cardiovascular Center, 1Research
Institute, Fujishirodai, Suita, Osaka 565, and 2Department of Hypertension
and Cardiorenal Medicine, Dokkyo University School of Medicine, Mibu, Tochigi 321-0293, Japan

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Nishikimi, Toshio, Fumiki Yoshihara, Akio Kanazawa, Ichiro Okano, Takeshi Horiio, Noritoshi Nagaya, Chikao Yutani, Hisayuki Matsuo, Hiroaki Matsuoka, and Kenji Kangawa. Role of increased circulating and renal adrenomedullin in rats with malignant hypertension. Am J Physiol Regulatory Integrative Comp Physiol 281: R2079–R2087, 2001.—Although it has been reported that the circulating adrenomedullin (AM) level is elevated in hypertension and renal failure, the pathophysiological significance of circulating and intrarenal AM in malignant hypertension remains unknown. We investigated the circulating and intrarenal AM system in rats with malignant hypertension by measuring the plasma level, renal tissue level, and mRNA abundance of AM and the mRNA abundance of AM receptor. We also investigated the effects of intravenously infused calcitonin gene-related peptide (CGRP)-(8–37), an antagonist of AM, on the hemodynamics and renal tubular function. We studied the following four groups: control Wistar-Kyoto rats (WKY), control spontaneously hypertensive rats (C-SHR), salt-loaded SHR (S-SHR), and DOCA-salt SHR (D-SHR). After 3 wk of DOCA treatment, D-SHR developed malignant hypertension. D-SHR were characterized by higher blood pressure, kidney weight, urinary protein excretion and blood urea nitrogen, and lower creatinine clearance compared with the other three groups. The plasma AM level and urinary excretion of AM were markedly higher in D-SHR than in the other three groups. In the kidney, the tissue AM level and the expression of AM mRNA in the renal medulla were significantly increased in D-SHR compared with the other three groups, whereas there were no significant differences in these levels in the renal cortex among the four groups. In the renal AM receptor system, the expression of the gene for receptor activity modifying protein 3 was significantly increased in the renal medulla in D-SHR compared with the other three groups. An immunohistochemical study revealed that AM immunostaining in renal collecting duct cells and distal tubules was more intense in D-SHR than in the other three groups. After CGRP-(8–37) infusion, blood pressure increased significantly and urinary sodium excretion and urine flow decreased significantly only in D-SHR. These results suggest that the increased circulating AM and renal AM and the increased expression of the mRNA for AM and its receptor may at least partly compensate for the malignant hypertensive state in certain forms of malignant hypertension via the hypotensive, natriuretic, and diuretic actions of AM.

ADRENOMEDULLIN (AM) is widely distributed in various tissues and organs, including the kidney (31). The AM gene and specific binding sites for the AM peptide are highly expressed in the kidney (8, 30, 31). The considerable colocalization between the expression of the AM peptide and AM mRNA and the expression of AM receptors in the kidney suggests that this peptide may influence renal function as an autocrine and/or a paracrine factor. Previous studies have shown that intrarenal infusion of AM increased the renal blood flow and glomerular filtration rate (GFR) (2, 18) and that low-dose intrarenal infusion of AM increased the urinary flow and sodium excretion without changing the GFR (2). Immunohistochemical studies of AM in the canine kidney have shown AM immunoreactivity in glomeruli, cortical distal tubules, and medullary collecting duct cells (11). Furthermore, plasma AM levels were significantly higher in patients with essential hypertension, malignant hypertension, and chronic renal failure than in normal subjects (9, 12). These findings suggest that AM in the kidney may be involved in the regulation of renal function and in the pathophysiology of renal impairment. However, the exact role of circulating and intrarenal AM in malignant hypertension is not fully understood.

