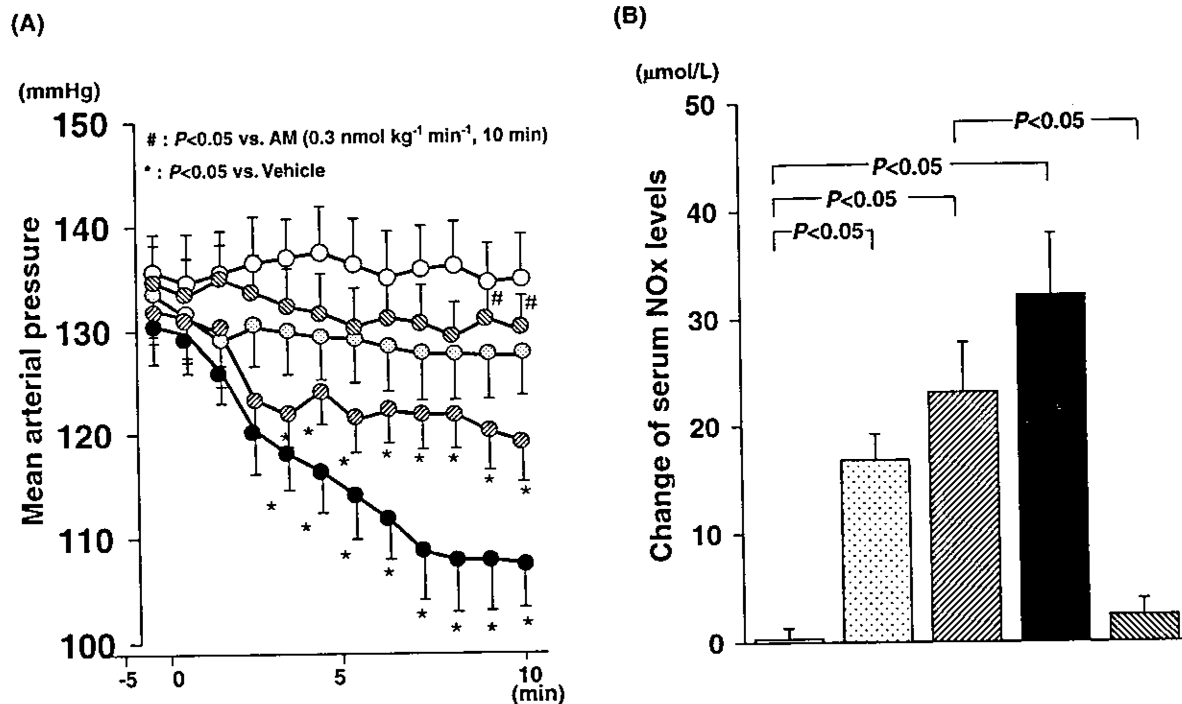

Fig. 5. Effects of the infusion of exogenous adrenomedullin (AM) on mean arterial pressure (A) and the change in serum NOx levels (B). Control rats were intravenously administered either vehicle (○, $n = 6$) or human AM (0.1 (◎), 0.3 (◍), 1.0 (●) nmol kg^{-1} min^{-1} for 10 min, $n = 6$, respectively). 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

