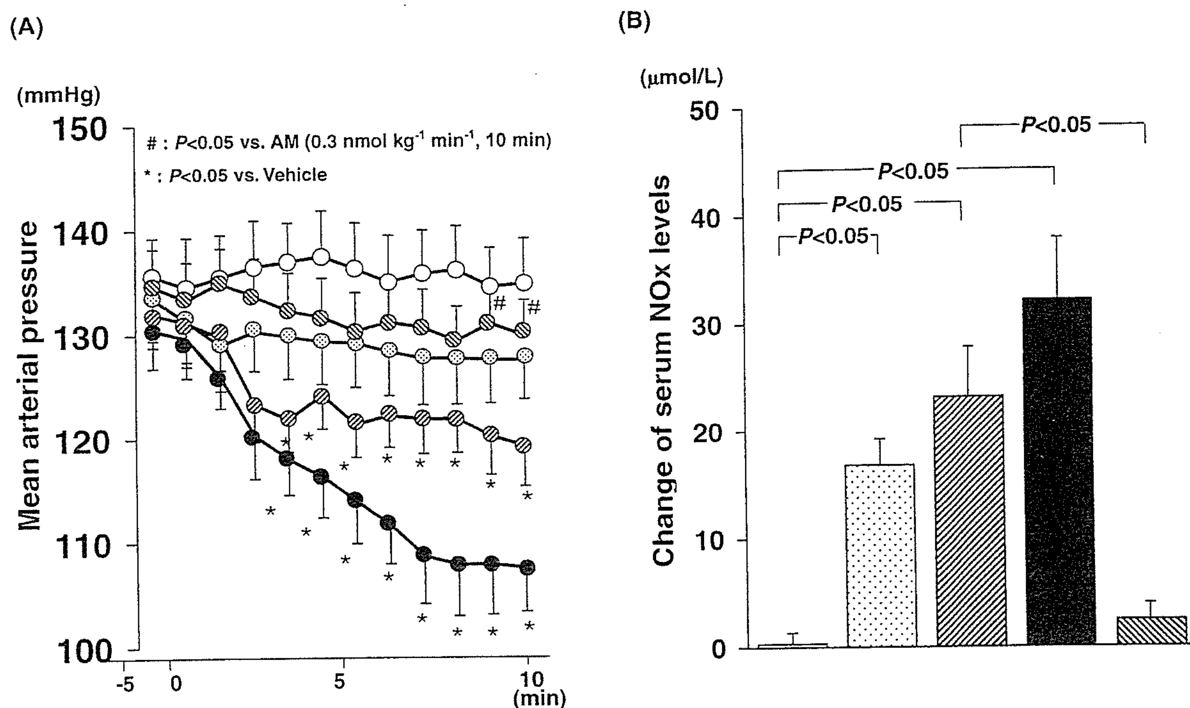

Fig. 5. Effects of the infusion of exogenous adrenomedullin (AM) on mean arterial pressure (A) and the change in serum NOx levels (B). Control rats were intravenously administered either vehicle (○, $n = 6$) or human AM (0.1 (◎), 0.3 (◍), 1.0 (●) nmol kg^{-1} min^{-1} for 10 min, $n = 6$, respectively). Another 6 control rats (◍) were intravenously injected the neutralizing antibody (500 μg/kg) and were thereafter infused with human AM (0.3 nmol kg^{-1} min^{-1} for 10 min). AM infusion reduced systemic arterial pressure and increased serum NOx levels in a dose-dependent manner. The changes following exogenous AM infusion were abolished by the pretreatment of neutralizing antibody. Values are presented as mean ± $s_{\bar{x}}$ (standard error of the mean).

fmol/mL, $P < 0.05$) (Fig. 1A). Plasma AM levels in cirrhotic rats with ascites (19.2 ± 5.4 fmol/mL) were especially conspicuous and showed an approximately 3-fold increase compared with those in controls. Plasma AM levels were negatively correlated with mean arterial pressure in cirrhotic rats ($r = -0.62$, $P < 0.05$) (Fig. 1B).

To investigate the origin of increased plasma AM levels in cirrhotic rats, tissue concentrations of AM in various organs were determined (Fig. 2). Tissue concentrations of AM widely varied from organ to organ and were highest in the lung. In every organ, cirrhotic rats with ascites showed higher AM concentrations compared with controls (liver: 41.5 ± 2.6 versus 16.3 ± 1.5, $P < 0.01$; kidney: 40.8 ± 4.6 versus 28.3 ± 1.9, $P < 0.05$; lung: 1042.3 ± 267.2 versus 381.4 ± 106.1 fmol/mg protein, $P < 0.05$). Hepatic and renal AM levels were higher in cirrhotic rats without ascites than in controls (liver: 30.6 ± 2.0, $P < 0.01$; kidney: 40.2 ± 3.1 fmol/mg protein, $P < 0.01$).

Gene expression of AM in various organs and vessels

Reverse-transcription polymerase chain reaction analysis showed a clear single band with the predicted size of 568 base pairs. In agreement with tissue concentrations, gene expression of AM was strongest in the lung, followed by the kidney and liver. Gene expression of AM in the liver, kidney and lung was enhanced in cirrhotic rats with ascites compared with controls (Fig. 3A). Because AM is known to be vigorously synthesized in vascular tissue (17, 18), gene expression of AM in systemic and splanchnic vessels was also evaluated. Gene expression of AM in the aorta and the portal vein was evidently enhanced in cirrhotic rats compared with in controls (Fig. 3B).

Effects of AM and neutralizing antibody against AM in phenylephrine-induced contraction of aortic rings

To elucidate the role of AM in the vascular hyporeactivity in cirrhotic rats, phenylephrine-induced contraction in the aortic rings of control rats and cirrhotic animals was evaluated in the presence of vehicle, human AM or neutralizing antibody against AM (Fig. 4). As shown in vehicle-treated aortas, the contractility to phenylephrine was blunted in cirrhotic aortas compared with controls (Rmax: 1.0 ± 0.1 versus 1.9 ± 0.2 g/mg tissue, $P < 0.05$), whereas the reactivity was similar between both aortas (EC_{50}: −7.1 ± 0.1 versus −7.2 ± 0.1). AM treatment caused a significant reduction in the contractility compared with vehicle treatment in both aortas, although the vascular effect induced by AM was less in cirrhotic aortas than in control aortas (Rmax: control, 1.3 ± 0.1 g/mg tissue, $P < 0.05$; cirrhosis, 0.7 ± 0.1 g/mg tissue, $P < 0.05$). In contrast to the contractility, AM did not affect the reactivity in either of the aortas (EC_{50}: control, −7.1 ± 0.1; cirrhosis, −7.0 ± 0.2). Neutralizing antibody

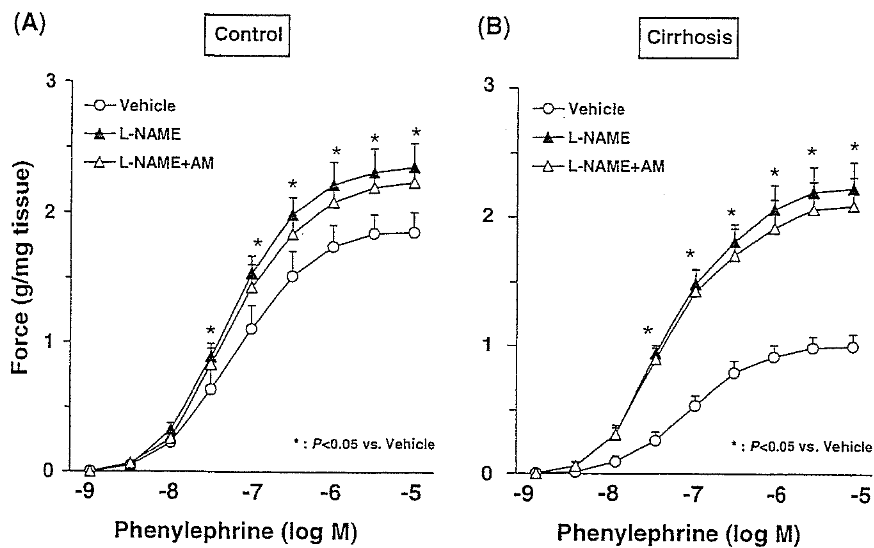

Fig. 6. Effects of nitric oxide synthase inhibitor in vascular hyporeactivity induced by adrenomedullin (AM) in the aortic rings of control (A) and cirrhotic rats (B). The effect of AM in phenylephrine-induced contraction was evaluated in the presence of nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). L-NAME potentiated the phenylephrine-induced contraction in both control and cirrhotic aortas, although the potentiation of the contractile response by L-NAME was remarkable in cirrhotic aortas. In the presence of L-NAME, AM could not blunt the contractile response in both aortas. Values are presented as mean $\pm s_{\bar{x}}$ (standard error of the mean) of 6 aortic rings.

against AM did not affect the contractile response of control aortas (Rmax: 1.9 ± 0.2 g/mg tissue, EC_{50}: -7.2 ± 0.1), whereas this antibody ameliorated the blunted contractility in cirrhotic aortas (Rmax: 1.5 ± 0.1 g/mg tissue, $P < 0.05$), although the contractile response did not reach to the value of vehicle-treated control aortas.