There has been considerable difficulty in identifying a distinct AM receptor (13). However, a recent study demonstrated that a seven-transmembrane receptor, the calcitonin receptor-like receptor (CRLR), can function as a receptor for either calcitonin gene-related peptide (CGRP) or AM, depending on the coexpression of a new family of three single transmembrane receptor activity modifying proteins (RAMP) (20). RAMP1 presents CRLR at the plasma membrane as a terminally

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glycosylated, mature glycoprotein and a CGRP receptor, whereas RAMP2 and RAMP3 present CRLR as an immature, core glycosylated AM receptor (20). To date, there have been few reports about the expression of CRLR and RAMP mRNAs in the kidney in mice (21). Nagae et al. (21) reported that RAMP3 is most abundant in the kidney in mice. After ureteral obstruction, expression of the RAMP1, RAMP2, and CRLR genes in the obstructed kidney was markedly upregulated, whereas RAMP3 expression was unchanged, suggesting that they play distinct roles in renal pathophysiology (21). However, there have been no studies investigating the expression of CRLR and RAMP mRNAs in the kidney of rats with malignant hypertension.

DOCA-salt spontaneously hypertensive rats (SHR) have been used extensively as a model of malignant hypertension accompanied by extensive end organ damage, including malignant nephrosclerosis (33). In the present study, to clarify the pathophysiological significance of AM in renal impairment, we measured the plasma, urine, and tissue AM levels and the abundance of AM, CRLR, and RAMP mRNAs and AM immunostaining in the kidney of DOCA-salt SHR. In addition, to examine the role of endogenous AM, we measured hemodynamics and renal tubular function before and after infusion of CGRP-(8–37), an AM antagonist, in control SHR and DOCA-salt SHR.

METHODS

Study 1

Materials and experimental design. All procedures were in accordance with our institutional guidelines for animal research and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Nine-week-old male Wistar-Kyoto rats (WKY) (n = 8) and SHR (Clea Japan, Tokyo, Japan; n = 27) weighing from 200 to 240 g were studied. SHR were randomly divided into the following three groups: control SHR group (n = 9), given drinking water ad libitum; salt-loaded SHR group (n = 9), given 1% NaCl drinking water ad libitum; DOCA-salt SHR group (n = 9), treated with DOCA (Sigma, St. Louis, MO) and given 1% NaCl drinking water ad libitum. DOCA was administered once a week by subcutaneous injection for 3 wk as previously reported (33). The rats were housed in metabolic cages for collecting 24-h urine samples for the measurement of urinary protein, sodium, creatinine, and AM and after an acclimatization period of at least 3 days. At the end of the 3 wk of DOCA treatment, all rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) and their body weights were measured. A polyethylene catheter (PE-50) was inserted into the thoracic aorta via the right carotid artery to measure heart rate (HR) and mean arterial pressure (MAP) as previously reported (23, 25). All procedures were done within 15 min. After these hemodynamic measurements were finished, 3 ml of blood was obtained from the carotid artery and transferred to a chilled glass tube for measurement of the serum levels of creatinine, urea nitrogen, and plasma AM. The heart was then arrested by an injection of 2 mmol KCl through the carotid artery, and the kidneys were excised, weighed, separated into the cortex and medulla (14), and frozen in liquid nitrogen and stored at −80°C until the RIA for AM.

RIA for tissue AM, plasma AM, and urinary AM. RIA for rat tissue, plasma, and urinary AM was performed as described previously (31).

Characterization of immunoreactive AM in rat urine. Rat urine was condensed with a Sep-Pak C_{18} cartridge and then separated by reverse-phase HPLC on a μ-Bondasphere C_{18} column (300 Å, 3.9 × 150 mm, Waters). Bound materials were eluted with a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid, and each collected fraction (1 ml) was submitted to the RIA for rat AM as previously reported (7).

Preparation of rat CRLR, RAMP2, RAMP3, and AM cDNA probes. An EcoRI/NaeI restriction fragment of rat cDNA corresponding to nucleotides –153 to 436 was used as the rat AM cDNA probe (23, 24). The rat (r) CRLR, RAMP2, and RAMP3 cDNA probes were synthesized by PCR using the following primers: rCRLR sense, 5'-AGG AGA TGG ACA AAC TAC AC-3'; rCRLR antisense, 5'-TTC CTT GAC CCT GTG GAA CG-3'; rRAMP2 sense, 5'-AAC ACA TGT CCT ACT TGC GTC-3'; rRAMP2 antisense, 5'-ACC GAG GAG GTG-3'; rRAMP3 sense, 5'-AGC GAC TGC ACC TTC TTC CAC-3'; and rRAMP3 antisense, 5'-CCG TCG GTA TCG TGC TCA GTC-3'. Amplification of cDNA by these primers should give 301-bp (rCRLR), 327-bp (rRAMP2), and 386-bp (rRAMP3) products. These PCR products have 85.4% (rCRLR), 82.1% (rRAMP2), and 84.2% (rRAMP3) nucleic acid identity with the corresponding human CRLR, RAMP2, and RAMP3, respectively.