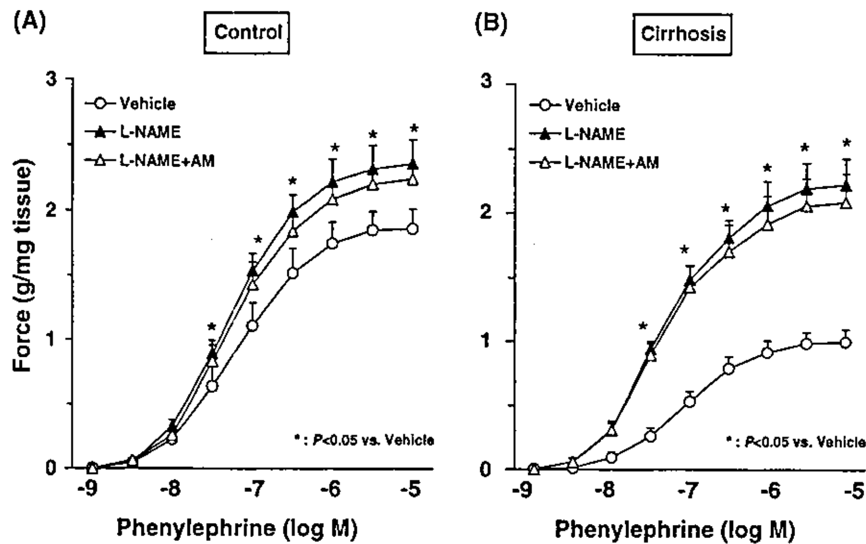

Fig. 6. Effects of nitric oxide synthase inhibitor in vascular hyporeactivity induced by adrenomedullin (AM) in the aortic rings of control (A) and cirrhotic rats (B). The effect of AM in phenylephrine-induced contraction was evaluated in the presence of nitric oxide synthase inhibitor, N^G-nitro-L-arginine 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.

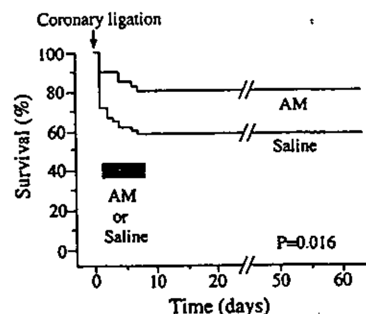

Figure 1. Kaplan-Meier survival curve of MI rats infused with saline (n=61) or 1.0 μg/h of human AM (n=42).

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

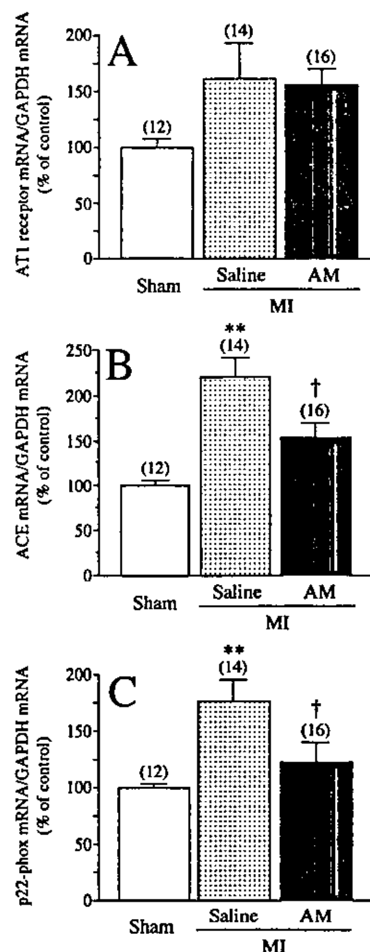

Figure 2. AT1 receptor (A), ACE (B), and p22-phox (C) mRNA levels of noninfarcted LV at day 7. **$P<0.01$ vs sham group; †$P<0.05$ vs saline-infused MI group; mean±SEM (n).

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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Plasma Concentrations of Adrenomedullin and Ghrelin in Hemodialysis Patients with Sustained and Episodic Hypotension

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Abstract. Sustained and/or episodic hypotension during hemodialysis (HD) is an important clinical issue. Plasma adrenomedullin (AM) is increased in HD patients with sustained hypotension, but little is known about its implications for episodic hypotension. Ghrelin may also contribute to the pathophysiology of hypotension in HD patients. We evaluated plasma levels of AM and total ghrelin in sustained hypotensive (SH; n = 23), episodic hypotensive (EH; n = 30) and normotensive (NT; n = 23) HD patients. In the EH group, the relationship between low blood pressure during HD and circulating levels of AM and ghrelin was also evaluated. Plasma levels of AM were significantly higher in SH (34.3 ± 8.3 fmol/ml, p<0.01) than in NT patients (27.6 ± 5.2 fmol/ml), but not in EH patients (30.8 ± 6.1 fmol/ml). There was no significant difference of plasma total ghrelin in SH (548.1 ± 426.5 fmol/ml) and in EH patients (544.6 ± 174.3 fmol/ml), compared with NT patients (400.0 ± 219.7 fmol/ml). On the other hand, in EH patients, the "suppressed blood pressure ratio" during HD significantly correlated with plasma AM (r = 0.77, p<0.001) and with total ghrelin levels (r = 0.44, p<0.05). Our results suggest that ghrelin, as well as AM, may play an important role as vasodilator local hormones and regulation of blood pressure during HD, especially the occurrence of EH. Further studies are necessary to clarify the implication of these hormones in the control of hypotension during HD.

