Abstract—We investigated whether adrenomedullin (AM) participates in the pathophysiology during the transition from left ventricular hypertrophy (LVH) to heart failure (HF). We used the Dahl salt-sensitive (DS) rat model, in which systemic hypertension causes LVH at the age of 11 weeks, followed by HF at the age of 18 weeks. Two molecular forms of AM levels in the plasma and myocardium at the LVH stage were significantly elevated compared with those in controls, and they were further increased at the HF stage. Interestingly, the LV tissue AM-mature/AM-total ratio was higher only in the HF group than in controls and LVH. The LV tissue AM-mature/AM-total ratio, AM-mature, and AM-total concentrations had close relations with the LV weight/body weight (r = 0.72, r = 0.79, and r = 0.70, respectively; all P < 0.001). AM gene expression was significantly increased at the LVH stage and was further increased at the HF stage. Furthermore, gene expression of AM receptor system components such as calcitonin receptor–like receptor (CRLR), receptor activity–modified protein 2 (RAMP2), and RAMP3 were significantly increased at the stage of LVH and HF. Regarding other neurohumoral factors, plasma renin and aldosterone levels were not increased at the LVH stage but were increased at the HF stage, whereas atrial natriuretic peptide was increased in both the plasma and myocardium at the LVH stage and was further increased at the HF stage. These results suggest that induction of the cardiac AM system, including the ligand, receptor, and amidating activity, may modulate pathophysiology during the transition from LVH to HF in this model. (Hypertension. 2003;41:512-518.)

Key Words: adrenomedullin • hypertension, sodium dependent • hypotrophy, left ventricular • heart failure • rats, Dahl

Many neurohumoral factors are involved in the pathophysiology of heart failure (HF).1 Therefore, it is very important to investigate the pathophysiological roles of newly identified neurohumoral factors in HF because these studies may lead to the development of a new drug.1 In fact, angiotensin-converting enzyme inhibitors, β-blockers, and angiotensin receptor blockers, which were produced in this field of research, are now used in many patients with HF and contribute to the improvement in prognosis and quality of life in this syndrome. Recent studies indicate that most patients with HF have a history of hypertension and/or left ventricular hypertrophy (LVH).2,3 Furthermore, it has been suggested that treating high blood pressure actually prevents HF.4 These results suggest that hypertension and LVH are the most common risk factors for HF, and they contribute a large proportion of the HF cases.5 Therefore, it seems important to assess the derangement of neurohumoral factors during the transition from LVH to HF.

Adrenomedullin (AM) is a 52–amino acid novel vasodilatory peptide that was originally discovered in human pheochromocytoma tissue.6 AM is widely distributed in various tissues and organs, including the heart.7 The plasma levels of AM are elevated in various pathophysiological states, eg, hypertension, pulmonary hypertension, myocardial infarction, and congestive HF.8 The elevated levels of AM in HF are closely associated with the severity of disease.9 We and other groups showed that short-term administration of AM improves neurohumoral factors and hemodynamics in humans and animals with HF.10–12 These findings suggest that AM plays a pathophysiological role as a circulating hormone in HF.

In addition to its action as a systemic hormone, AM has a number of actions as a local factor in the heart. Indeed, we and other groups demonstrated that cardiac myocytes and fibroblasts produce and secrete AM, and AM inhibits cardiac hypertrophy in myocytes and collagen synthesis in cardiac fibroblasts.13–15 In addition, previous studies reported that the expression of AM in the heart is accelerated after pressure overload, volume overload, and myocardial infarction16–18 and that chronic elevation of AM by AM gene delivery or
peptide infusion improves cardiac hypertrophy in a hypertension model.16,19,20

However, several questions remain to be defined with regard to the local roles of AM. First, because no precise serial evaluation of the AM receptor system has been performed, it is unclear at present whether the cardiac AM receptor system is upregulated even in the hypertrophied heart or in association with HF. A recent study demonstrated that a 7-transmembrane receptor, the calcitonin receptor–like receptor (CRLR), can function as an AM receptor with the coexpression of single transmembrane receptor activity–modifying proteins (RAMPs), RAMP2 or RAMP3.21,22 Second, the roles of the different molecular forms of AM are unclear at present. Recent studies revealed that 2 molecular forms of AM, an active, mature form of AM (AM-m), and an intermediate, inactive form of glycine-extended AM (AM-Gly), circulate in human plasma.23 However, there have been no reports of studies investigating the molecular forms of AM in plasma and cardiac tissues in the normal state, LVH, and HF.

