Alterations of Intrarenal Adrenomedullin and Its Receptor System in Heart Failure Rats

Fumiki Yoshihara, Toshio Nishikimi, Ichiro Okano, Takeshi Horio, Chikao Yutani, Hisayuki Matsuo, Shuichi Takishita, Tohru Ohe, Kenji Kangawa

Abstract—Calcitonin receptor–like receptor/receptor activity–modifying protein 2 (CRLR/RAMP2) and CRLR/RAMP3 complexes have been reported to be specific adrenomedullin (AM) receptors. In the present study, we evaluated the pathophysiological significance of renal AM and its receptor system in aortocaval shunt (ACS) rats. Renal AM levels were measured serially during 5 weeks after the operation. Renal gene expressions of AM, CRLR, RAMP2, and RAMP3 were measured at 2 weeks (decompensated phase) and 5 weeks (compensated phase) after the operation. Immunohistochemical localizations of renal AM were also evaluated. Furthermore, the relations between urinary sodium excretion (UNaV) and renal AM levels were evaluated. Renal AM levels were higher in ACS than in control animals only at 1, 2, and 3 weeks after the operation. At 2 weeks after the operation, renal AM mRNA expression was also higher in ACS than in control animals. CRLR, RAMP2, and RAMP3 mRNAs were expressed in the kidney, but there were no differences between the 2 groups. Immunohistochemistry revealed the positive AM immunostaining within the renal tubular cells, and it was more intense in ACS than in control animals. There were significant correlations between UNaV and renal AM levels. At 5 weeks after the operation, there were no differences in mRNA levels of AM, CRLR, RAMP2, and RAMP3 between the 2 groups. There was a significant correlation between UNaV and medullary AM levels. The present findings suggest that increased renal AM levels in decompensated heart failure, presumably due to increased AM production in renal tubules, in part, are involved in the regulation of sodium excretion. (Hypertension. 2001;37:216-222.)

Key Words: adrenomedullin receptors, adrenomedullin kidney heart failure sodium

Adrenomedullin (AM) and its gene expression have been reported to be observed in the glomerulus, distal tubules, and medullary collecting duct cells with respect to limitation to the kidney.1-4 The AM infusion studies revealed that AM increased renal blood flow, urine flow, and urinary sodium excretion (UNaV) and decreased proximal and distal fractional reabsorption of sodium.5-9 Furthermore, AM has been shown to inhibit DNA synthesis and endothelin production and to stimulate cAMP formation in mesangial cells and renal tubular cells.10,11,14,15 These findings suggest that AM may elicit its action as an autocrine and/or a paracrine factor as well as a circulating factor in the kidney. However, the specific AM receptor that mediates these many AM functions was only recently elucidated. McLatchie et al16 demonstrated that the calcitonin receptor–like receptor (CRLR), a receptor with 7 transmembrane domains, can function as either a calcitonin gene–related peptide (CGRP) receptor or an AM receptor, depending on which members of a new family of single-transmembrane-domain proteins, which are called receptor-activity-modifying proteins (RAMPs) are expressed. RAMP1 presents the receptor at the cell surface as a CGRP receptor, whereas RAMP2- or RAMP3-transported receptors are AM.

The findings of AM infusion studies suggest that AM may be involved in the pathophysiology of heart failure. However, few reports have focused on the role of renal AM in heart failure. One study has reported that immunoreactive AM was increased in the glomerulus, distal tubules, and medullary collecting duct cells in the kidney in dogs with overt heart failure induced by rapid ventricular pacing.17 However, serial changes and the pathophysiological significance of renal AM and renal specific AM receptor in the development of heart failure have not been elucidated. In the present study, we evaluated the pathophysiological significance of increased renal AM in heart failure and whether renal specific AM receptors (CRLR/RAMP2 and CRLR/RAMP3 complexes) are involved in the pathophysiology of heart failure.

Methods

This study was performed in accordance with the guidelines of the Animal Care Committee of the National Cardiovascular Center Research Institute.

Received March 8, 2000; first decision March 23, 2000; revision accepted August 17, 2000.