Key words: Adrenomedullin, Chronic hypotension, Episodic hypotension, Ghrelin, Hemodialysis, Sustained hypotension
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SIGNIFICANT hypotension is a major cardiovascular complication in patients with end-stage renal disease undergoing hemodialysis (HD). Two types of hypotension are recognizable in the setting of maintenance HD: episodic hypotension (EH) during HD is the most common manifestation of hemodynamic instability, and occurs in around 30–40% of the dialysis population [1]. A second form is sustained hypotension (SH), characterized by a systolic blood pressure (SBP) lower than 100 mmHg, during the interdialysis period and is present in approximately 5–10% of patients [2, 3]. Both groups of patients require a substantial amount of medical and nursing care during and after HD to control hypotension-related symptoms. Although several clinical factors, such as autonomic dysfunction, reduced pressor response to vasopressor agents and cardiac dysfunction, have been shown to be responsible for the occurrence of EH and SH [1], the pathophysiology of chronic hypotension in dialysis patients has yet to be fully clarified.

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Adrenomedullin (AM) is a novel vasodialator peptide that has been recently isolated from human pheochromocytoma cells by monitoring the elevated activity of platelet cyclic adenosine monophosphatase [4]. AM is present in several normal tissues, such as adrenal medulla, lungs, kidneys and cardiac atrium, and has a potent and long-lasting hypotensive effect [5]. In 2000, Cases *et al.* [6] reported that plasma AM levels and nitrate levels were increased in HD patients, but only AM levels were higher in sustained hypotensive than in normotensive (NT) and hypertensive HD patients. Based on these results, they suggested the involvement of AM in the pathophysiology of SH in HD patients. Although several studies supported the association between AM and SH, there is no report to describe the association between AM and EH, which is more frequent among the dialysis population.

Ghrelin is a novel growth hormone (GH)-releasing peptide, originally isolated from the rat stomach, which was identified as an endogenous ligand for an orphan receptor termed GH secretagogue receptor [7]. Human ghrelin is a 28-amino acid peptide with a fatty acid chain modification on the N-terminal third amino acid. The hydroxygen atom of the hydroxyl group of the N-terminal third amino acid serine residue is replaced by a hydrophobic moiety, $C_7H_{15}CO$; in other words, the hydroxyl group of Ser^3 is octanoylated [7]. The n-octanoyl group at this position of the ghrelin molecule seems to be essential for some of the hormone's activity, including GH release and appetite. Non-acylated (desoctanoyl or desacyl) ghrelin circulates in far greater amounts than the acylated form and does not displace ghrelin from its hypothalamic and pituitary binding site [7].

Ghrelin is synthesized in several organs, such as intestine and kidney, as well as stomach [8, 9]. Recent studies reported that peripheral administration of ghrelin or GH secretagogue causes not only GH release from the pituitary gland, but also improvement in cardiac function, increase in food intake, fat accumulation and decrease in blood pressure [10, 11]. Furthermore, Wiley and Davenport investigated the vasodilator function of ghrelin by using human internal mammary artery *in vitro*, and concluded that ghrelin was an effective, endothelin-independent vasodilator of the long-lasting constrictor endothelin-1 in human arteries producing responses similar to those of AM [12]. From this point of view, it is speculated that accumulated ghrelin, as well as AM, before dialysis may cause hypotensive status in HD patients through its vasodilator effect. However, to date there is no study to clarify the association between plasma ghrelin concentration and SH and/or EH.

Based on the above background, we hypothesized that increased production of AM and ghrelin might be the underlying mechanism of SH and EH in HD patients. This study was designed to assess the possible role of AM and ghrelin in the pathogenesis of SH and EH in hemodialyzed patients.