RNA preparation and Northern blot analysis. Total RNA for the evaluation of AM mRNA expression was extracted from the renal cortex and renal medulla by the acid guanidinium thiocyanate-phenol-chloroform method as previously described (24). Furthermore, poly(A)^+ RNA for the evaluation of CRLR and RAMP mRNA expression was obtained from total RNA using Oligotex-dT_{30} (Takara Shuzou, Kyoto, Japan) according to the previously described procedure (17). Total RNA (20 μg/lane) for AM mRNA evaluation and poly(A)^+ RNA (2 μg/lane) for CRLR and RAMP mRNA evaluation were denatured with formaldehyde and formamide and electrophoresed on a 1% agarose gel containing formaldehyde. RNA in the gel was then transferred to a nylon membrane (Zeta-Probe blotting membrane, BioRad Laboratories, Hercules, CA) and crosslinked by ultraviolet irradiation. The conditions for prehybridization, hybridization, and membrane washing have been described previously (24). Band intensity was estimated using a radiolimage analyzer (BAS 5000, Fuji Film, Tokyo, Japan). To normalize the signals for the amount of RNA loaded and the transfer efficiencies, the same membrane was rehybridized with an oligonucleotide probe for 18S rRNA in the case of the AM signal and with a GAPDH cDNA probe in the cases of the CRLR and RAMP signals, respectively. Rehybridization was carried out after probes were stripped off by boiling in 0.1% SDS for 2 min.

Immunohistochemistry. For immunohistochemical analysis, kidney tissues obtained from WKY and control SHR, salt-loaded SHR, and DOCA-salt SHR were immediately fixed with 10% formalin. The tissues were embedded in paraffin, and 4-μm-thick sections were cut and mounted on slide glasses treated with silica. The slides were incubated overnight at 60°C and deparaffinized with graded concentrations of xylene and ethanol. Immunohistochemical analysis was performed using a monoclonal antibody recognizing AM (46–52) (dilution of ascites 1:200) as previously described (19). Nonimmune mouse IgG was used as a control.

Other analyses. Twenty-four-hour urine electrolytes were measured using an automated analyzer (System E2, Beck-
Table 1. Body weight, ventricular weights, heart rate, and mean arterial pressure in WKY and control, salt-loaded, and DOCA-salt SHR in study 1

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>Control SHR</th>
<th>Salt-Loaded SHR</th>
<th>DOCA-Salt SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats, n</td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>300 ± 9</td>
<td>296 ± 7</td>
<td>291 ± 5</td>
<td>289 ± 13</td>
</tr>
<tr>
<td>Left ventricular wt/body wt, g/kg</td>
<td>1.88 ± 0.15</td>
<td>2.31 ± 0.05a</td>
<td>2.47 ± 0.06a</td>
<td>3.69 ± 0.41abc</td>
</tr>
<tr>
<td>Right ventricular wt/body wt, g/kg</td>
<td>0.46 ± 0.06</td>
<td>0.49 ± 0.02</td>
<td>0.59 ± 0.02ace</td>
<td>0.71 ± 0.08abc</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>410 ± 16</td>
<td>465 ± 10</td>
<td>450 ± 11</td>
<td>430 ± 15</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>113 ± 16</td>
<td>165 ± 11b</td>
<td>170 ± 5b</td>
<td>193 ± 17b</td>
</tr>
</tbody>
</table>

Values are means ± SD. WKY, Wistar-Kyoto; SHR, spontaneously hypertensive rats. "P < 0.05 vs. control WKY, "P < 0.01 vs. control WKY, "P < 0.05 vs. control SHR, "P < 0.01 vs. control SHR, "P < 0.05 vs. salt-loaded SHR, "P < 0.01 vs. control SHR.