Effect of AM infusion in systemic arterial pressure and serum NOx levels

To investigate the role of NO in the vasodilator effect of AM, exogenous AM was infused in control rats with or without neutralizing antibody against AM and the effects on systemic arterial pressure and serum NOx ($NO_2^- + NO_3^-$) levels were evaluated. AM infusion reduced systemic arterial pressure (Fig. 5A) and increased serum NOx levels (Fig. 5B) in a dose-dependent manner (vehicle: 135 ± 4 mmHg and 0.3 ± 1.0 μmol/L; AM 0.1 nmol kg^{-1} min^{-1}: 128 ± 4 mmHg, $P < 0.01$ and 16.8 ± 2.4 μmol/L, $P < 0.01$ versus vehicle; AM 0.3 nmol kg^{-1} min^{-1}: 119 ± 4 mmHg, $P < 0.05$ and 23.2 ± 4.7 μmol/L, $P < 0.01$ versus vehicle; AM 1.0 nmol kg^{-1} min^{-1}: 106 ± 3 mmHg, $P < 0.01$ and 32.2 ± 5.8 μmol/L, $P < 0.01$ versus vehicle). These changes following AM infusion (0.3 nmol kg^{-1} min^{-1} for 10 min) were abolished by the pretreatment of neutralizing antibody (130 ± 3 mmHg and 2.4 ± 1.5 μmol/L).

Effects of NO synthase inhibitor in vascular hyporeactivity induced by AM

To investigate the role of NO in the vascular hyporeactivity induced by AM, the effect of AM in phenylephrine-induced contraction was evaluated in the presence of NO synthase inhibitor, L-NAME (Fig. 6). L-NAME potentiated the phenylephrine-induced contraction in both control and cirrhotic aortas (control: Rmax; 2.4 ± 0.2 g/mg tissue, $P < 0.05$, EC_{50}; -7.3 ± 0.2, $P = 0.05$, versus vehicle-treated control aortas, cirrhosis: Rmax; 2.2 ± 0.2 g/mg tissue, $P < 0.05$, EC_{50}; -7.3 ± 0.1, $P < 0.05$, versus vehicle-treated cirrhotic aortas), although the potentiation of the contractile response by L-NAME was remarkable in cirrhotic aortas. In the presence of L-NAME, AM could not blunt the contractile response in both aortas (control: Rmax; 2.4 ± 0.2 g/mg tissue, EC_{50}; -7.3 ± 0.1, cirrhosis: Rmax; 2.1 ± 0.2 g/mg tissue, EC_{50}; -7.3 ± 0.1).

Discussion

In several clinical studies it is suggested that AM, a potent vasodilator peptide, may be implicated in hemodynamic derangement in cirrhotic patients (22–25). This suggestion is based on the observations that plasma AM levels are increased along with progression of liver disease and correlate with hemodynamic parameters and the activation of vasoconstrictor systems in cirrhosis. We and other groups of investigators have shown that plasma AM levels correlate positively with the Child-Pugh score and negatively with glomerular filtration rate in cirrhotic patients (22–25). Therefore, the increased circulating AM levels in cirrhotic patients could be attributed to a reduced degradation of AM in liver and/or kidney. However, no significant differences in AM levels were found among plasma samples obtained from

hepatic vein, renal vein, pulmonary artery and femoral artery in cirrhotic patients (23). The lack of significant arteriovenous difference in AM levels in various vascular territories suggests that the increased circulating AM in cirrhotics is not the result of a reduced clearance in a specific organ. In this study, cirrhotic rats showed a significant increase in plasma AM levels compared with controls (Fig. 1A) and AM expression in various organs (liver, kidney, lung) and vessels (portal vein, aorta) was enhanced in cirrhotic rats compared with that in control animals (Figs. 2 and 3). These findings indicate that the increased plasma AM levels in cirrhotic rats are derived from systemic overproduction. AM is processed from its precursor as glycine-extended AM, an inactive intermediate form of AM. Subsequently, mature AM is converted from glycine-extended AM by enzymatic amidation (33). Kitamura et al. showed that most of the circulating AM is occupied by glycine-extended AM, an intermediate form of AM, and reflects the process of AM production in tissue (33). Because plasma AM levels correlated negatively with systemic arterial pressure in cirrhotic rats (Fig. 1B), the systemic production of AM reflected by plasma levels may be associated with arterial vasodilatation in cirrhosis.

The specific receptor antagonist which can abolish the vascular effect of AM is not available at present, because AM exerts a vasodilator effect via the CGRP receptor as well as the AM receptor (14–16). In a recent study it was demonstrated that the calcitonin receptor-like receptor (CRLR) could function as either a CGRP receptor or AM receptor, depending on the co-expression of receptor activity-modifying proteins (RAMPs). The co-expression of CRLR and RAMP1 results in a CGRP receptor, whereas the association of RAMP2 or RAMP3 with CRLR gives an AM receptor (34). We, therefore, used the neutralizing antibody against AM to abolish the vascular effect of AM. This neutralizing antibody possesses an extremely effective neutralizing potency against AM in vitro (26) which may allow us to elucidate a role of AM in vascular hyporeactivity in cirrhosis. In this study, exogenous AM reduced the contractile response to phenylephrine in both control and cirrhotic aortas (Fig. 4). Interestingly, the magnitude of vascular hyporeactivity induced by AM was lower in cirrhotic aortas than in controls. Considering that AM expression was enhanced in cirrhotic aortas and that vascular endothelial and smooth muscle cells vigorously produce AM (17, 18), there is a possibility that the endogenous AM overproduced in cirrhotic aortas may occupy more receptors for AM in the aorta, resulting in a decrease in unbound receptors and vascular hyporeactivity to exogenous AM. Moreover, a role of endogenous AM in vascular hyporeactivity in cirrhosis was evaluated using the neutralizing antibody against AM. This antibody ameliorated the blunted phenylephrine-induced contraction in cirrhotic aortas, whereas it did not affect the vascular response in controls (Fig. 4). Together with the up-regulation of AM in aortas of cirrhotic rats (Fig. 3B), these findings indicate that AM may regulate the vascular tonus of cirrhotic rats in a paracrine and/or autocrine manner. It is of interest that the contractile response in cirrhotic aortas did not reach to the value of vehicle-treated control aortas despite the use of the neutralizing antibody against AM, indicating that factors other than AM may be involved in the vascular hyporeactivity in liver cirrhosis, as well.

We focused on the role of NO in the vascular effect of AM, because AM causes a potent vasodilatation via NO release from vascular endothelial cells together with an increased intracellular cAMP in vascular smooth muscle cells (14–16) and NO plays a major role in vascular hyporeactivity in cirrhosis (9, 10). In this study, the infusion of exogenous AM caused a reduction of systemic arterial pressure along with an increase in serum NOx levels in a dose-dependent manner, which was abolished by the pretreatment of neutralizing antibody against AM (Fig. 5). In the presence of NO synthase inhibitor, AM could not affect the vascular response to vasoconstrictors in both control and cirrhotic aortas (Fig. 6). It is reported that the denudation of rat aortic endothelium and the inhibition of guanylate cyclase substantially inhibit AM-induced vasodilatation (16) and that AM increases NO release from rat perfused kidneys and NO synthase inhibitor decreases both NO release and AM-induced vasodilatation (15). These findings, together with the existence of the receptors for AM in vascular endothelial cells (14), indicate that AM may regulate the vascular tonus via NO release from the endothelium. Recent study has demonstrated that NO stimulates AM secretion and gene expression in endothelial cells (35), indicating that NO and AM may cooperatively regulate the vascular tonus.

In conclusion, plasma AM levels were significantly higher in cirrhotic rats than in controls and were derived from a generalized overproduction in various organs and vessels. An overproduced AM may contribute to the vascular hyporeactivity in cirrhosis via NO release as a paracrine and/or autocrine regulator of vascular tonus.

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Adrenomedullin Administration Immediately After Myocardial Infarction Ameliorates Progression of Heart Failure in Rats

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Background—Adrenomedullin (AM) is expressed in cardiac tissue, and plasma AM levels increase in patients with acute myocardial infarction (MI). This study was performed to determine whether AM administration immediately after acute MI inhibits progression of heart failure in rats.

Methods and Results—Rats were infused with 1.0 μg/h IP AM or saline over 7 days immediately after MI inducted by left coronary ligation and were examined 9 weeks after MI. Compared with the saline infusion, AM infusion significantly improved survival (59% versus 81%; $P<0.05$) and body weight gain (32%; $P<0.01$) and reduced heart weight (−28%; $P<0.01$), lung weight (−26%; $P<0.01$), left ventricular (LV) end-diastolic pressure (11.4±2.0 versus 4.0±0.6 mm Hg, mean± SEM; $P<0.01$), collagen volume fraction of noninfarcted LV (−39%; $P<0.05$), and plasma levels of endogenous rat AM (−38%; $P<0.05$) without affecting infarct size. To investigate the mechanism of AM actions, another series of MI rats infused with AM were killed on day 7. AM infusion had no effect on organ weights and hemodynamic parameters on day 7 of MI but significantly reduced urinary excretion of isoprostane (−61%; $P<0.01$) and noninfarcted LV mRNA levels of ACE (−31%; $P<0.05$) and p22-phox (−30%; $P<0.05$).

Conclusions—AM administration during the early period of MI improved the survival and ameliorated progression of LV remodeling and heart failure. This beneficial effect was accompanied by reductions in oxidative stress and ACE mRNA expression in noninfarcted LV in the AM infusion period. (*Circulation*. **2004;110:426-431.**)

Key Words: adrenomedullin ■ heart failure ■ myocardial infarction ■ remodeling

Myocardial infarction (MI) frequently produces left ventricular (LV) dilatation with hypertrophy and collagen deposition in the noninfarct myocardium, changes referred to as LV remodeling, which leads to depressed cardiac performance.[1] Previous studies have demonstrated that LV remodeling was an important factor in determining not only development of heart failure but also long-term survival after acute MI.[2] It is therefore essential to inhibit LV remodeling for better clinical outcomes of patients with MI. The underlying mechanisms responsible for LV remodeling have been shown to be hemodynamic stress to the heart and activation of neurohumoral factors, including the renin-angiotensin-aldosterone (RAA) system.[3,4] Meanwhile, according to recent studies, oxidative stress appears to be another important factor participating in the progression of heart failure after MI.[5] Adrenomedullin (AM), originally isolated from human pheochromocytoma,[6] is known as a peptide having a wide spectrum of biological actions such as vasodilatation, natriuresis, and diuresis.[7] Plasma AM levels were found to increase immediately after the onset of acute MI and to return to the basal level within 1 week.[8] Previous studies have suggested that AM counteracts the systemic or local RAA system in vitro and in vivo.[9,10] Recently, Shimosawa et al,[11] using mice lacking 1 copy of the AM gene, reported an antioxidative action of AM. Thus, it is possible that AM plays an important role as a cardioprotective factor in acute MI by counteracting excessive vasoconstrictors or oxidative stress. We have already shown that continuous administration of AM has beneficial effects on LV remodeling and hemodynamics in MI rats[12]; however, it remained to be explored whether AM administered in the early period of MI improves long-term outcome. If AM has a cardioprotective effect in acute MI, an AM supplement in the early period would be beneficial in inhibiting the progression of LV remodeling and

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heart failure. The aim of this study was to examine the effect of AM administered during the early period of MI on the progression of heart failure in rats.