Methods

Study participants

The study subjects were 76 patients with renal diseases on maintenance HD (23 SH, 30 EH and 23 NT patients). SH was defined as SBP less than 100 mmHg at predialysis in at least 80% of blood pressure measurements in the previous three months [1]. EH was defined as decreases of more than 25 mmHg during HD and/or as hypotension requiring medication during HD [13]. NT was defined as SBP less than 145 mmHg and DBP less than 90 mmHg at predialysis at least 80% of blood pressure measurements in the previous three months [1]. The causes of renal disease were chronic glomerulonephritis (n = 61), diabetic nephropathy (n = 12), Crohn's disease (n = 1) and undefined (n = 2). None of the patients were anephric, had evidence of cardiac disease, such as myocardial infarction, or suffered from chronic obstructive pulmonary disease or hepatic dysfunction. None of the patients received antihypertensive treatment and vasodilatory drugs. Before the study, ethical approval was obtained from the special committee of Nagasaki University School of Medicine (project registration no. 15052224). Blood samples from patients were collected at three hospitals in Nagasaki city (see Acknowledgment). In all cases, a signed informed consent was obtained before the study.

Measurement of plasma AM and ghrelin concentrations

Blood samples were collected in tubes with 2 mg/ml of ethylenediaminetetraacetic acid (EDTA)-2Na and 500 KIU/ml aprotinin just before the dialysis. After collection, the samples were promptly centrifuged at 4°C. Plasma total AM was measured by immunoradiometric assay using a specific kit for each form

(adrenomedullin RIA Shionogi, adrenomedullin mature RIA Shionogi; Cosmic Corporation, Tokyo, Japan).

Plasma total ghrelin was measured by our specific radioimmunoassay (RIA) system as described previously [9]. Since the active form of ghrelin is unstable in non-acidified normal plasma, the total amount of ghrelin was used in this study.

We defined "suppressed blood pressure ratio" in patients with EH as [(systolic blood pressure at predialysis) – (minimum systolic blood pressure at the episode of hypotension during HD)/systolic blood pressure at predialysis] × 100 (%), and determined its correlation with plasma levels of AM and ghrelin.

Statistical analysis

Statistical analysis was performed using software package SPSS 9.0 for Windows (Chicago, IL). Data are expressed as means ± SD. One-way ANOVA was used for statistical comparisons between SH and NT groups, and EH and NT groups. Pearson correlation analysis was performed for correlation between variables, and a p value less than 0.05 was accepted as statistically significant.

Results

Table 1 summarizes the hemodynamic and laboratory values for the three groups of HD patients. Systolic, diastolic, and mean blood pressures were significantly different between SH and NT patients, and EH and NT patients ($p<0.001$ and $p<0.001$, respectively). On the other hand, there was no significant difference between SH and EH patients. Duration of HD was significantly longer in SH than in NT patients ($p<0.001$). There was no significant difference of hematocrit (Ht) in SH (34.5 ± 8.1%) and in EH patients (31.8 ± 3.2%), compared with NT patients (30.9 ± 2.4%).

When data of all patients were analyzed, plasma levels of AM correlated with those of ghrelin ($r = 0.29$, $p = 0.003$, Fig. 1). Plasma levels of AM in HD patients were significantly higher in SH (34.3 ± 8.3 fmol/ml, $p<0.01$) than in NT patients (27.6 ± 5.2 fmol/ml, Table 1), but not in EH patients (30.8 ± 6.1 fmol/ml). There was no significant difference of plasma total ghrelin in SH (548.1 ± 426.5 fmol/ml) and in EH patients

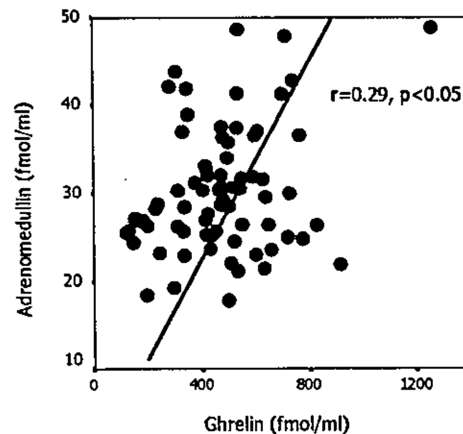

Fig. 1. Relationship between ghrelin and AM using data of all HD patients ($r = 0.29$, $p<0.05$).