RESULTS

Study 1

Systemic and renal characteristics in WKY rats and in control, salt-loaded, and DOCA-salt SHR. The body weight, both ventricular weights, HR, and MAP in the four groups are presented in Table 1. The left ventricular weights and MAP were higher in control SHR and salt-loaded SHR than in WKY, and they were further increased in DOCA-salt SHR. The right ventricular weights were higher in salt-loaded SHR than in WKY and control SHR, and they were further increased in DOCA-salt SHR. There were no differences in HR among the four groups. Table 2 shows the renal characteristics of the four groups. DOCA-salt SHR had higher kidney weight, urea nitrogen, and urinary protein excretion, and lower creatinine clearance compared with the other three groups. Urinary sodium excretion was significantly higher in salt-loaded SHR and DOCA-salt SHR than in WKY and control SHR.

Plasma, urinary, and renal AM levels in WKY rats and in control, salt-loaded, and DOCA-salt SHR. The plasma AM, urinary excretion of AM, and tissue AM concentrations in the renal medulla and cortex in the four groups are presented in Fig. 1, A-D. The plasma AM concentration and urinary excretion of AM were markedly higher in DOCA-salt SHR than in the other three groups. The tissue AM concentration in renal medulla was also significantly higher in DOCA-salt SHR than in the other three groups, whereas there were no significant differences in the tissue AM concentrations in the renal cortex among the four groups. The plasma AM concentration was inversely correlated with Ccr (r = −0.75, P < 0.01) and positively correlated with the tissue AM concentrations in the renal cortex (r = 0.48, P < 0.05) and renal medulla (r = 0.45, P < 0.01).

Table 2. Renal characteristics of WKY and control, salt-loaded, and DOCA-salt SHR in study 1

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>Control SHR</th>
<th>Salt-Loaded SHR</th>
<th>DOCA-Salt SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney wt/body wt, g/kg</td>
<td>3.52 ± 0.20</td>
<td>3.62 ± 0.14</td>
<td>3.96 ± 0.39</td>
<td>5.78 ± 0.65abc</td>
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<tr>
<td>Urea nitrogen, mg/dl</td>
<td>23.5 ± 0.7</td>
<td>24.5 ± 0.8</td>
<td>26.0 ± 0.9</td>
<td>37.6 ± 4.0ace</td>
</tr>
<tr>
<td>Creatinine clearance, ml·min⁻¹·g kidney⁻¹</td>
<td>2.7 ± 0.3</td>
<td>2.6 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>1.2 ± 0.1ace</td>
</tr>
<tr>
<td>U_{Na}V, mmol/day</td>
<td>1.2 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>10.8 ± 3.8bd</td>
<td>12.4 ± 7.6bd</td>
</tr>
<tr>
<td>Urinary protein excretion, mg/day</td>
<td>20.0 ± 6.9</td>
<td>23.6 ± 2.9</td>
<td>23.2 ± 4.5</td>
<td>179 ± 95bd</td>
</tr>
</tbody>
</table>

Values are means ± SD. "P < 0.05 vs. control WKY, "P < 0.01 vs. control WKY, "P < 0.05 vs. control SHR, "P < 0.01 vs. control SHR, "P < 0.05 vs. salt-loaded SHR, "P < 0.01 vs. control SHR. U_{Na}V, urinary excretion of sodium.
The tissue AM level in the renal medulla was positively correlated with the urinary AM excretion ($r = 0.83$, $P < 0.01$). In addition, urinary AM excretion was positively correlated with urinary Na excretion ($r = 0.69$, $P < 0.01$) and negatively correlated with CCr ($r = -0.58$, $P < 0.05$).

Reverse-phase HPLC of rat urine. Immunoreactive AM in the urine was characterized by reverse-phase
HPLC. The immunoreactive AM consisted of one major and several minor peaks, and the major peak was eluted with a retention time identical to that of synthetic rat AM, suggesting that urinary AM is authentic AM consisting of 50 amino acids.