To address these questions, we used the Dahl salt-sensitive (DS) rat. In this rat maintained on a high-salt diet, systemic hypertension induces compensated concentric LVH at the age of 11 weeks, which is followed by congestive heart failure (CHF) with marked LV dilatation and global hypokinesis at the age of 16 to 18 weeks.24 Here, we investigated the role of the AM system in the transition from LVH to CHF in this animal model.

Methods

Experimental Animals and Protocols

All procedures were in accordance with institutional guidelines for animal research. Male inbred DS rats (Eisai Co Ltd, Tokyo, Japan) were used. After weaning, DS rats were fed a 0.3% NaCl (low-salt) diet until the age of 6 weeks. Thereafter, they were fed a diet containing 9% NaCl (high salt). Age-matched male Dahl salt-resistant (DR) rats fed the same diet served as a control group. At 11 and 18 weeks of age, the body weight (BW), heart rate (HR), mean arterial pressure (MAP), and left ventricular end-diastolic pressure (LVEDP) were measured under anesthesia as previously reported,16,17 and then blood samples were collected. Immediately after the heart was arrested by injection of 2 mmol KCl, it was excised, weighed, frozen in liquid nitrogen as early as possible, and stored at −80°C until the radioimmunoassays (RIAs) for AM and atrial natriuretic peptide (ANP).

Assay for Plasma and LV Tissue Levels of Rat AM-m, AM-Total, ANP, and Other Factors

Rat plasma and tissue AM-m and AM-total (AM-m+AM-Gly) were measured by immunoradiometric assays with specific kits (Shionogi Co Ltd) with some modifications as previously reported.26 The assay’s minimal detectable quantity of AM-m or AM-total is 0.5 pmol/L in both kits. The coefficients of variation of the intra-assay and interassay in several blood samples were 4.4% to 8.2% and 5.5% to 8.3%, respectively, in the AM-m kit and 3.4% to 7.3% and 5.3% to 9.0%, respectively, in the AM-total kit. The plasma concentration of ANP was determined by RIA as described previously.27 The plasma renin concentration (PRC) was measured after adding an excess of angiotensinogen provided by binephrectomized rat plasma by a gamma coat plasma renin activity kit (Dade Behring Co).28 The plasma aldosterone concentration was measured by an RIA, ie, an SPAC-S aldosterone kit (Daiichi Radioisotope Labs).29 Extraction of LV tissue was performed as previously reported.30 RIAs for rat AM-m, AM-total, and ANP were measured as mentioned previ-

ouously. We measured these hormone levels in tissue or plasma samples from DS and DR rats at the same time.

RNA Preparation and Northern Blot Analysis

Total mRNA was prepared from the LV by use of TRizol (Life Technologies Inc). Northern blot analysis was performed as described in detail in our previous reports.16–18 The density of each mRNA band was measured with a Bioimaging analyzer (BAS-2000, Fuji Photo Film Co).

Reverse Transcription (RT)–Polymerase Chain Reaction Analysis for Rat CRLR, RAMP2, and RAMP3

After total RNA was extracted, synthesis of first-strand complementary DNA, polymerase chain reaction (PCR) with appropriate primers, and quantification of PCR products were performed as described in detail in our previous report.26 As an internal control, we measured glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the same manner.26 The quantity of each species of mRNA was expressed by using the following formula: amount of original template of each molecule/amount of original template of GAPDH.

Statistical Analysis

All values are expressed as mean±SD. Multiple comparisons were performed with 1-way ANOVA followed by the Bonferroni test. Correlation coefficients were calculated by linear regression analysis. A value of P<0.05 was considered significant.