Table 1. Demographic, hemodynamic, and laboratory values of the three groups of HD patients.

	SH patients	EH patients	Normotensive patients
Number of patients	23	30	23
Males (%)	30.4	53.1	69.5
Age (years)	57.3 ± 12	56.7 ± 14.7	64.4 ± 13.8
Duration of HD (years)	18.7 ± 11.1[b]	10.6 ± 8.3	8.0 ± 5.4
Systolic BP (mmHg)	87.1 ± 11.6[b]	104.1 ± 11.5[b]	119.1 ± 16.6
Mean BP (mmHg)	62.1 ± 8.5[b]	79.4 ± 12.2[b]	94.1 ± 8.7
Diastolic BP (mmHg)	49.5 ± 7.9[b]	64.0 ± 13.7[b]	71.5 ± 8.9
Interdialysis weight gain (g)	1218.3 ± 1102.7	2690.7 ± 1221.5	2269.7 ± 993.4
Hematocrit (%)	34.5 ± 8.1	31.8 ± 3.2	30.9 ± 2.4
Plasma AM (fmol/ml)	34.3 ± 8.3[a]	30.8 ± 6.1	27.6 ± 5.2
Plasma total ghrelin (fmol/ml)	548.1 ± 426.5	544.6 ± 174.3	400.0 ± 219.7

Data are mean ± SD.
[a]$p<0.01$, [b]$p<0.001$, *vs* normotensive patients.
HD, hemodialysis; BP, blood pressure.

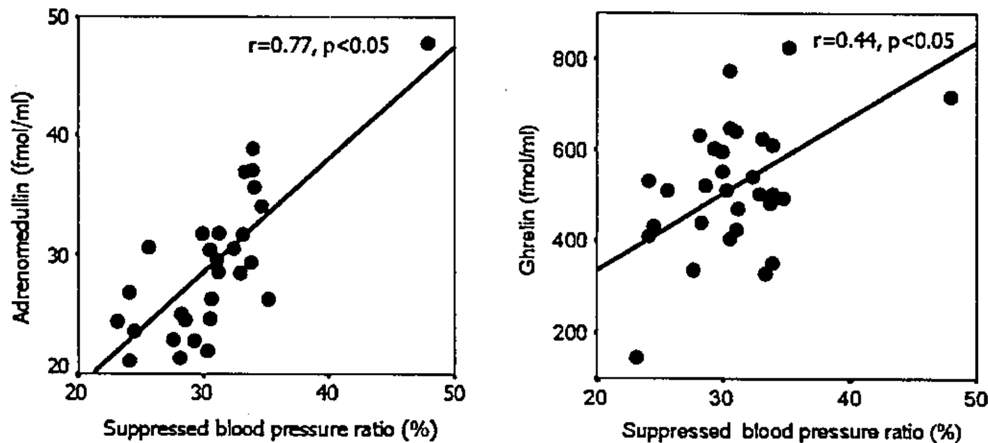

Fig. 2. Relationships between AM and depressed mean blood pressure ($r = 0.77$, $p<0.05$, left), and ghrelin ($r = 0.44$, $p<0.05$, right) and "suppressed blood pressure ratio" in EH patients.

(544.6 ± 174.3 fmol/ml), compared with NT patients (400.0 ± 219.7 fmol/ml). On the other hand, in EH patients, the "suppressed blood pressure ratio" significantly correlated with plasma levels of AM ($r = 0.77$, $p<0.001$) and of ghrelin ($r = 0.44$, $p<0.05$, Fig. 2). On the other hand, there was no correlation between "suppressed blood pressure ratio" and Ht.