**Level of renal AM mRNA.** The expression of rat AM mRNA in the renal cortex and renal medulla of WKY and control, salt-loaded, and DOCA-salt SHR was investigated by Northern blot analysis. Figure 2A shows representative results of RNA blot analysis from the cortex and medulla in the kidney. The bands hybridizing to the rat AM cDNA probe were found at the position of ~1.6 kb. The results of quantitative analysis of these blots corrected for the levels of 18S rRNA as an internal control are shown in Fig. 2B. The expression of AM mRNA in the renal medulla was higher in DOCA-salt SHR than in the other three groups. However, the expression of AM mRNA in the renal cortex did not differ significantly among the four groups.

**Levels of renal CRLR, RAMP2, and RAMP3 mRNAs.** The expression of CRLR, RAMP2, and RAMP3 mRNA in the renal cortex and renal medulla of WKY and control, salt-loaded, and DOCA-salt SHR was investigated by Northern blot analysis. Representative blots are presented in Fig. 3A. The results of quantitative analysis of these blots corrected for the levels of GAPDH as an internal control are shown in Fig. 3B. There were no differences in the level of CRLR, RAMP2, and RAMP3 mRNAs in the cortex or medulla between WKY and control SHR. There were no differ-

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**A**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>Control SHR</th>
<th>Salt-loaded SHR</th>
<th>DOCA-salt SHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRLR</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>RAMP2</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>RAMP3</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
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<tr>
<td>GAPDH</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
</tr>
</tbody>
</table>

**B**

**Medulla**

- CRLR mRNA/GAPDH (%): ![Image](image17) ![Image](image18) ![Image](image19) ![Image](image20)
- RAMP2 mRNA/GAPDH (%): ![Image](image21) ![Image](image22) ![Image](image23) ![Image](image24)
- RAMP3 mRNA/GAPDH (%): ![Image](image25) ![Image](image26) ![Image](image27) ![Image](image28)

**Cortex**

- CRLR mRNA/GAPDH (%): ![Image](image29) ![Image](image30) ![Image](image31) ![Image](image32)
- RAMP2 mRNA/GAPDH (%): ![Image](image33) ![Image](image34) ![Image](image35) ![Image](image36)
- RAMP3 mRNA/GAPDH (%): ![Image](image37) ![Image](image38) ![Image](image39) ![Image](image40)

Fig. 3. A: representative Northern blot analysis of rat calcitonin receptor-like receptor (CRLR), receptor activity modifying protein (RAMP)2, and RAMP3 mRNAs in renal cortex and medulla of WKY and control, salt-loaded, and DOCA-salt SHR. B: quantitative analysis of rat CRLR, RAMP2, and RAMP3 mRNAs in the renal cortex and medulla of WKY and control, salt (S)-loaded, and DOCA-salt SHR. GAPDH was used as an internal control. *P < 0.05 vs. WKY, †P < 0.05 vs. control SHR, #P < 0.05 vs. salt-loaded SHR.
ences in the levels of CRLR, RAMP2, and RAMP3 mRNAs in the cortex or in the levels of CRLR and RAMP2 in the medulla between control SHR and DOCA-salt SHR, whereas DOCA-salt SHR had a higher level of RAMP3 mRNA in the medulla than in WKY and control SHR and a higher level of RAMP2 mRNA in the medulla than in WKY. Salt-loaded SHR had lower levels of CRLR and RAMP2 mRNAs and a higher level of RAMP3 mRNA in the cortex and a lower level of RAMP2 mRNA in the medulla than WKY and control SHR.

**Immunohistochemistry.** Hematoxylin-eosin staining of the kidney of a DOCA-salt SHR revealed marked wall thickening and obstruction of the small arteries, with hemorrhage and fibrinoid necrosis, consistent with the findings of malignant hypertension. Representative immunohistochemical staining for AM in the renal cortex and medulla from WKY and control, salt-loaded, and DOCA-salt SHR is shown in Fig. 4. Weak AM immunostaining in the cortical distal tubules and medullary collecting duct cells was observed in WKY and control and salt-loaded SHR. The AM immunoreactivity in the medullary collecting duct cells and cortical distal tubules was significantly more intense in the DOCA-salt SHR than in the control and salt-loaded SHR. Intense AM immunostaining in glomeruli was not found in any group. Control slides with nonimmune mouse IgG were negative for AM immunoreactivity (Fig. 4). The sections treated with preabsorbed anti-serum showed no immunoreactivity for AM either (data not shown).