Methods

Animals and Peptide

Male Wistar rats (Charles River Inc) weighing 220 to 280 g were used in this study. All rats were housed in a temperature- and humidity-controlled room with free access to standard rat chow and tap water. The recombinant human AM used in this study was provided by Shionogi & Co, Ltd.[12]

Experimental Protocols

MI was induced in rats by ligation of the left coronary artery as described previously,[12] and sham-operated animals underwent an identical surgical procedure without the actual coronary artery ligation. Experiments were performed under the regulations of the Animal Research Committee of Miyazaki Medical College (2002–013).

Long-Term Study

To examine the effects of AM on the progression of heart failure and LV remodeling after MI, rats receiving the coronary ligation were randomly divided into 3 groups: 2 groups infused with AM at a low (0.3 μg/h; n=12) or a high (1.0 μg/h; n=11) dose and 1 group infused with saline that served as controls (n=16). The rats were intraperitoneally implanted with osmotic minipumps (model 2001, DURECT Co) filled with recombinant human AM dissolved in 0.9% saline to release 0.3 or 1.0 μg/h peptide. Similarly, both the control group and the sham-operated rats (n=7) were infused with saline by an identical method. The infusion was discontinued on day 7 after the surgery by removing the pump from the rats. After an observational period of 8 weeks, rats were examined for the hemodynamic and hormonal parameters, organ weights, and histological evaluation of the heart.

Short-Term Study

To investigate the mechanisms of AM actions on LV remodeling and heart failure, we performed a separate series of experiments in which sham-operated (n=18) or MI rats infused with 1.0 μg/h AM (n=31) or saline (n=45) were examined during or at the end of the 7-day AM infusion period. To evaluate the systemic and local RAA system, 3 groups of sham-operated or MI rats infused with AM or saline were killed by decapitation on day 7 of MI. For measurements of plasma renin activity and aldosterone concentration, blood samples were collected into chilled tubes with 1.5 mg/mL of EDTA-2Na and centrifuged at 2000*g* for 15 minutes at 4°C. Plasma renin activity and aldosterone concentration were measured with radioimmunoassay kits as described previously.[13] After collection of blood samples, hearts were resected to measure the mRNA levels of ACE, angiotensin II type 1 (AT1) receptor, and p22-phox, a critical component of NADH/NADPH oxidase.[14]

The other 3 groups of sham-operated and MI rats infused with AM or saline were placed in individual metabolic cages to collect urine samples every 24 hours for measurement of urinary 8-iso-prostaglandin F2α (isoprostane), a marker of oxidative stress,[15] during the AM infusion period of 7 days. Urinary isoprostane excretion was measured by an enzyme immunoassay according to the manufacturer's instructions (Assay Design, Inc). On day 7 of the AM infusion, rats were subjected to hemodynamic, hormonal, and histological studies.

Hemodynamic Studies and Peptide Measurements

The animals were anesthetized by injection of 50 mg/kg IP pentobarbital sodium on day 7 or 9 weeks after the MI induction. Hemodynamic parameters were measured with a micromanometer-tipped catheter (SPC-320, Millar Instruments, Inc) as described previously.[12] After the hemodynamic measurements, blood samples were collected and plasma levels of endogenous rat AM and ANP were measured with an immunoradiometric assay[12] and a radioimmunoassay,[13] respectively.

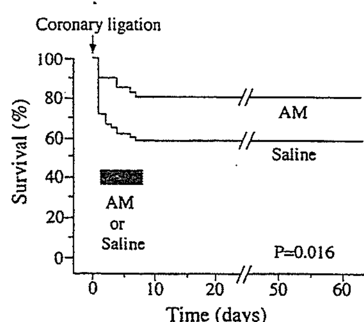

Figure 1. Kaplan-Meier survival curve of MI rats infused with saline (n=61) or 1.0 μg/h of human AM (n=42).

Determination of Infarct Size and Collagen Volume Fraction

After collection of the blood samples, 30 mmol/L potassium chloride was injected from the catheter to arrest the hearts in diastole. Then, the hearts were weighed, fixed in 10% formalin, and embedded in paraffin. Infarct size was measured by hematoxylin and eosin staining, and the collagen volume fraction of the noninfarcted LV area was determined by staining with Sirius red, a collagen-specific dye, as described previously.[12]

Measurement of ACE, AT1 Receptor, and p22-phox mRNA in Noninfarcted LV

Total RNA was extracted from the noninfarcted LV with TRIzol (Invitrogen, Inc) according to the manufacturer's protocol and then subjected to reverse transcription by means of SuperScript reverse transcriptase (Gibco-BRL, Life Technologies, Inc) into cDNA. To measure rat ACE, AT1 receptor, p22-phox, and GAPDH mRNA levels, we used the quantitative reverse transcription–polymerase chain reaction method, real time–quantitative polymerase chain reaction (Prism 7700 Sequence Detector; Applied Biosystemics).[16] Oligonucleotides used as probes and primers for the ACE, AT1 receptor, and GAPDH measurements were previously described by us[16] and Naito et al,[17] and those used for p22-phox were as follows: probe, 5′-TGTCCTCCACTTACTGCTGTCCGTGCCTGC-3′; forward primer, 5′-TGTCCTCCACTTACTGCTGTCCGTGCCTGC-3′; and reverse primer, 5′-GCTCATCTGTCTGCTGGAGTA-3′. The mRNA levels were compared after they had been normalized relative to those of GAPDH.

Statistical Analysis

All data are expressed as mean±SEM. Multiple comparisons were evaluated by 1-way ANOVA, followed by the Scheffé F test, and differences were considered significant at $P<0.05$.

Results

Survival Rate

To assess the AM effect on mortality, we compared the survival rates between the 2 MI groups infused with saline or 1.0 μg/h AM (Figure 1). Eight rats in the AM-infused and 25 in the saline-infused MI group died during the long- and short-term experimental periods, whereas no rats in the sham group died. As shown in Figure 1, the survival rate of the AM infusion group (81%) was significantly ($P<0.05$) higher than that of the control group (59%) by Kaplan-Meier survival analysis, whereas the effect of 0.3 μg /h AM did not reach statistical significance (data not shown).

TABLE 1. Infarct Size, Body and Organ Weights, and Collagen Volume Fraction of the Noninfarcted LV Area at 9 Weeks

		MI		
	Sham	Saline	0.3 μg/h AM	1.0 μg/h AM
Rats, n	7	9	9	9
Infarct size, %	0	43.3±2.5	41.1±1.8	43.8±1.8
Body weight, g	460±6	411±10†	441±11	458±7§
Body weight gain, g/d	3.7±0.1	2.8±0.2†	3.3±0.2	3.7±0.1§
Heart weight/body weight, mg/g	3.0±0.1	4.7±0.2†	4.5±0.4†	3.4±0.1§
Lung weight/body weight, mg/g	3.2±0.1	6.5±0.6†	4.8±0.5*	3.7±0.1§
Collagen volume fraction, %	3.4±0.2	8.3±0.7†	7.0±1.1*	5.1±0.6‡

Values are mean±SEM.
*$P<0.05$, †$P<0.01$ vs sham rats; ‡$P<0.05$, §$P<0.01$ vs MI rats infused with saline.

Infarct Size, Body Weight, Organ Weights, and Collagen Volume Fraction

As shown in Table 1, no significant differences were noted in the infarct sizes among the 3 MI groups. Both the body weight and body weight gain of the saline-infused MI rats were lower ($P<0.01$) than those of the sham group, although the impaired weight gain was significantly ($P<0.01$) improved by the 1.0 μg/h AM infusion. The heart, lung, and body weights in the MI groups infused with saline were heavier ($P<0.01$) than in the sham group but were significantly ($P<0.01$) lightened by 1.0 μg/h AM. An increase ($P<0.01$) in the collagen volume fraction in the noninfarcted LV area was observed in the saline-infused MI group compared with the sham, and the increased collagen volume was significantly ($P<0.05$) reduced by the 1.0 μg/h AM infusion.

Hemodynamic and Hormonal Parameters

No significant differences were noted in heart rate and mean arterial and central venous pressures among the 4 groups at 9 weeks (Table 2). The LV end-diastolic pressure in the saline-infused MI group was raised ($P<0.01$) compared with the sham group but was significantly ($P<0.01$) lowered by the 1.0 μg/h AM infusion. The plasma level of rat endogenous AM was increased ($P<0.05$) in the saline-infused MI group compared with the sham group, and the increased level was significantly ($P<0.05$) reduced by the AM infusion. A similar tendency was observed in plasma ANP, although the differences were not statistically significant.

Results of the Short-Term Experiment

To investigate the mechanism(s) responsible for the beneficial effect of AM on the late outcome described above, we examined the sham-operated or MI rats infused with saline or 1.0 μg/h AM during or at the end of the 7-day infusion period. There were no differences in infarct size, heart rate, and mean and central venous pressures among the groups (Table 3). The LV end-diastolic pressure was elevated in 2 MI groups compared with the sham group, but at this time point, the AM infusion had no significant effect on LV end-diastolic pressure. In addition, no differences were observed in body, heart, and lung weights on day 7 (data not shown).