Results

Physiological Profiles

Both ventricular weights and MAP were higher in DS-LVH and DS-CHF than in 11-week-old and 18-week-old DR rats. LV weight/BW was elevated in DS-CHF than in DS-LVH rats. Lung weight, as an index of pulmonary congestion, and LVEDP, as an index of LV dysfunction, were increased in DS-CHF rats only compared with the other 3 groups. Differences in HR were observed between DS-LVH and DS-CHF rats only. BW was higher in 11-week-old and 18-week-old DR rats than in DS-LVH and DS-CHF animals. BW was further elevated in 18-week DR compared with 11-week DR rats (Table).

Levels of Molecular Forms of Plasma AM and Other Neurohumoral Factors

The plasma AM-total, AM-m, and ANP levels were higher in DS-LVH and DS-CHF than in the other 2 DR groups. These indexes were further elevated in DS-CHF compared with DS-LVH. In contrast, the PRC and aldosterone levels were increased in DS-CHF animals only compared with the other 3 groups. There were no differences in the AM-m/AM-total ratio among the 4 groups. Thus, the major molecular forms of AM in plasma is an inactive form, AM-Gly (Figures 1A through 1F).

Molecular Forms of Ventricular AM and ANP

The LV AM-total, AM-m, and ANP levels were higher in DS-LVH and DS-CHF than in the other 2 DR groups. These indexes were further elevated in DS-CHF compared with DS-LVH animals. Interestingly, the mean value of the AM-m/AM-total ratio was higher in LV tissue than in plasma (0.77±0.17 vs 0.26±0.08, P<0.001), suggesting that the major molecular forms of AM in the LV is the active form,
AM-m. In addition, in contrast to the findings in plasma, the LV AM-m/AM-total ratio was higher in DS-CHF than in the other 3 groups (Figures 2A through 2D). The LV tissue AM-total levels, AM-m levels, and AM-m/AM-total ratio were significantly correlated with LV weight/BW (Figures 3A through 3C).

**Levels of Gene Expression of AM and ANP in the LV**

Representative results of Northern blot analysis from the LV and the results of quantitative analysis of these blots, corrected for the levels of GAPDH mRNA as an internal control, are shown in Figures 4A and 4B. The expression of AM/GAPDH mRNA in the LV was slightly but significantly higher in DS-LVH and DS-CHF than in the other 2 groups, and it was further elevated in DS-CHF compared with DS-LVH animals (Figure 4A). In contrast, the expression of ANP/GAPDH mRNA in the LV was considerably higher in DS-LVH and DS-CHF than in the other 2 DR groups, and it was further elevated in DS-CHF compared with DS-LVH (Figure 4B).

**Levels of Gene Expression of CRLR, RAMP2, and RAMP3 in the LV**

Representative electrophoretic profiles of RT-PCR products (323, 164, and 416 bp) and quantitative analysis of the levels of these products, corrected for the levels of the GAPDH-specific product as an internal control, are shown in Figures 5A through 5C. The CRLR/GAPDH mRNA level, RAMP2/GAPDH mRNA level, and RAMP3/GAPDH mRNA level in the LV were higher in DS-LVH and DS-CHF than in the 2 DR groups (Figures 5A through 5C), whereas there were no significant differences in these mRNA levels between DS-LVH and DS-CHF.

**Discussion**

To investigate the role of AM in the transition from compensated LVH to CHF, we used a DS rat model. In this model maintained on a high-salt diet, systemic hypertension results in the establishment of LVH at the age of 11 weeks, followed by marked ventricular dilatation and neurohumoral derangement at the age of 18 weeks. We found that in rats with established LVH, cardiac and circulating AM levels and its
receptor system were significantly activated. Along with the transition to HF, the cardiac and circulating AM system showed further activation. Furthermore, the active/inactive ratio of AM in cardiac tissue was further elevated as part of this activated AM system, in parallel with the progression to HF. The present results show for the first time that not only circulating AM, but also the cardiac AM system, including ligands, receptors, and the active-form/inactive-form ratio, plays a role in the transition from LVH to CHF.