**Study 2**

Body weight, ventricular weight, and kidney weight in the control SHR-CGRP-(8–37), DOCA-salt SHR, and DOCA-salt SHR-CGRP-(8–37) groups are presented in Table 3. DOCA-salt SHR had higher left and right ventricular weights and kidney weight and lower body weight compared with control SHR-CGRP-(8–37). The effect of CGRP-(8–37) or vehicle infusion on hemodynamics and renal excretory function in control and DOCA-salt SHR is shown in Table 4. DOCA-salt SHR had higher MAP, urine flow, and urinary sodium excretion before infusion compared with control SHR-CGRP-(8–37). These hemodynamic and renal excretory function parameters did not change after CGRP-(8–37) infusion in control SHR or vehicle infusion in DOCA-salt SHR, whereas MAP significantly increased and urine flow and urinary sodium excretion significantly decreased after CGRP-(8–37) infusion only in DOCA-salt SHR.

**DISCUSSION**

In the present study we showed for the first time that the tissue AM and AM mRNA levels in the renal medulla were increased with the increased plasma AM levels in DOCA-salt SHR, thereby at least partly com-

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**Table 3. Body weight, ventricular weights, and kidney weight in control and DOCA-salt SHR in study 2**

<table>
<thead>
<tr>
<th></th>
<th>Control SHR CGRP-(8–37)</th>
<th>DOCA-Salt SHR Vehicle</th>
<th>DOCA-Salt SHR CGRP-(8–37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats, n</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>330 ± 17</td>
<td>288 ± 17*</td>
<td>294 ± 11*</td>
</tr>
<tr>
<td>Left ventricular wt/body wt, mg/g</td>
<td>2.61 ± 0.31</td>
<td>3.59 ± 0.35*</td>
<td>3.42 ± 0.18*</td>
</tr>
<tr>
<td>Right ventricular wt/body wt, mg/g</td>
<td>0.55 ± 0.05</td>
<td>0.71 ± 0.10*</td>
<td>0.69 ± 0.06*</td>
</tr>
<tr>
<td>Kidney wt/body wt, mg/g</td>
<td>3.44 ± 0.24</td>
<td>4.53 ± 0.63*</td>
<td>4.38 ± 0.41*</td>
</tr>
</tbody>
</table>

Values are means ± SD. CGRP, calcitonin gene-related peptide. *P < 0.05 vs. control SHR, **P < 0.01 vs. control SHR.
pensating via AM’s hypotensive, natriuretic, and diuretic actions for the malignant hypertensive state in DOCA-salt SHR.

DOCA-salt SHR has been employed extensively as a model of malignant hypertension accompanied by extensive end organ damage. A previous study showed that the mean arterial pressure, total peripheral resistance index, cardiac and renal weights, and levels of blood urea nitrogen and serum creatinine were increased in DOCA-salt SHR (33), consistent with our present findings. The histological examination of the DOCA-salt SHR kidneys revealed obstruction of the small arteries with fibrinoid necrosis and glomerular sclerosis, indicating severe renal damage by malignant hypertension. Thus DOCA-salt SHR is a good model for studying the acute development of renal impairment (33). Using this model, we investigated the pathophysiological significance of AM in renal impairment.

It is well established that the plasma AM level is increased in patients with essential hypertension, malignant hypertension, and renal failure and is correlated with the severity of these diseases (9, 12). In the current study, the plasma AM level was markedly increased in the DOCA-salt SHR and was inversely correlated with creatinine clearance, consistent with the results of our previous study (9). In addition, we measured tissue AM levels in the renal cortex and medulla and found that the tissue AM level in the renal medulla was increased and that the plasma AM level was correlated with the tissue AM levels in the renal cortex and medulla in DOCA-salt SHR. These results suggest that the increased plasma AM level in DOCA-salt SHR may be due not only to decreased clearance of AM in the kidney, but also to increased production of AM in the kidney.