As shown in Table 4, no differences were noted in the plasma renin activity and aldosterone concentration of the sham-operated and MI groups. Meanwhile, the urinary excretion of isoprostane was increased ($P<0.01$) in the saline-infused MI rats compared with the shams. Interestingly, the AM infusion significantly ($P<0.01$) reduced the urinary isoprostane excretion to the control level during the 7-day period. To detect any change in the local RAA system and oxidative stress, we measured AT1 receptor, ACE, and p22-phox mRNA levels in the noninfarcted LV (Figure 2). No significant differences were noted in the AT1 receptor expressions in the noninfarcted LV (Figure 2A), but as shown in Figure 2B, ACE expression in the saline-infused MI rats was increased by 121% ($P<0.01$) compared with the sham rats. This augmentation was significantly ($P<0.05$) reduced in the AM-infused MI group by 31%. Similarly, p22-phox

TABLE 2. Hemodynamic and Hormonal Parameters at 9 Weeks

		MI		
	Sham	Saline	0.3 μg/h AM	1.0 μg/h AM
Heart rate, bpm	415±10	394±17	405±18	422±6
Mean arterial pressure, mm Hg	85±6	91±4	82±8	90±6
Central venous pressure, mm Hg	0.2±0.1	2.9±1.8	0.5±0.3	0.3±0.3
LV end-diastolic pressure, mm Hg	2.3±0.3	11.4±2.0†	6.5±1.8	4.0±0.6§
Rat endogenous AM, fmol/mL	3.5±0.3	5.3±0.8*	3.7±0.6	3.3±0.3‡
Rat ANP, fmol/mL	11±3	61±29	53±16	19±3

Values are mean±SEM. The number of rats examined is given in Table 1.
*$P<0.05$, †$P<0.01$ vs sham rats; ‡$P<0.05$, §$P<0.01$ vs MI rats infused with saline.

TABLE 3. Infarct Size and Hemodynamics at Day 7

		MI	
	Sham	Saline	1.0 μg/h AM
Rats, n	8	9	9
Infarct size, %	0	43.3±2.5	41.1±1.8
Heart rate, bpm	449±11	438±16	455±9
Mean arterial pressure, mm Hg	96±2	93±6	104±6
Central venous pressure, mm Hg	2.0±0.7	2.2±0.5	3.0±1.0
LV end-diastolic pressure, mm Hg	2.4±0.5	11.6±2.0†	8.9±1.6*

Values are mean±SEM.
*$P<0.05$, †$P<0.01$ vs sham group.

expression in the MI group infused with saline was increased by 76% ($P<0.01$), and the increased expression was significantly ($P<0.05$) decreased in the AM infusion group by 30% (Figure 2C).

Discussion

In the present study, we showed that intraperitoneal AM infusion over 7 days immediately after MI induction reduced the LV end-diastolic pressure, collagen volume fraction of the noninfarcted LV, and heart and lung weights, which were determined at 9 weeks of MI, in rats. A dose setting of 1.0 μg/h of human recombinant AM was chosen through reference to our previous study[10,12]; in this study, we also used a lower dose of 0.3 μg/h AM and found milder effects that did not reach statistically significant levels. We previously reported that plasma AM levels in patients with heart failure progressively increased in relation to disease severity and that the elevated levels were gradually reduced by successful treatment.[18] Accordant with this, the increased endogenous rat AM levels in the MI rats were significantly reduced by human AM infusion in the present study. Thus, AM administration during the early period of MI ameliorated chronic progression of LV remodeling and heart failure in rats.

Although AM has been shown to possess a wide spectrum of biological actions,[7] we may first need to discuss whether the vasodilator and natriuretic actions of AM contributed to the beneficial effects observed in the present study. Long-term infusion of AM has been found to lower blood pressure in a rat model of renovascular hypertension.[10] However, in the present study, the mean arterial pressure in the AM-infused MI group remained unchanged at day 7 of the infusion, a finding consistent with our previous observation.[12] To examine the natriuretic and diuretic effects in the AM

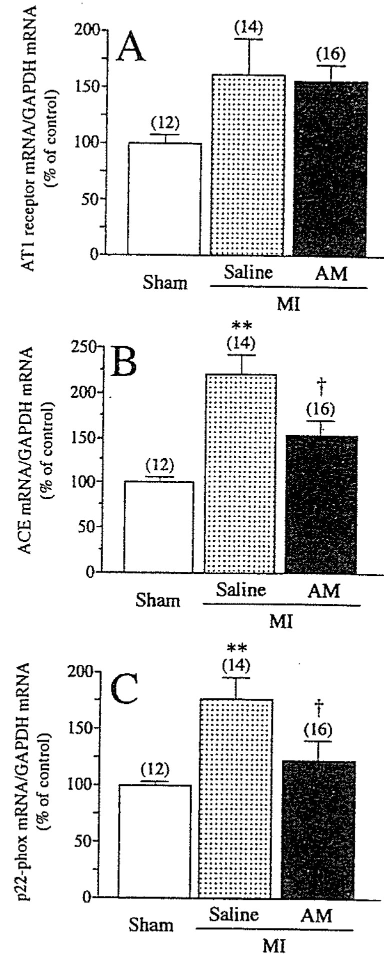

Figure 2. AT1 receptor (A), ACE (B), and p22-phox (C) mRNA levels of noninfarcted LV at day 7. **$P<0.01$ vs sham group; †$P<0.05$ vs saline-infused MI group; mean±SEM (n).

infusion period, we measured the LV end-diastolic pressures on day 7 of MI. As shown, the elevated LV end-diastolic pressure was lowered by AM infusion, but at this time point, the difference was not statistically significant. To further examine the natriuretic and diuretic actions, we measured urine volume and urinary sodium excretion during the AM infusion period using individual metabolic cages but again failed to detect a significant increase in urinary output and sodium excretion (data not shown). Thus, either an afterload or preload reduction during the AM infusion period may be unlikely as the major mechanism alleviating chronic progression of LV remodeling and heart failure, although we should

TABLE 4. Plasma Renin Activity and Aldosterone Concentration at Day 7 and Urinary 8-Iso-Prostaglandin F2α Excretion During 7-Day AM Infusion Period

		MI	
	Sham	Saline	1.0 μg/h AM
Plasma renin activity, ng · mL^{-1} · h^{-1} (n)	6.7±0.7 (12)	7.0±1.0 (12)	9.9±2 (12)
Plasma aldosterone concentration, pg/mL (n)	122±19 (12)	126±51 (12)	85±23 (12)
8-Iso-prostaglandin F2α, ng/d (n)	0.95±0.15 (8)	2.39±0.40* (9)	0.92±0.17† (9)

Values are mean±SEM.
*$P<0.01$ vs sham group; †$P<0.01$ vs MI rats infused with saline.

not totally exclude the possibility that the slightly lower LV end-diastolic pressure contributed to the beneficial effects.

A number of neurohumoral factors acting systematically or locally are thought to be involved in the progression of LV remodeling or heart failure after MI.[19] Among them, the RAA system has an important role,[3,4] and blockade of the RAA system with ACE inhibitors or AT1 receptor blockers improves the cardiac function or prognosis of patients with MI.[20,21] We reported that AM continuously infused for 2 weeks reduced plasma renin activity and aldosterone concentration in rats with renovascular hypertension.[10] In the present study, plasma renin activity was not reduced in the MI group at day 7 of the AM infusion, with a slightly lower concentration of plasma aldosterone. We are unable to attribute the beneficial actions of AM largely to the inhibition of the systemic RAA system, but considering the role of aldosterone in LV remodeling,[22] even a slight reduction should be raised as a possibility. In an effort to see the local RAA system, we measured ACE and AT1 mRNA levels in the noninfarcted LV on day 7 of the infusion. The ACE mRNA level increased in the noninfarcted LV of the saline-infused MI group, and the elevated level was significantly reduced at this time point, without a significant change in AT1 mRNA. Although the role of the local RAA system in LV remodeling or heart failure remains unclear, these findings suggest the possible modulation of the cardiac RAA system by AM.

An important finding is the reduced oxidative stress. Urinary excretion of 8-isoprostane, a marker of systemic oxidative stress,[15] was decreased in the MI rats during the AM infusion period. This finding is comparable to that of Shimosawa et al,[11] who recently showed an antioxidative effect of AM using mice lacking the AM gene. In addition, we found that the AM infusion reduced expression of p22-phox, an essential component of NADH/NADPH oxidase,[14] in noninfarcted LV. Because the NADH/NADPH oxidase system is known to be a major source of superoxide anion production in cardiac myocytes,[23] the AM infusion might have reduced not only systemic but also local oxidative stress in the MI rats. Both animal and human studies suggest that an increase in free radical formation or oxidative stress is associated with the progression of heart failure.[5,24] In fact, antioxidant therapies have been found to have beneficial effects on heart failure and LV remodeling after MI.[25,26] Although the precise mechanism in the AM-induced reduction of urinary 8-isoprostane and p22-phox expression remains to be explored, reduced oxidative stress may have contributed in part to the beneficial effects of AM observed in this study.

Recent studies suggest that AM exerts antiapoptotic effects in a rat model of myocardial ischemia-reperfusion injury probably through an Akt-dependent mechanism.[27,28] We currently have no data on gene expression related to apoptosis, although it is possible that an antiapoptotic effect of AM participates in inhibiting LV remodeling. On the other hand, AM was reported to increase endothelial nitric oxide synthase expression or increase nitric oxide production in vascular walls via a phosphatidylinositol 3-kinase/Akt-dependent pathway.[29] We measured endothelial nitric oxide synthase expression mRNA levels in noninfarcted LV, but no differences were noted in the saline- and AM-infused groups compared with controls (data not shown).