Cardiac AM System Is Activated in Compensated Hypertrophy

AM is an antihypertrophic peptide able to inhibit hypertrophy induced by angiotensin II or endothelin-1 in cultured neonatal cardiac myocytes and fibroblasts. Stimulation by angiotensin II or stretch induces the expression of AM in cultured cardiac tissue. This study found that the expression of AM in cardiac tissue was further elevated as part of the activated AM system, in parallel with the progression to HF. The present results show for the first time that not only circulating AM, but also the cardiac AM system, including ligands, receptors, and the active-form/inactive-form ratio, plays a role in the transition from LVH to CHF.
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ible with the report by Morimoto et al16 showing that those in the age-matched DR rats. Our findings are compat-
plasma and myocardial AM peptide and mRNA levels in DS compensated hypertrophy against increased afterload. The DS rats at 11 weeks had a marked increase in blood pressure and LV mass with normal LVEDP, indicating an established, activated even in hypertrophied hearts. We demonstrated that AM exerts antihypertrophic effects in vivo.16 Moreover, the AM receptor system was significantly activated at the LVH stage in this study. Although the exact role of increased cardiac AM is still unknown at present, a previous study showed that chronic AM infusion significantly reduced LVH induced by aortic banding compared with those in the age-matched DR rats. Our findings are compatible with the report by Morimoto et al16 showing that myocardial AM levels were increased in LVH induced by aortic banding compared with those in sham-operated rats. Furthermore, the AM receptor system was significantly activated at the LVH stage in this study. The present study, we first showed that the major circulating form of AM in the failing heart. A recent study reported that endothelin-1 significantly increases CRLR and RAMPs expression in cultured cardiac myocytes.30 Because it is well known that endothelin-1 is increased in the failing heart,24 increased endothelin-1 may in part have contributed to the increase in CRLR and RAMPs in this study. Thus, gene expression of AM receptor components may also be upregulated in the failing heart with concomitant increases of the ligand and its mRNA.

In the present study, we also analyzed the molecular forms of AM. In the biosynthesis of AM, the AM precursor is converted to C-terminal glycine-extended AM (AM-Gly), which is an inactive, intermediate form of AM. Subsequently, inactive AM-Gly is converted to the active form, mature AM (AM-m), by enzymatic amidation.13 Recent studies showed that (1) 2 molecular forms of plasma AM circulate in human plasma, (2) the major circulating form of AM is AM-Gly, and (3) both plasma AM-m and AM-Gly levels are increased in parallel in patients with hypertension and HF.23,31 In the present study, we first showed that the major circulating form of rat AM is AM-Gly in normotensive and hypertensive rats and in rats with HF and that both plasma AM-m and AM-total levels were increased in parallel in hypertension with LVH and HF. In the current study, we further analyzed the molecular forms of ventricle12 tissue AM in LVH and the failing heart. We first found that the AM-m/AM-total ratio was higher in cardiac tissue than in plasma and that the AM-m/AM-total ratio was increased not in LVH but only in failing myocardium compared with that in DR rats. These results indicate that LV tissue amidating enzyme activity may increase in AM peptide levels was associated with a concomitant increase in the AM mRNA levels in the LV. These findings are consistent with previous observations in DS rats at the CHF stage.27 In the present study, we extended our investigation to include the AM receptor system and 2 molecular forms of AM in the failing LV and found that the AM receptor components, CRLR, RAMP2, and RAMP3, were maintained at higher levels in the progression to HF. Recent studies showed that gene expression of RAMP2, CRLR, and AM in the ventricles of rats with old myocardial infarction were increased compared with those of sham-operated rats.28,29 The present findings are in good agreement with that study. There are a few studies regarding the mechanism of increased CRLR and RAMPs in the failing heart. A recent study reported that endothelin-1 significantly increases CRLR and RAMP3 mRNA expression in cultured cardiac myocytes.30 Because it is well known that endothelin-1 is increased in the failing heart,24 increased endothelin-1 may in part have contributed to the increase in CRLR and RAMPs in this study. Thus, gene expression of AM receptor components may also be upregulated in the failing heart with concomitant increases of the ligand and its mRNA.