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TABLE 1. Body Weight, Hemodynamics, and Heart Weights in Control and ACS Rats

<table>
<thead>
<tr>
<th></th>
<th>1 wk</th>
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<tr>
<td></td>
<td>C</td>
<td>ACS</td>
<td>C</td>
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<td>118±11†</td>
<td>142±10</td>
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<tr>
<td>LVEDP, mm Hg</td>
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<td>15±4‡</td>
<td>8±3</td>
<td>16±4‡</td>
<td>7±3</td>
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<tr>
<td>RVSP, mm Hg</td>
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<td>55±4‡</td>
<td>37±4</td>
<td>56±8§</td>
<td>40±3</td>
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<tr>
<td>RV/BW, mg/g</td>
<td>0.59±0.05</td>
<td>0.91±0.16‡</td>
<td>0.58±0.09</td>
<td>0.95±0.22‡</td>
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<tr>
<td>LV+SEP/BW, mg/g</td>
<td>2.01±0.28</td>
<td>2.41±0.32‡</td>
<td>1.92±0.15</td>
<td>2.37±0.32†</td>
<td>1.96±0.22</td>
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<td>Lung/BW, mg/g</td>
<td>3.88±0.58</td>
<td>5.17±1.21*</td>
<td>3.99±0.44</td>
<td>5.30±1.30*</td>
<td>4.08±1.22</td>
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</table>

MAP indicates mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; RVSP, right ventricular systolic pressure; RV, right ventricular weight; BW, body weight; LV, left ventricular weight; SEP, septal weight. Values are mean±SD.

*P<0.05, †P<0.01, ‡P<0.001 vs C.

Study 1
Seven- to 8-week-old male Wistar rats, weighing 250 to 300 g, were used for aortocaval shunt (ACS)-induced heart failure. ACS was produced in rats via a method previously described and modified in our laboratory. Control (C) rats underwent an identical operation, but no shunt was established (n=36). Approximately 30% of ACS rats died. As a result, 55 AC shunt rats were studied.

Hemodynamic Study
Hemodynamic studies were performed at 1, 2, 3, 4, and 5 weeks (1 week: ACS n=14, C n=11; 2 weeks: ACS, n=8, C n=8; 3 weeks: ACS n=14, C n=6; 4 weeks: ACS n=9, C n=6; 5 weeks: ACS n=10, C n=5) after the ACS operation as previously described. The rats were then killed, and their kidneys and hearts were excised. Each kidney was immediately separated into medulla and cortex as previously described. Heart and lung weights were also measured as previously described.

Metabolic Study
Twenty-two rats (ACS n=14, C n=8) were housed in metabolic cages for collection of 24-hour urine samples for measurements of UNaV at 1, 2, 3, 4, and 5 weeks after the operation. Hemodynamic studies were also performed in these 22 rats after the metabolic study, and the results were entered into the data for hemodynamic studies in study 2.

Radioimmunoassay for Renal AM
AM levels in the renal medulla and cortex during the 5-week time course after the operation were measured serially in ACS and C rats. Radioimmunoassay for rat AM was performed as described previously.

Study 2
Because study 1 revealed that the period of 3 weeks after the ACS operation was the decompensated heart failure phase and that the period at and after 4 weeks was the compensated phase, we evaluated the parameters as follows at 2 and 5 weeks after the operation.

Metabolic Study
An additional 18 rats (ACS, n=10; C, n=8) were housed in metabolic cages for collecting 24-hour urine samples for measurements of UNaV at 2 weeks after the operation as described above.

Hemodynamic Study
Hemodynamic studies were performed in ACS and C rats at 2 weeks: ACS n=18; ACS n=10, C n=8) and 5 weeks (n=22; ACS n=14, C n=8) after the operation as described earlier. Animals were anesthetized with pentobarbital sodium (40 mg/kg IP), and blood (1 mL) was withdrawn through the femoral vein for plasma renin concentration (PRC) and atrial natriuretic peptide (ANP) measurements.
After hemodynamic measurements, blood (4 mL) was withdrawn again through the catheter for plasma AM measurement.