Finally, it should be noted that the mortality rate in MI rats was reduced by the AM infusion. Of interest, this effect was observed during the AM infusion period in the present study; we could not specify the cause of death despite postmortem examination. It has been reported that overexpression of AM by adenovirus-mediated gene delivery reduced ventricular arrhythmia after reperfusion injury in rats.[27] Reduced fatal arrhythmia can be raised as a possibility for improved survival by AM infusion, but this hypothesis should be carefully tested by future experiments.

In summary, AM administration during the early period of MI reduced the mortality rate and alleviated the progression of LV remodeling and heart failure in rats. These beneficial effects were accompanied by reductions in oxidative stress and ACE expression in noninfarcted LV in the AM infusion period. The present findings suggest the possibility of AM as a new therapeutic tool for the treatment of acute MI.

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Alteration of renal adrenomedullin and its receptor system in the severely hypertensive rat: effect of diuretic

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Abstract

Objective: We investigated the pathophysiological role of the renal adrenomedullin (AM) system, including the ligand, receptor, and amidating activity, in severe hypertensive rats.
Method: We studied three groups: control Wistar Kyoto rats (WKY), spontaneously hypertensive stroke-prone rats (SHR-SP), and diuretic-treated SHR-SP. We measured AM-mature, active form, and AM-total (active form+inactive form) in plasma and renal tissues, and mRNA levels of AM and AM receptor system components such as calcitonin receptor-like receptor (CRLR), receptor activity-modifying protein (RAMP) 2, and RAMP3 in renal tissues.
Results: SHR-SP had higher blood pressure, plasma neurohumoral factors, and lower renal function than WKY. SHR-SP had higher AM-mature and AM-total levels in plasma and renal tissues than WKY. Although the plasma AM-mature/AM-total ratio was similar in the two groups, AM-mature/AM-total ratio in renal tissues was higher in SHR-SP than in WKY. In addition, mRNA levels of AM in the renal cortex and medulla and the mRNA levels of CRLR, RAMP2, and RAMP3 in the renal cortex were higher in SHR-SP than in WKY. Chronic diuretic treatment decreased blood pressure and improved kidney function and neurohumoral factors, with reductions in plasma and renal AM system.
Conclusion: Upregulation of circulating and renal AM system may modulate pathophysiology in SHR-SP.
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Keywords: Adrenomedullin; Hypertension; Renal cortex; Renal medulla; Renal impairment

1. Introduction

The 52-amino-acid peptide adrenomedullin (AM), discovered in human pheochromocytoma tissues [1], has potent hypotensive activity in a variety of species [2,3]. In addition to its vascular effects, AM has natriuretic and diuretic actions [2,3]. The AM gene and its peptide are distributed in a broad range of tissues, including the kidneys [1,4,5] The AM gene and specific binding sites for AM peptide are expressed in the kidney [6]. Plasma levels of AM are increased in a variety of disorders, including hypertension [7], renal impairment [7,8], and congestive heart failure [9,10]. Thus, AM may be involved in the pathophysiology of cardiovascular disease. However, the pathophysiological implications of AM in renal impairment associated with malignant hypertension are not fully understood.

Considerable colocalization between the expression of AM peptide and AM mRNA and the expression of AM receptors in the kidney suggests that this peptide may act as an autocrine or paracrine factor (or as both) and influence

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renal function. McLatchie et al. [11] have described novel receptor complexes, consisting of calcitonin receptor-like receptors (CRLRs) and receptor activity-modifying proteins (RAMPs). RAMPs are required to transport CRLRs to the plasma membrane to generate receptors specific for calcitonin gene-related peptide (CGRP) and AM. RAMP1/CRLR complex generates a CGRP-specific receptor, and both RAMP2/CRLR and RAMP3/CRLR complexes generate an AM-specific receptor. We and others have recently reported that gene expression of AM, RAMP2, RAMP3, and CRLR is upregulated in the heart in cardiac hypertrophy and congestive heart failure [12–15]. Furthermore, recent studies have shown that RAMP1, RAMP2, and CRLR gene expression is markedly upregulated in the kidney obstructed by ureteral ligation [16], whereas RAMP2, RAMP3, and CRLR gene expression in the kidney does not differ between a rat model of heart failure and control rats [17]. Thus, expression of AM and its receptor complex components appears to be differentially regulated in a tissue-specific fashion in various pathologic conditions. Consistent with this hypothesis, we previously reported that gene expression of AM and its receptor component in the renal cortex is differently regulated from that in the renal medulla [17]. However, whether the expression of AM and the AM receptor system in the renal cortex and medulla is induced by malignant hypertension remains unknown.

AM is produced from AM precursor by a two-step enzymatic pathway. First, AM precursor, consisting of 185 amino acids, is converted to glycine-extended AM (AM-Gly), a 53-amino-acid peptide that is an inactive intermediate form of AM. Subsequently, AM-Gly is converted to active mature AM (AM-m), a 52-amino-acid peptide with a C-terminal amide structure, by enzymatic amidation [18]. Recent studies have shown that two molecular forms of AM, AM-m and AM-Gly, circulate in human plasma [18,19]. To our knowledge, however, no study has investigated the molecular forms of AM in plasma and renal tissues in normal and malignant hypertensive rats.

Chronic malignant hypertension causes structural and functional modifications in the cardiovascular system that are associated with increased mortality and morbidity. The spontaneously hypertensive stroke-prone rat (SHR-SP) is an experimental model of malignant hypertension in which the animals have severe cerebral and renal dysfunction/damage and die of stroke [20]. To clarify the pathophysiological significance of AM in renal impairment, we measured two molecular forms of AM in plasma and in the renal cortex and medulla of SHR-SP. We also measured the abundance of AM, CRLR, and RAMP mRNAs in the renal cortex and medulla. Moreover, we investigated the effect of antihypertensive treatment with the diuretic, trichlormethiazide, on the two molecular forms of AM, abundance of AM mRNA, and the AM receptor system in these tissues.

2. Methods

2.1. Materials and experimental design

All procedures were in accordance with our institutional guidelines for animal research and with the NIH Guide for the Care and Use of Laboratory Animals. Six-week-old male SHR-SPs (Clea Japan, Tokyo, Japan) (n=15) were studied. Wistar Kyoto rats (WKY) were used as control (n=9). The rats were housed three per cage under controlled conditions of temperature, humidity, and light. They were fed a low-salt diet (0.12% NaCl) for 2 weeks to permit acclimatization. The rats were then given a high-salt diet (8% NaCl). SHR-SPs were randomly divided into the following two groups: (1) the control SHR-SP group (n=8): untreated, given drinking water ad libitum; and (2) the diuretic-treated (n=7) group. The diuretic, trichlormethiazide, was dissolved in drinking water (trichlormethiazide, 20 mg/750 ml). Our preliminary study showed that the rats drank about 60 ml of drinking water per day. Thus, the diuretic-treated group was given 1.6 mg/kg/day trichlormethiazide.

2.2. Urine collection

Twenty-four-hour urine samples were collected from rats in metabolic cages about 8 weeks after the start of treatment for measurement of electrolytes, protein, and creatinine levels [17]. Urine was collected and centrifuged at 3000 rpm to remove particles. The volume of the supernatant was measured, and the samples were analyzed. Urinary electrolytes, urinary protein, creatinine in serum and urine, and serum blood urea nitrogen were analyzed by standard methods. Creatinine clearance was calculated using standard formulae.

2.3. Hemodynamic measurements and blood sampling

At the end of the 8-week treatment, all rats were anesthesized 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), mean arterial pressure (MAP), systolic blood pressure (SBP), and diastolic blood pressure (DBP), as reported previously [14,17]. All procedures were done within 15 min. After these hemodynamic measurements were completed, 5 ml of blood was obtained from the carotid artery and transferred to a chilled glass tube for measurement of serum creatinine and blood urea nitrogen, plasma levels of two molecular forms of AM and aldosterone, and plasma renin concentration (PRC). The heart was then arrested by an injection of 2 mmol of KCl into the carotid artery, and the kidneys were removed, weighed, separated into the cortex and medulla, frozen in liquid nitrogen, and stored at −80 °C until radioimmuno-

assay (RIA) for the two molecular forms of AM or gene expression analysis.

2.4. Assay for plasma and left ventricular tissue levels of rat AM-m, AM-T, ANP, and others

Both AM-m and AM-T (AM-m+AM-Gly) in rat plasma were measured by immunoradiometric assays using specific kits (AM mature RIA SHIONOGI, AM RIA SHIONOGI; Shionogi, Osaka, Japan), with some modifications. Each of these assay kits contains two monoclonal antibodies against human AM: one recognizing the ring structure of human AM in both kits and the other recognizing the carboxy-terminal sequence in the AM-m kit or AM (25–36) in the AM-T kit [21,22]. These assays measure human AM-m or AM-T by sandwiching it between two antibodies without the extraction of plasma. Because the amino acid sequences in the ring structures of rat and human AM differ slightly, we used a specific monoclonal antibody recognizing the ring structure of rat AM instead of the kits' antibody against the ring structure of human AM.

The renal cortex and medulla tissue were weighed and boiled in 10 vol of 1 mol/l acetic acid. Then, the tissues were homogenized with a Polytron mixer. The homogenate was centrifuged at $3000\times g$, and the supernatant was centrifuged again at $15{,}000\times g$. The supernatant was evaporated under a vacuum until dry. The immunoradiometric assays for rat AM-T and AM-m were performed as described above. The PRC was measured as previously reported [23] after adding an excess of angiotensinogen in the form of plasma from rats undergoing bilateral nephrectomy. The plasma aldosterone level was measured by RIA as previously reported [24].

2.5. RNA preparation and Northern blot analysis

Total RNA for the evaluation of AM mRNA expression was extracted from the renal cortex and medulla by the acid guanidinium thiocyanate–phenol–chloroform method as described previously [25]. Northern blot analysis was performed as described in detail in our previous report [23]. In brief, 20 μg of total RNA samples from individual renal tissues was subjected to 1% agarose gel electrophoresis and transferred to nylon membranes, and hybridization was carried out with [^{32}P]dCTP-labeled cDNA probes for AM and GAPDH. The density of each mRNA band was measured with a bioimaging analyzer (BAS-2000; Fuji Photo Film, Tokyo, Japan).