Discussion

Our study showed that plasma AM levels are significantly increased in patients with SH. In addition, we showed that in patients with EH, plasma AM levels correlated positively with the "suppressed blood pressure ratio". Furthermore, we showed for the first time that the levels of plasma total ghrelin also correlated with the "suppressed blood pressure ratio" in EH patients. These results suggest that as vasodilator peptides, these two hormones may be involved in the pathophysiology of hypotension in HD patients, especially in EH.

Several groups have investigated the mechanism(s) of high levels of vasodilator agents during HD [1, 14–16]. Imai *et al.* [14] conducted a hemodynamic study and reported that while cardiac index, heart rate or stroke volume were similar in hypotensive and normotensive HD patients, total peripheral vascular resistances were lower in the former group. They suggested that increased biosynthesis and/or release of vasodilator agents might be critical in the pathogenesis of hypotension during HD. Plasma atrial natriuretic peptide levels have been reported to be similar in hypotensive and normotensive dialysis patients [15, 16], but the possible role of this molecule in chronic hypotension in uremia is controversial [6]. In addition, it has been shown that plasma levels of adenosine, a strong hypotensive agent, are increased, while the activity of intracellular adenosine deaminase, the enzyme that metabolizes this agent, is reduced in HD patients, but not in predialysis or peritoneal dialysis patients [17]. However, the possible role of this agent in hypotension in dialysis patients has yet to be fully evaluated.

In addition to these peptides, AM was reported to be increased in patients with SH [6, 18]. However, there is little or no information regarding the relationship between AM and EH. In the present study, we found that plasma AM levels are increased in patients with SH. In addition, we showed that AM levels positively correlated with the "suppressed blood pressure ratio" in patients with EH. Thus, the present results suggest that AM may be involved in the pathophysiology of EH and SH in HD patients.

The exact mechanism of the increased production of vasodilators including AM is still unknown. However, it is likely that the inflammatory state of uremia plays some role [19]. The production of both nitric oxide and AM is induced by cytokines, such as hepatocyte growth factor (HGF), which induces endothelial proliferation and nitric oxide-mediated vasodilation, and other studies showed that HGF was increased in hypotensive HD patients [20]. Several studies also suggested the possible roles of microinflammatory state in chronic hypotension of dialysis patients, through the

induction of synthesis of several vasodilator substances [21, 22]. Although further studies are needed, similar mechanism(s) may be associated with the pathophysiology of EH during dialysis.

Besides vasodilator effect, it is suggested that AM may also be associated with circulating blood volume in HD patients [23]. In our current study, there was no relationship between Ht, one of the markers of circulating blood volume, and hypotension. Also there was no relationship between Ht and AM. Further studies will be needed to clarify the contribution of AM to EH and SH through the change of blood volume in HD patients.

Ghrelin, an endogenous peptide recently linked to growth hormone secretagogue receptor [7], is a potent, endothelium-independent vasodilator of human arteries, effectively reversing endothelin-1 (ET-1)-mediated constriction. Ghrelin is present in human plasma at approximately 100 pmol/L [7], a concentration considerably higher than other vasoactive peptides. Yoshimoto *et al.* demonstrated that plasma ghrelin in patients with renal disease is increased in parallel with the severity of renal damage [24]. They also revealed that approximately half of the plasma ghrelin, as well as half of the serum creatinine or blood urea nitrogen, are removed from the blood by a single course HD, and that bilateral nephrectomy in mice causes marked increase in plasma ghrelin concentrations. They concluded that increased plasma ghrelin in renal failure may result from decreased clearance or degradation in the kidney. Although overproduction of ghrelin in organs other than the stomach may contribute to higher plasma concentrations [25, 26], a similar pathophysiological changes may occur in patients in HD. In our current study, we showed a positive correlation between plasma AM and ghrelin concentrations in HD patients, and found a positive correlation between "suppressed blood pressure ratio" and plasma levels of ghrelin in EH patients. These results suggest that ghrelin, in cooperation with AM, may contribute to the development of EH through its vasodilatory effect. On the other hand, we could not reveal the significant difference of total ghrelin levels between SH and NT patients, and EH and NT patients. Although this may merely reflect our small sample size, ghrelin may not play an important role in the occurrence of hypotension in chronic state.