To our knowledge, no previous study has investigated the role of endogenous AM in renal impairment in vivo using the AM antagonist CGRP-(8–37), although several studies have demonstrated that the hemodynamic and renal actions of AM were antagonized by CGRP-(8–37) (4, 6, 26–28). In this study, to examine the role of the endogenously increased plasma AM level in rats with malignant hypertension, we measured blood pressure before and after infusion of CGRP-(8–37). We found that MAP significantly increased after CGRP-(8–37) infusion only in DOCA-salt SHR, suggesting that endogenous AM plays a compensatory role in the regulation of blood pressure in this malignant hypertension model. Previous studies have shown that continuous infusion of human AM in the hypertensive rats significantly reduces blood pressure with an increase of the plasma AM level within the physiological range (15, 16). Furthermore, a very recent study showed that human AM gene delivery significantly reduced blood pressure with an increase of the plasma AM level within the pathophysiological range (1). Taken together, these findings suggest that increased circulating AM may compensate for severe hypertension via its hypotensive action in DOCA-salt SHR. In contrast, the effect of CGRP-(8–37) on the MAP in SHR was not significant in this study. Several previous studies have shown that the plasma AM level is increased in human essential hypertension (9). However, in this study the plasma AM level in SHR was not increased compared with the level in normotensive rats, which is consistent with a previous report (34).

These results suggest that endogenous circulating AM may not play an important role in the blood pressure regulation in SHR. Further studies will be necessary to elucidate the exact role of circulating AM in the regulation of blood pressure in mild to moderate hypertension.

Previous reports have shown that urine contains AM (32); however, the origin and the pathophysiological significance of urinary AM remain unknown. In the present study, we demonstrated that the excretion of urinary AM was increased in malignant hypertension and that AM peptide and mRNA levels in the renal medulla were significantly increased in DOCA-salt SHR compared with the other three groups. We also showed that the AM immunoactivity in the medullary collecting duct cells was significantly more intense in the DOCA-salt SHR than in the control SHR. A close relationship was observed between urinary AM excretion and the tissue AM level in the renal medulla. These results suggest that urinary AM is derived from the kidney and that increased urinary excretion of AM in DOCA-salt SHR may reflect the increased production of AM in the renal medulla. Regarding the pathophysiological significance of increased AM in the renal medulla, it has been established that the inner medullary collecting ducts (IMCD) are the final arbiter of renal sodium excretion and that sodium transport in this segment is controlled by a wide variety of hormones (37). AM has been reported to increase sodium excretion in the IMCD via prostaglandin (10), suggesting a role of AM in the regulation of sodium excretion. Indeed, urinary AM

Table 4. Effect of CGRP-(8–37) or vehicle infusion on hemodynamics and renal excretory function in control and DOCA-salt SHR in study 2

<table>
<thead>
<tr>
<th></th>
<th>Control SHR CGRP-(8–37)</th>
<th>DOCA-Salt SHR Vehicle</th>
<th>DOCA-Salt SHR CGRP-(8–37)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>156 ± 11</td>
<td>154 ± 10</td>
<td>167 ± 14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>395 ± 26</td>
<td>400 ± 35</td>
<td>376 ± 57</td>
</tr>
<tr>
<td>Urine flow, µL·min&lt;sup&gt;-1&lt;/sup&gt;·kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.57 ± 0.45</td>
<td>2.67 ± 0.66</td>
<td>3.83 ± 1.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>UNaV, neq·min&lt;sup&gt;-1&lt;/sup&gt;·kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>2.19 ± 0.41</td>
<td>2.17 ± 0.27</td>
<td>7.39 ± 1.39&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SD. <sup>a</sup>P < 0.05 vs. control SHR, <sup>b</sup>P < 0.01 vs. control SHR, <sup>c</sup>P < 0.05 vs. before infusion.
excretion was significantly correlated with urinary sodium excretion in this study. These observations suggest that increased AM in the renal medulla of DOCA-salt SHR may be involved in the sodium regulation. To test this hypothesis, we measured urinary sodium excretion and urine flow before and after CGRP(8–37) infusion. Urinary sodium excretion and urine flow were slightly but significantly decreased after CGRP(8–37) infusion. These findings indicate that increased renal AM in malignant hypertension may at least partially compensate renal impairment via its natriuretic and diuretic actions.