2.6. Reverse transcription polymerase chain reaction (RT-PCR) analysis for rat CRLR, RAMP2, RAMP3, TGF-β, collagen I, and peptidyl-glycine α-amidating monooxygenase (PAM)

After total RNA was extracted and the RNA concentration was determined, first-strand complementary DNA was synthesized as previously reported [26]. PCR and quantification of PCR products were performed as described in detail in our previous report [26]. The sets of primers used except for PAM were as previously described [24,27]. In PAM, the following primers were used: (forward) 5′-GCC CTC GTC CAC TGG AAG TTA C-3′ and (reverse) 5′-CCT GGT GGT ATG ACA GTG TCA ACA. The numbers of PCR cycles for the three genes examined were as follows: CRLR, 27; RAMP2, 29; RAMP3, 32; TGF-β, 29; collagen I, 31; and PAM, 29. For these numbers of PCR cycles, RT-PCRs were all in the linear range. As an internal control, we measured glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in a similar manner, using GAPDH-specific primers [27]. The number of PCR cycles used for GAPDH was 22. Each species of mRNA was quantified by the following formula: amount of original template of each molecule/amount of original template of GAPDH.

2.7. Statistical analysis

All values are expressed as mean±S.D. Statistical comparisons among three groups were performed by analysis of variance (ANOVA) followed by Bonferroni's method. Log transformation was used to normalize the distributions of plasma aldosterone and PRCs. A P value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Physiological profiles of WKY, SHR-SP, and SHR-SP+Diuretic

The body weight, MAP, SBP, DBP, HR, and kidney weight in the three groups are presented in Table 1. Body weight was significantly lower, and MAP, SBP, DBP, and

Table 1
Physiological profiles of the three experimental groups

Variables	WKY	SHR-SP	SHR-SP+ Diuretic
Rats, n	9	8	7
Body weight (g)	361±10	231±12*	243±7*
Mean arterial pressure (mm Hg)	150±5	250±12*	215±5*,#
Systolic blood pressure (mm Hg)	163±6	291±13*	241±9* #
Diastolic blood pressure (mm Hg)	132±5	219±9*	188±3* #
Heart rate (beats/min)	337±31	364±37	386±36
Kidney weight/body weight (g/kg)	3.44±0.16	4.57±0.46*	4.25±0.26* #

WKY, Wistar Kyoto rats; SHR-SP, spontaneously hypertensive stroke-prone rats; SHR-SP+Diuretic, trichlormethiazide-treated SHR-SP. Values are mean±S.D.

* $P<0.01$ vs. WKY.

# $P<0.05$ vs. SHR-SP.

Table 2
Renal characteristics of the four experimental groups

	WKY	SHR-SP	SHR-SP+Diuretic
Blood urea nitrogen (mg/dl)	11.6±1.7	20.7±4.4**	18.7±2.1**
Creatinine (mg/dl)	0.23±0.02	0.34±0.08**	0.20±0.01###
Creatinine clearance (ml/min)	3.0±0.3	1.7±0.4**	2.5±0.2##
Urine volume (ml/day)	61±23	62±9	23±9**,##
Urinary protein excretion (mg/day)	1.0±0.3	9.5±5.9**	1.1±0.3##

WKY, Wistar Kyoto rats; SHR-SP, spontaneously hypertensive stroke-prone rats; SHR-SP+Diuretic, trichlormethiazide-treated SHR-SP. Values are mean±S.D..

** $P<0.01$ vs. WKY.

$P<0.05$ vs. SHR-SP.

$P<0.01$ vs. SHR-SP.

kidney weight/body weight ratio were significantly higher in SHR-SP than in WKY. Treatment with diuretic attenuated the increases in kidney weight, MAP, SBP, and DBP in SHR-SP. HR did not differ significantly among the three groups.

3.2. Renal variables

Renal variables are shown in Table 2. Serum creatinine level, serum blood urea nitrogen level, and urinary protein excretion were significantly higher in SHR-SP than in WKY, whereas creatinine clearance was significantly lower in SHR-SP than in WKY. Long-term diuretic treatment with trichlormethiazide significantly reduced the serum creatinine level, urine volume, and urinary protein excretion, and increased the creatinine clearance in SHR-SP.

3.3. Plasma AM-m, AM-T, AM-m/AM-T ratio, aldosterone levels, and PRC in WKY, SHR-SP, and SHR-SP+Diuretic

Neurohumoral factors in plasma in the three groups are presented in Fig. 1. The plasma AM-T, AM-m, and aldosterone levels, and PRC were significantly higher in SHR-SP than in WKY, whereas the plasma AM-m/AM-T ratio did not differ significantly between the two groups. Diuretic treatment normalized the plasma AM-m and AM-T levels in SHR-SP and significantly attenuated the increases in aldosterone and PRC. However, the aldosterone level and

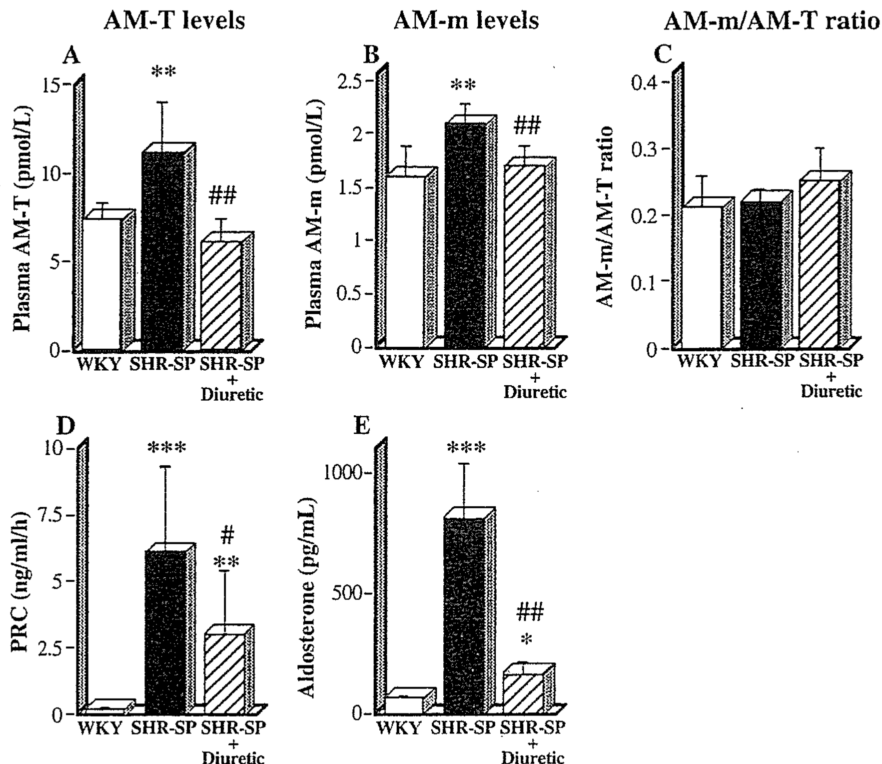

Fig. 1. Plasma AM-T level (A), AM-m level (B), AM-m/AM-T ratio (C), and PRC (D), and plasma aldosterone level (E) in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Data are expressed as mean±S.D. *$P<0.05$ vs. WKY; **$P<0.01$ vs. WKY; ***$P<0.001$ vs. WKY; #$P<0.05$ vs. SHR-SP; ##$P<0.01$ vs. SHR-SP.

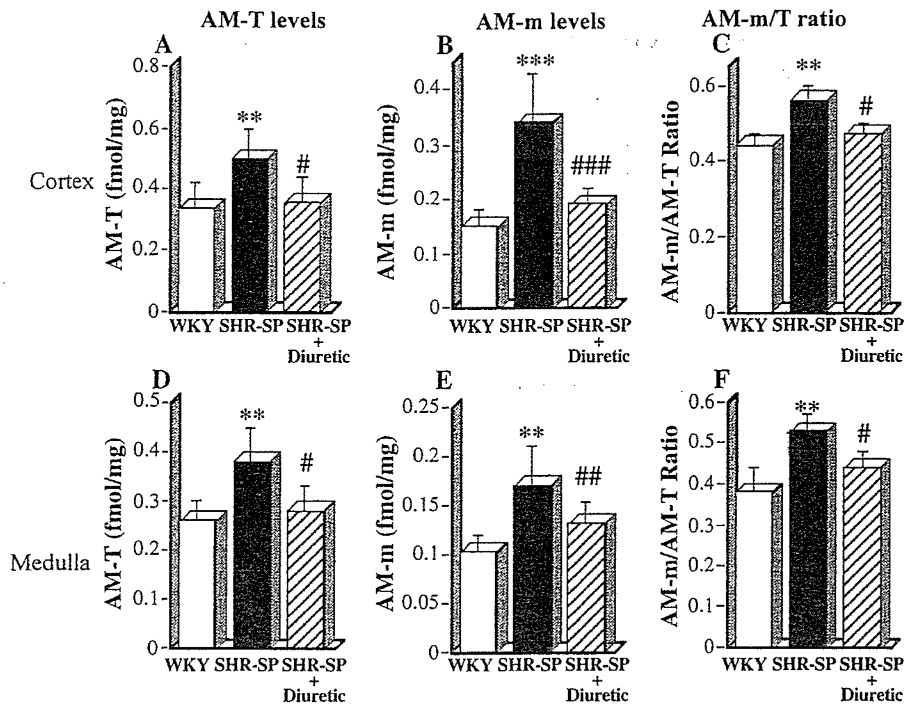

Fig. 2. Tissue AM-T levels (A and D), AM-m levels (B and E), and AM-m/AM-T ratio (C and F) in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Data are expressed as mean±S.D. $^{**}P<0.01$ vs. WKY; $^{***}P<0.001$ vs. WKY; $^{\#}P<0.05$ vs. SHR-SP; $^{\#\#}P<0.01$ vs. SHR-SP; $^{\#\#\#}P<0.001$ vs. SHR-SP.