In addition to this effect, ghrelin is associated with changes in body composition in patients on HD. Ayala *et al.* investigated patients with end-stage renal disease and found markedly high plasma ghrelin concentrations in this group, and its level correlated significantly with plasma insulin, body mass index, log serum leptin levels and truncal fat mass [27]. These findings suggest that the kidney is an important site for clearance and/or degradation of ghrelin.

There are several limitations in this study. First, we measured AM and ghrelin only just before, not during and after HD. Observation on the dynamics of AM and ghrelin in a series of HD will be available to clarify the contribution of these hormones, more precisely. Second, we could not measure plasma levels of atrial natriuretic peptide and adenosine, which have been suggested to have possible roles in SH patients [19, 28]. Third, our sample size was relatively small to completely identify the roles of AM and ghrelin in SH and EH patients. Additional sampling may overcome the insufficient statistical significance in our study.

In this study, we observed that duration of HD was significantly longer in SH than in NT patients. It is known that longer duration of HD is associated with autonomic neuropathy, which is one of the major causes of hypotension in HD patients [29]. Besides vasodilator agents, such clinical factors should be also considered to be key factors for the occurrence of hypotension in HD patients. In conclusion, our results suggested the possibility that ghrelin and AM might play important roles as vasodilator local hormones and control of blood pressure during HD. Further studies are clinically important to clarify the implication of these hormones in the clinical control of hypotension during HD.

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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 anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg), and their body weights were measured. A polyethylene catheter (PE-50) was inserted into the thoracic aorta via the right carotid artery to measure heart rate (HR), 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

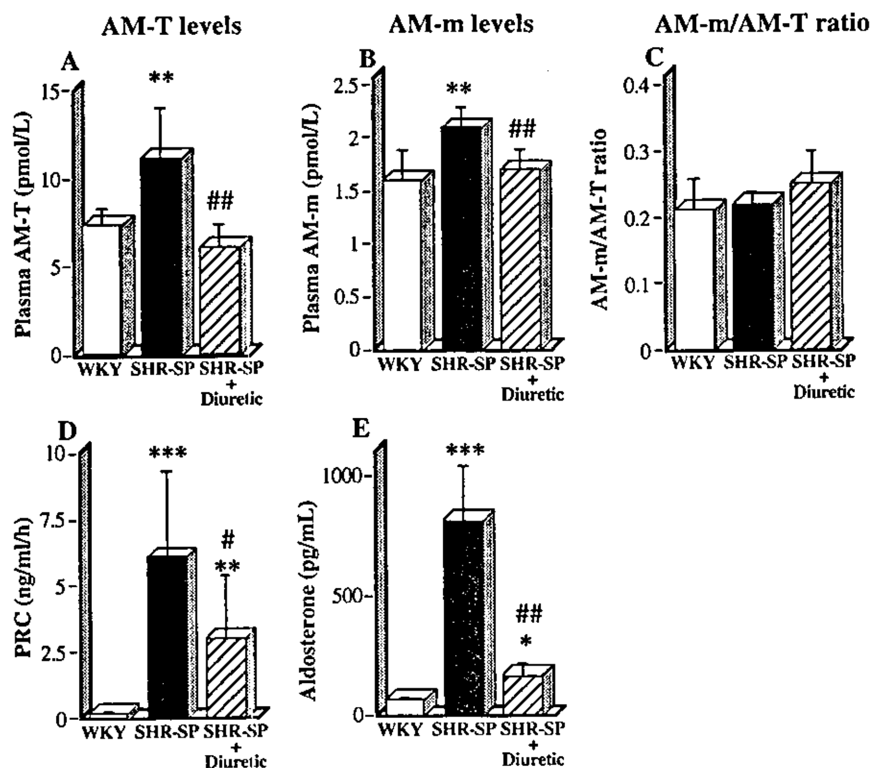

Fig. 1. Plasma AM-T level (A), AM-m level (B), AM-m/AM-T ratio (C), and PRC (D), and plasma aldosterone level (E) in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Data are expressed as mean±S.D. *$P<0.05$ vs. WKY; **$P<0.01$ vs. WKY; ***$P<0.001$ vs. WKY; #$P<0.05$ vs. SHR-SP;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