RIA for Plasma AM, Plasma ANP, and PRC
RIA for rat AM was performed as described earlier. Plasma ANP and PRC were also measured with the specific RIA as previously reported.

cDNA Probes and Radiolabeling of Probes
An EcoRI/NaeI restriction fragment of rat AM cDNA corresponding to nucleotides 153 to 436 was used as the rat AM cDNA probe. The rat CRLR, RAMP2, and RAMP3 cDNA probes were synthesized with PCR with the following primers: CRLR sense, 5'-AGGACATGGACAAACTACAC-3'; CRLR antisense, 5'-GAA-TGAACTGGGACACCTTGC-3'; RAMP2 sense, 5'-AACACATGGACAAACTACAC-3'; RAMP2 antisense, 5'-GAA-TGAACTGGGACACCTTGC-3'; RAMP3 sense, 5'-AGCGACTGCACCTTCCA-3'; and RAMP3 antisense, 5'-GCCAGCCATAGC-CACAGTCAG-3'. Amplification of cDNA with these primers should result in 301-bp (CRLR), 327-bp (RAMP2), and 386-bp (RAMP3) PCR products. These PCR products have 85.4% (CRLR), 82.1% (RAMP2), and 84.2% (RAMP3) nucleic identity with the corresponding human CRLR, RAMP2, and RAMP3, respectively. These probes were radiolabeled by random priming with [α-32P]dCTP (Amersham), and the labeled probes were purified by column chromatography (NICK column; Pharmacia Biotech).

Northern Blot Analysis
Total RNA (20 μg/lane) for AM mRNA evaluation and poly(A)+ RNA (2.5 μg/lane) for CRLR, RAMP2, and RAMP3 mRNA evaluation were denatured, electrophoresed, and transferred to a nylon membrane. For hybridization with the cDNA probes, conditions for hybridization and washing have been previously described.

Immunohistochemistry
Immunohistochemical analysis was performed with a monoclonal antibody that recognizes AM-46–52 (dilution of ascites, 1:200) as previously reported.

Statistical Analysis
All values are presented as mean±SD. Comparisons of renal AM concentrations in the time course after the operation were performed by ANOVA with Fisher’s post hoc test. Comparisons between 2 groups were performed by unpaired t-test. Differences were considered statistically significant at a level of P<0.05. Correlation coefficients were calculated using linear regression analysis.

Results

Study 1
There were no significant differences in body weight and heart rate between the 2 groups during the 5 weeks after the operation (Table 1). Mean arterial pressure was significantly lower in ACS than in C animals during the 5 weeks after the operation (Table 1). Left ventricular end-diastolic and right

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<th>TABLE 2. Body Weight, Hemodynamics, Humoral Factors, and Heart Weights in Control and ACS Rats</th>
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<td>LVEDP, mm Hg</td>
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<td>PRC, ng AngI · mL⁻¹ · h⁻¹</td>
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<td>ANP, pg/mL</td>
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<td>AM, fmol/mL</td>
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<td>LV+SEP/BW, mg/g</td>
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<td>Lung/BW, mg/g</td>
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MAP indicates mean arterial pressure; LVEDP, left ventricular end-diastolic pressure; PRC, plasma renin concentration; ANP, plasma ANP concentration; AM, plasma AM concentration; RV, right ventricular weight; BW, body weight; LV, left ventricular weight; SEP, septal weight. Values are mean±SD.

*P<0.05, †P<0.01, ‡P<0.001 vs C.
ventricular systolic pressures and right ventricular, left ventricular, and septal weights were significantly higher in ACS than in C animals during the 5 weeks after the operation (Table 1). Urinary sodium excretion was significantly lower in ACS than in C animals at only 1, 2, and 3 weeks after the operation (Figure 1). Tissue AM levels in the renal medulla and cortex were significantly higher in ACS than in C animals at 1, 2, and 3 weeks after the operation but not at 4 and 5 weeks (Figure 1).