PRC still differed significantly between WKY and diuretic-treated SHR-SP.

3.4. Tissue AM-m level, AM-T level, and AM-m/AM-T ratio in renal cortex and medulla in WKY, SHR-SP, and SHR-SP+Diuretic

The tissue AM-m levels, AM-T levels, and AM-m/AM-T ratios in the renal cortex and medulla in the three groups are presented in Fig. 2. The tissue AM-m levels, AM-T levels, and AM-m/AM-T ratios in the renal cortex and medulla were significantly higher in SHR-SP than in WKY. Treatment with diuretic significantly decreased the tissue AM-m levels, AM-T levels, and AM-m/AM-T ratios in the renal cortex and medulla of SHR-SP. There were no significant differences in these indices between WKY and diuretic-treated SHR-SP.

3.5. Levels of gene expression of AM in renal cortex and medulla

The expression of rat AM mRNA in the renal cortex and medulla in the three groups was measured by Northern blot analysis. Representative results of Northern blot analysis of AM mRNA from the renal cortex and medulla and the results of quantitative analysis of these blots corrected for the levels of GAPDH mRNA, serving as an internal control, are shown in Fig. 3A and B. The expression of AM mRNA in the renal cortex and medulla was slightly but significantly higher in SHR-SP than in WKY (Fig. 3A and B). Treatment with diuretic decreased the expression of AM mRNA in SHR-SP.

3.6. Levels of gene expression of CRLR, RAMP2, and RAMP3 in renal cortex and medulla

The expression of CRLR, RAMP2, and RAMP3 mRNA in the renal cortex and medulla in the three groups was investigated by RT-PCR analysis with specific CRLR, RAMP2, and RAMP3 primers. Specific bands of the

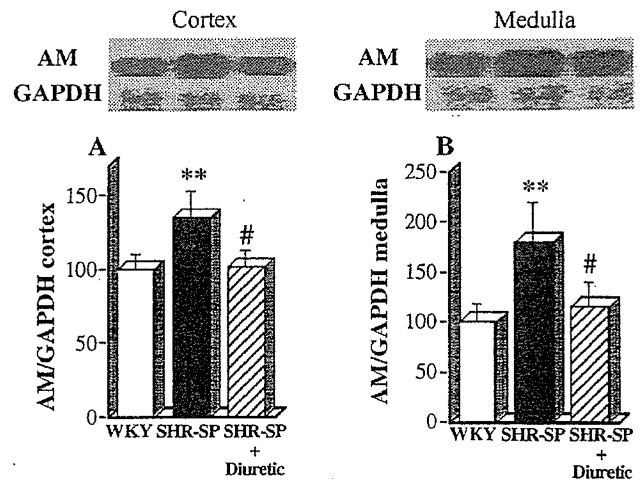

Fig. 3. Levels of AM gene expression in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Representative autoradiograms of AM and GAPDH mRNA bands in (A) renal cortex and (B) medulla (upper). AM mRNA levels corrected for GAPDH mRNA levels in the renal cortex and medulla (lower). Data are expressed as mean±S.D. $^{**}P<0.01$ vs. WKY; $^{\#}P<0.05$ vs. SHR-SP.

predicted length (323, 164, and 416 bp) were obtained with each CRLR-, RAMP2-, and RAMP3-specific primer. Representative electrophoretic profiles of RT-PCR products and quantitative analysis of the levels of these products corrected for the levels of the GAPDH-specific product, serving as an internal control, are shown in Fig. 4A–F. The CRLR/GAPDH, RAMP2/GAPDH, and RAMP3/GAPDH mRNA levels in the renal cortex were higher in SHR-SP than in WKY (Fig. 4A–C); CRLR/GAPDH, RAMP2/GAPDH, and RAMP3/GAPDH mRNA levels in the renal medulla did not differ significantly (Fig. 4D–F). Treatment with the diuretic reduced the CRLR/GAPDH, RAMP2/GAPDH, and RAMP3/GAPDH mRNA levels in the renal cortex of SHR-SP (Fig. 4A–C), but did not alter these levels in the renal medulla.

3.7. Levels of gene expressions of TGF-β, collagen I, and PAM in renal cortex and medulla

The TGF-β/GAPDH mRNA level and collagen I/GAPDH mRNA level in the renal cortex and medulla were higher in SHR-SP than in WKY (Fig. 5A–D). Long-term diuretic treatment significantly reduced the TGF-β/GAPDH and collagen I/GAPDH mRNA levels. In contrast, there were no differences in the mRNA level of PAM in the renal cortex or medulla among the three groups (data not shown).

4. Discussion

To investigate the pathophysiological role of the renal AM system in renal impairment associated with severe hypertension, we used a model of malignant hypertension, SHR-SP. In this model, a high-salt diet causes severe systemic hypertension, resulting in the establishment of renal impairment at the age of 16 weeks. We found that both molecular forms of AM in the plasma and the renal cortex and medulla tissue were higher in SHR-SP than in WKY. In addition, AM-m/AM-T ratio and AM gene expression in the renal cortex and medulla, and gene expression of CRLR, RAMP2, and RAMP3 in the renal cortex were also higher in SHR-SP than in WKY. Long-term diuretic treatment

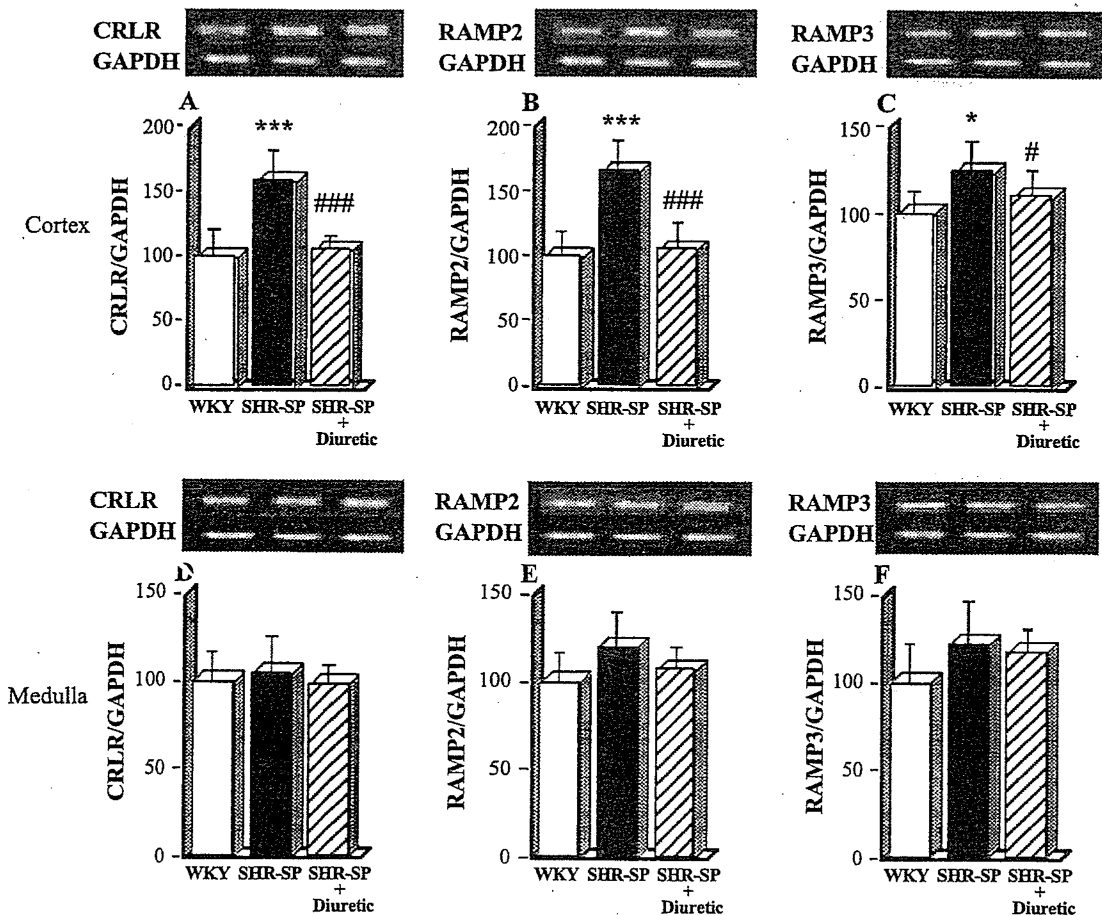

Fig. 4. Gene expression levels of CRLR (A and D), RAMP2 (B and E), and RAMP3 (C and F) in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Representative ethidium bromide-stained agarose gels of RT-PCR products for CRLR, RAMP2, RAMP3, and GAPDH (upper). Quantitative analysis of CRLR, RAMP2, and RAMP3 mRNA levels normalized relative to the GAPDH mRNA level (lower). Data are expressed as mean±S.D. *P<0.05 vs. WKY; ***P<0.001 vs. WKY; $^{\#}P$<0.05 vs. SHR-SP; $^{\#\#\#}P$<0.001 vs. SHR-SP.