Further Activation of AM System During Transition to CHF
In human CHF, it has been demonstrated that the increase in circulating AM levels is correlated with functional class and alterations in hemodynamics.9 The present study demonstrated that plasma and myocardial AM levels markedly increased during the transition from LVH to CHF. The increase in AM peptide levels was associated with a concomitant increase in the AM mRNA levels in the LV. These findings are consistent with previous observations in DS rats at the CHF stage.27 In the present study, we extended our investigation to include the AM receptor system and 2 molecular forms of AM in the failing LV and found that the AM receptor components, CRLR, RAMP2, and RAMP3, were maintained at higher levels in the progression to HF. Recent studies showed that gene expression of RAMP2, CRLR, and AM in the ventricles of rats with old myocardial infarction were increased compared with those of sham-operated rats.28,29 The present findings are in good agreement with that study. There are a few studies regarding the mechanism of increased CRLR and RAMPs in the failing heart. A recent study reported that endothelin-1 significantly increases CRLR and RAMP3 mRNA expression in cultured cardiac myocytes.30 Because it is well known that endothelin-1 is increased in the failing heart,24 increased endothelin-1 may in part have contributed to the increase in CRLR and RAMPs in this study. Thus, gene expression of AM receptor components may also be upregulated in the failing heart with concomitant increases of the ligand and its mRNA.

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be enhanced in the failing myocardium. A recent study showed that amidating enzyme mRNA expression is detected in the atria and ventricle; however, the precise mode of regulation of the mRNA of this enzyme in vivo is not fully understood. Whether the increase in this enzyme activity is due to mechanical overload and/or neurohumoral factors needs further study. Taken together, these findings suggest that the cardiac AM system, including ligands, AM mRNA, mRNAs of receptor components, and amidating activity, is activated during the transition from LVH to CHF, and it may modulate the pathophysiology during the transition from LVH to CHF.

Regarding other neurohumoral factors, plasma and ventricular ANP was further elevated during the transition from LVH to HF, whereas plasma renin activity and aldosterone were elevated at the CHF stage only. Previously, Lee et al reported that ANP participates in the suppression of activation of the renin-angiotensin-aldosterone system in the acute phase of HF. The present results are consistent with their report. Thus, increased ANP at the LVH stage may in part serve to inhibit suppression of the renin-angiotensin-aldosterone system until later stages of HF when presumably additional stimuli to renin overcome this inhibitory action of ANP. Furthermore, a recent study revealed that endogenous ANP plays an important role as an autocrine and paracrine antiremodeling factor in the heart. Thus, both the AM and ANP systems are activated at the LVH stage, are further increased at the CHF stage, and play an important role not only as circulating hormones but also as cardiac autocrine and paracrine factors.

There are limitations to this study. To date, there seems to be a consensus that CRLR coexpressed with RAMP2 and RAMP3 composes the AM receptor and that CRLR coexpressed with RAMP1 composes the calcitonin gene-related peptide (CGRP) receptor; however, all of the experimental data are not necessarily consistent with this theory. In some culture systems, CRLR coexpressed with RAMP1 responds not only to CGRP but also to AM, elevating intracellular cAMP levels. Thus, we cannot deny the possibility that CRLR coexpressed with RAMP1 also may be partly involved in the activated AM system in the failing heart. Further studies are necessary to elucidate the role of this unique and complicated receptor system.

**Perspectives**

Previous studies revealed that LV tissue AM levels are increased in LVH and the failing heart. However, no precise serial evaluation of the AM receptor system or the different molecular forms of AM in the normal state, LVH, and the failing heart had been performed. In the present study, we demonstrated that the plasma and cardiac AM levels, gene expression of AM, and expression of the AM receptor system were all increased in advanced LVH, and that they were further increased during the transition to HF, with an increase in the active-inactive ratio in tissue. These findings, together with the direct inhibitory effect of AM on the proliferation and DNA synthesis of myocytes and nonmyocytes, imply that increases in the intracardiac AM system in the hypertrophied and failing myocardium may be an endogenous mechanism that protects the heart from remodeling. We propose that these increases in the intracardiac AM system in LVH and the failing heart may be a compensatory mechanism against activation of the intracardiac renin-angiotensin system, like ANP. Further studies are necessary to elucidate the exact role of increased AM in LVH and the failing heart.

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