Study 2
Mean arterial pressure was significantly lower and left ventricular end-diastolic pressure was higher in ACS than in C animals at both 2 and 5 weeks after the operation (Table 2). Although the plasma ANP level was higher in ACS than in C animals at both 2 and 5 weeks after the operation, the PRC and the plasma AM level were higher only at 2 weeks (Table 2). AM mRNA expressions in the renal medulla and cortex were also higher in ACS than in C animals only at 2 weeks after the operation (Figures 2 and 3). CRLR, RAMP2, and RAMP3 mRNA were expressed in the renal medulla and cortex, and there were no differences between the 2 groups at both 2 and 5 weeks after the operation (Figures 2 and 3). Immunohistochemistry revealed that positive AM immunostaining within the tubular cells in the renal medulla and cortex and AM immunoreactivity was more intense in ACS than in C animals only at 2 weeks after the operation (Figure 4). Tissue AM levels in the renal medulla (0.29±0.04 versus 0.22±0.02 fmol/mg, P<0.001) and cortex (0.39±0.02 versus 0.32±0.02 fmol/mg, P<0.0001) were significantly higher in ACS than in C animals at 2 weeks after the operation. In contrast, tissue AM levels in the kidney were comparable between the 2 groups (medulla 0.19±0.03 versus 0.20±0.02 fmol/mg, cortex 0.36±0.04 versus 0.32±0.05 fmol/mg) at 5 weeks. Finally, there were significant correlations not only between UNaV and tissue AM levels in the renal medulla and cortex at 2 weeks after the operation but also between UNaV and the medullary AM levels at 5 weeks (Figure 5).

Discussion
In the present study, we serially examined AM levels in the renal medulla and cortex in ACS rats and sham-operated C rats during 5 weeks after the operation. The expressions of AM and its receptor mRNA in the renal medulla and cortex were also measured to determine whether AM and its receptor system exist in the rat kidney and whether this system is modulated by transcriptional regulation after the ACS operation. In addition, the cellular localization of increased AM in the renal medulla and cortex was determined with immunohistochemical analysis. Furthermore, we examined the relationship between the renal AM levels and UNaV. We demonstrate for the first time that (1) AM and its receptor gene expression exist in rat kidney, (2) increased renal AM levels in decompensated heart failure may reflect increased AM production in renal tubules and collecting duct cells, and (3) increased renal AM in decompensated heart
failure and nonincreased medullary AM in compensated heart failure may be in part involved in the regulation of sodium excretion.

CRLR was identified in 1993 as a member of the 7-transmembrane-domain, G protein–coupled receptors. It has 55% overall identity with the calcitonin receptor. CGRP had been a candidate ligand for CRLR, although a previous report demonstrated that CRLR alone cannot function as a CGRP receptor. Recently, McLatchie et al isolated and cloned a new family of single-transmembrane-domain proteins that they called RAMP1, RAMP2, and RAMP3. RAMPs are required to transport CRLR to the plasma membrane. RAMP1 presents CGRP receptor due to terminal glycosylation of CRLR. RAMP2 and RAMP3 present AM receptors due to core glycosylation of CRLR. Although previous reports demonstrated that AM exists in the kidney and that AM has many renal functions, it has not previously been elucidated whether renal AM receptor is modulated by transcriptional regulation in heart failure or whether the modulated receptor may be involved in the pathophysiology of heart failure. Therefore, we examined the gene expression of CRLR, RAMP2, and RAMP3 in the kidney after the ACS operation in the present study. CRLR, RAMP2, and RAMP3 mRNAs were expressed in the renal cortex and medulla, but there were no significant differences in these gene expressions between ACS and C. However, the increased AM levels in the renal medulla and cortex in ACS rats were accompanied by increased AM gene expression at 2 weeks after the ACS operation. These findings suggest that increased renal AM may contribute to the pathophysiology of heart failure in acute phase and that this increased renal AM is probably caused by increased renal AM production.