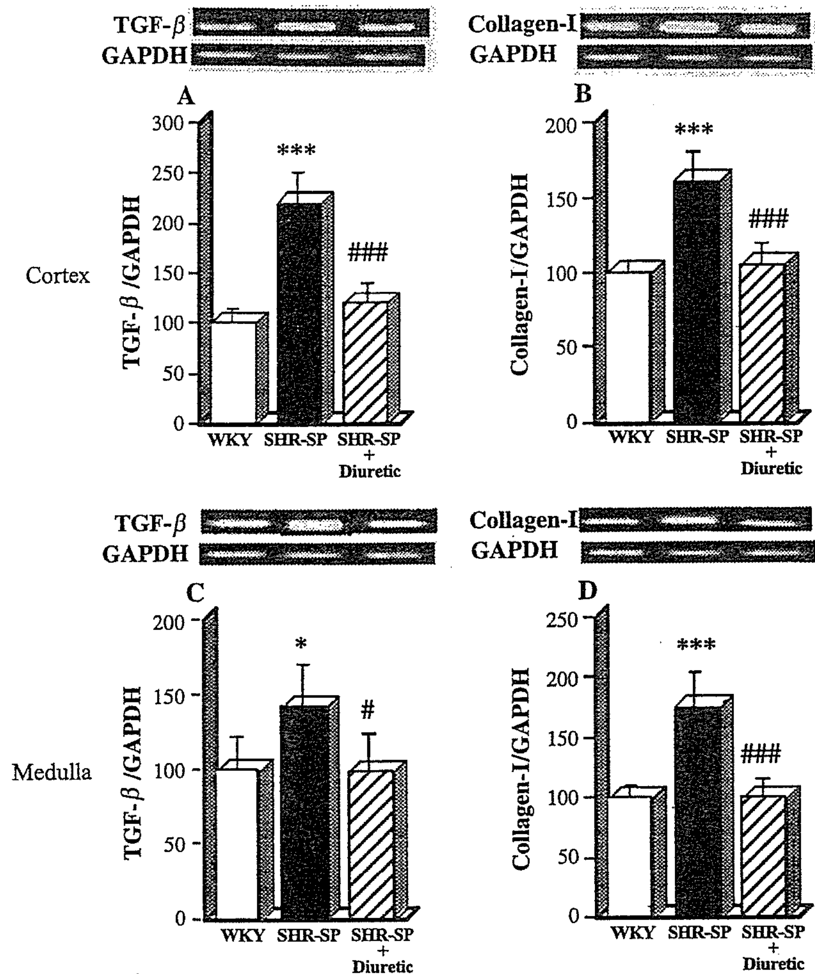

Fig. 5. Gene expression levels of TGF-β (A and C) and collagen I (B and D) in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Representative ethidium bromide-stained agarose gels of RT-PCR products for TGF-β, collagen I, and GAPDH (upper). Quantitative analysis of TGF-β and collagen I mRNA levels normalized relative to the GAPDH mRNA level (lower). Data are expressed as mean±S.D. *$P<0.05$ vs. WKY; ***$P<0.001$ vs. WKY; #$P<0.05$ vs. SHR-SP; ###$P<0.001$ vs. SHR-SP.

reduced blood pressure and improved kidney weight/body weight, serum creatinine level, serum blood urea nitrogen level, urinary excretion of protein, plasma aldosterone level, and PRC in SHR-SP. These improvements were associated with reductions in plasma AM, renal tissue AM, and mRNA of AM and AM receptor component in renal tissues. These results suggest that the AM system, including the ligand, receptor, and amidating activity in the kidneys, is activated in SHR-SP and modulates the pathophysiology of nephrosclerosis in this model.

The active mature form of AM is produced from AM precursor by a two-step enzymatic pathway. First, the AM precursor is converted to C-terminal glycine-extended AM, an inactive intermediate form of AM. Subsequently, inactive AM-Gly is converted by enzymatic amidation to the active form of mature AM, a 52-amino-acid peptide with a C-terminal amide structure [18]. Recent studies have shown that two molecular forms of AM circulate in human plasma and that the major circulating form of AM is AM-Gly [18,19]. We and others have reported that both plasma AM-m and AM-Gly levels increase in parallel in patients with hypertension, chronic renal failure, heart failure, and pulmonary hypertension [18,19,28–30]. In the rat hypertension model, plasma AM level in SHR is not increased compared with WKY [31]. However, it is increased in deoxycorticosterone acetate salt SHR, a severe hypertensive rat model with organ damage [31]. With regard to SHR-SP, plasma level of AM is low at 7 weeks compared with WKY; however, it is increased at 17 weeks [13]. The present study showed that the major circulating form of AM is AM-Gly in WKY and SHR-SP, and that both plasma AM-m and AM-T levels increased in parallel in SHR-SP, as compared with those in WKY. These results are consistent with the previous data [12,13]. Interestingly, long-term diuretic treatment reduced blood pressure levels and circulating AM-m and AM-T levels in salt-loaded SHR-SP. A recent study has reported that chronic salt load increases plasma AM levels in rat [32]. This finding suggests that reduced blood pressure, increased natriuresis, or both effects induced by diuretic treatment may account for the reduction in

circulating AM levels. Thus, plasma AM may be a noninvasive biochemical marker that can be used to monitor blood pressure control during antihypertensive therapy in severe hypertension.

In this study, we also analyzed the molecular forms of AM in renal tissue in WKY and SHR-SP. AM-m/AM-T ratio was obviously higher in renal tissue than in plasma. The reason for this difference is not fully understood. However, if AM-m/AM-T ratio is higher in tissues and AM acts as an autocrine or paracrine factor (or as both), most AM-m produced in tissues may be consumed, with release of primarily an inactive form into the circulation. Therefore, the major molecular form of AM in plasma is inactive. Our study also demonstrated that the AM-m/AM-T ratio in the renal cortex and medulla tissues was higher in SHR-SP than in WKY. This finding suggests that amidating enzyme activity is activated in the impaired kidney. A previous study reported that amidating enzyme mRNA expression is detected in collecting ducts and distal tubules [33]. Our study showed that antihypertensive therapy reduced AM-m and AM-T levels and AM-m/AM-T ratio. The amidating enzyme is an enzyme complex denoted collectively as peptidyl-glycine amidating monooxygenase [34]. In fact, amidation is a two-step process catalyzed by two separate enzyme activities: peptidyl-glycine-hydroxylating monooxygenase and peptidyl-hydroxyglycine-amidating lyase, both encoded by the same gene [35]. In the present study, we measured mRNA levels of PAM and found that there were no differences in mRNA levels of PAM in the renal cortex or medulla between WKY and SHR-SP. Thus, amidating enzyme activity might depend on posttranslational modification, although we did not measure the amidating enzyme activity in this study. Further study is required to elucidate the mechanism of regulation and posttranslational modification and the roles of amidating enzyme in the normal and diseased kidney.

A new family of single-transmembrane-domain proteins, which are called RAMP1, RAMP2, and RAMP3, has been cloned [11]. RAMPs are required to transport CRLR to the plasma membrane [11]. Previous studies demonstrated that the RAMP1/CRLR complex serves as a CGRP receptor, while RAMP2/CRLR and RAMP3/CRLR serve as AM receptors [11,36,37]. Several groups of investigators have reported that mRNA, binding sites, and immunoreactivity of AM exist in the kidney, and that AM has many physiological effects on the kidney [2,3]. However, whether the level of renal AM receptor is modulated by transcriptional regulation in hypertensive nephrosclerosis remains unknown. Furthermore, few studies have examined the expression of the mRNAs of CRLR and RAMPs in cardiovascular disease. One such study showed that RAMP1, RAMP2, and CRLR gene expression is markedly upregulated in obstructive nephropathy, whereas RAMP3 expression is unchanged [16]. We previously reported that mRNA levels of RAMP2 and RAMP3 in renal medulla were upregulated, whereas mRNA levels of CRLR in renal medulla or CRLR, RAMP2, and RAMP3 in renal cortex were not upregulated in deoxycorticosterone acetate salt SHR compared with WKY [31]. The present study showed that the mRNA levels of CRLR, RAMP2, and RAMP3 in the renal cortex were higher in SHR-SP than in WKY. Thus, the changes of mRNA levels of AM receptor component appear to be model-dependent. Further study is required to elucidate the exact mechanism of the regulation and role of AM receptor component in hypertensive nephrosclerosis. A recent study demonstrates that CRLR immunoreactivity is found in the distal tubules and glomerulus in renal cortex [38]. Previous studies have also shown that AM immunoreactivity is present in the distal tubules and glomerulus in renal cortex [2,3]. Taken together, the combined upregulation of receptor and ligand seems to enhance the effect of AM in the impaired kidney. Thus, not only upregulation of the ligand and amidating activity of AM, but also upregulation of the receptor system of AM may be involved in the pathophysiology of nephrosclerosis in severe hypertension.

To date, the pathophysiological role of increased AM system activity in the impaired kidney in hypertension is not fully understood. Previous studies demonstrated that AM is not only a natriuretic and diuretic peptide [2,3], but also an antigrowth peptide able to inhibit angiotensin II-induced proliferation of cultured mesangial cells, fibroblasts, and vascular smooth muscle cells [2,3,39]. Stimulation by IL-1β or TNF-α induces the expression of AM in these cells. These findings suggest a possible role of AM as an autocrine or paracrine cytoprotective factor (or as both) for nephrosclerosis [40]. In addition, we recently have shown that chronic AM infusion significantly improves renal function, histological findings, and biochemical and molecular markers in malignant hypertensive rats and salt-induced hypertensive rats without changing blood pressure [24,25]. Moreover, recent studies have indicated that AM gene delivery markedly increases plasma and renal tissue AM levels and attenuates the renal fibrosis induced by hypertension in rats [41,42] Furthermore, a very recent study shows that angiotensin II infusion causes more pronounced renal damage in AM+/− mice than AM+/+ mice, suggesting that endogenous AM exerts a protective effect against stress-induced renal injury [43] Taken together, these results suggest that increased activity of the AM system, including peptide, mRNA, and receptor, may have a compensatory effect on renal impairment in hypertensive nephrosclerosis.

In conclusion, our study shows that plasma AM levels, renal AM levels, renal gene expression of AM, and renal expression of the AM receptor system are all upregulated in impaired kidneys in severe hypertension. Diuretic therapy may attenuate increased activity of the AM system. These results suggest that induction of the AM system, including ligand, receptor, and amidating activity, as observed here, may modulate the pathophysiology of nephrosclerosis in the severely hypertensive rat.

Acknowledgments

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