Recently, McLatchie et al. (20) isolated and cloned a new family of single-transmembrane domain proteins, which they called RAMP1, RAMP2, and RAMP3. RAMPs are required to transport CRLR to the plasma membrane. The RAMP1/CRLR complex serves as a CGRP receptor due to terminal glycosylation of CRLR, whereas RAMP2/CRLR and RAMP3/CRLR serve as AM receptors due to core glycosylation of CRLR (5, 20). Although previous studies have demonstrated that AM exists in the kidney and that AM has many renal functions, whether the renal AM receptor is modulated by transcriptional regulation in renal impairment remains unknown. In the current study, we showed that CRLR, RAMP2, and RAMP3 were expressed in the renal cortex and medulla. There were no significant differences in the expressions of CRLR, RAMP2, or RAMP3 genes in the cortex and in that of the CRLR and RAMP2 genes in the medulla between control SHR and DOCA-salt SHR. However, RAMP3 gene expression in the renal medulla was slightly but significantly upregulated in DOCA-salt SHR. These results suggest that the RAMP family genes are differently regulated in the renal impairment in this model. Because AM has natriuretic and diuretic actions, upregulation of the AM and AM receptor gene expression in the renal medulla may partly favor the protective response against sodium and water retention in DOCA-salt SHR. On the other hand, only chronic salt loading significantly reduced RAMP2 mRNA expression both in the renal cortex and medulla and increased RAMP3 mRNA expression in the renal cortex. As noted above, the increase in RAMP3 mRNA in the kidney may lead to the potentiation of AM-induced natriuresis and diuresis and may serve to maintain blood pressure at a constant level. In contrast, the decrease of RAMP2 mRNA may account for the weakness of the AM response. The lack of difference of blood pressure between the control and salt-loaded SHR suggests that increase in the RAMP3 mRNA and decrease of RAMP2 mRNA may counteract each other. Elucidation of the details of the transcriptional regulation and the pathophysiologic significance of the intrarenal RAMPs and CRLR system in salt-loaded and DOCA-salt SHR will require further study.

This study has several limitations. Whether the effect of CGRP(8–37) is mediated by blockage of the action of AM or blockage of the action of CGRP, or both, is not clear at present. The plasma CGRP level has been reported to be decreased in human and rat hyper-tension compared with that in normal controls (3, 38). In contrast, neuronal levels of CGRP are reported to be increased in a volume-overloaded hypertension model (35). Because AM and CGRP can interact with each receptor, it is impossible to block only the AM receptor. Therefore, we cannot rule out the possibility that the present results obtained with CGRP(8–37) might be partly mediated by inhibition of the endogenous action of CGRP.

In conclusion, plasma AM, urinary AM, intrarenal AM peptide, AM mRNA, and AM receptor mRNA are significantly increased in DOCA-salt SHR. These increased levels of plasma and intrarenal components of the AM system seem, at least in part, to compensate for the malignant hypertensive state via the hypotensive, natriuretic, and diuretic actions of AM in certain forms of malignant hypertension.

Perspectives

Previous studies revealed that the plasma AM level is increased in severe hypertension (9, 12); however, the exact role of the increased plasma AM is not fully understood. The present study demonstrated that the endogenously increased AM participates in the blood pressure regulation in malignant hypertensive rats. Furthermore, increased renal AM, mainly in the tubules, also participates in the sodium and water handling in malignant hypertension. These findings, together with the direct inhibitory effect of AM on the proliferation and DNA synthesis of mesangial cells (29, 36), imply that long-term AM infusion may be a new therapeutic approach for the treatment of severe hypertension. Thus it is interesting to speculate that chronic AM infusion would have renoprotective effects against progressive renal injury in severe hypertension. The long-term effects of AM on glomerular injury should be clarified in further studies.

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