Previous studies reported that AM gene expression and AM immunoreactivity existed not only in distal tubules and medullary collecting duct cells but also in glomeruli. Furthermore, AM has many effects not only on renal tubules but also on renal mesangial cells. Thus, the cellular localization of increased AM production might occur in the renal glomeruli, distal tubules, and collecting duct cells. However, the remarkable increased intensities of AM immunoreactivity occurred only in the renal tubules and collecting duct cells in the present study, suggesting that renal tubules and collecting duct cells might be the main sites for increased AM production in rats with ACS-induced heart failure. AM secretion from renal tubular cell lines was reported to be mediated vasopressin via V2 receptors. AM production increased in the renal medulla and cortex only during the decompensated heart failure phase, during which PRC was increased, suggesting that the renin-angiotensin-aldosterone system may be one of the stimulators of AM production in the kidney in rats with heart failure. Furthermore, we previously reported that cytokines such as tumor necrosis factor-α and interleukin-1β regulate the AM production in cultured rat cardiac myocytes and nonmyocytes. Because these cytokines are involved in the pathophysiology of heart failure, these cytokines may contribute to the production of renal AM. Further study is necessary to reveal the exact mechanism for renal AM production. In addition, the mechanism for the regulation of AM receptor gene expression is still unknown. The discrepancy between the upregulation of AM gene expression and the lack of changes in AM receptor gene expression at 2 weeks after the operation in the present study suggest that there was a different regulatory mechanism of gene expression between AM and AM receptor in rats with ACS-induced heart failure.

AM increases cAMP more potently than CGRP and amylin in rat renal tubular basolateral membranes, indicating that renal tubules may be one of the target cells for AM. The intravenous infusion of AM exerted diuresis and natriuresis due to the increased glomerular filtration rate and effective renal plasma flow. Furthermore, low doses of exogenous AM infusion appeared to increase UNaV due to the decreased proximal and distal fractional reabsorption of sodium. These findings confirmed that exogenous AM infusion in-

![Figure 4. AM immunoreactivity with a monoclonal antibody that recognizes the C-terminal structure of AM in the renal medulla and cortex. A, Sham-operated medulla. B, Aortocaval shunting medulla. C, Control medulla (nonimmune mouse IgG was used). D, Sham-operated cortex. E, Aortocaval shunting cortex. F, Control cortex (nonimmune mouse IgG was used). G, Sham-operated medulla. H, Aortocaval shunting medulla. I, Control medulla (nonimmune mouse IgG was used). J, Sham-operated cortex. K, Aortocaval shunting cortex. L, Control cortex (nonimmune mouse IgG was used) at 5 weeks after the operation.](http://hyper.ahajournals.org/content/vol37/issue2)
creased UNaV in rats. However, the role of increased AM in renal tubules was still unknown. In the present study, the tissue AM levels in the renal medulla and cortex were increased in ACS rats compared with control rats at 1, 2, and 3 weeks after the operation, and there were significant correlations between renal AM levels and UNaV at 2 weeks after the operation. Although renal AM levels were not increased in ACS rats at 5 weeks after the operation, medullary AM levels were significantly correlated with UNaV. Taken together, these findings suggest that increased endogenous renal AM in decompensated heart failure may be involved in the regulation of sodium excretion as a defense mechanism and that nonincreased renal medullary AM in the compensated phase may also contribute to the regulation of sodium excretion.

The present study has limitations. The lack of significant increases in PRC and plasma AM levels and moderately increased plasma ANP levels in ACS rats at 5 weeks after the operation suggested that rats with chronic heart failure were relatively well compensated. Further study is necessary to reveal the pathophysiological significance of renal AM in the development of heart failure.

In conclusion, in the present study we demonstrated that AM and its receptor system exist in the kidney. The present findings suggest that increased renal AM in decompensated heart failure may be mediated via increased AM synthesis in renal distal tubules and collecting duct cells and that increased renal AM may in part be involved in the regulation of sodium excretion in rats with ACS-induced heart failure.

Acknowledgments

This work was supported in part by Special Coordination Funds for Promoting Science and Technology (Encouragement System of COE) from the Science and Technology Agency of Japan, the Ministry of Health and Welfare, and the Human Science Foundation of Japan. We thank Yoko Saito for technical assistance.

References


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Hypertension. 2001;37:216-222
doi: 10.1161/01.HYP.37.2.216

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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