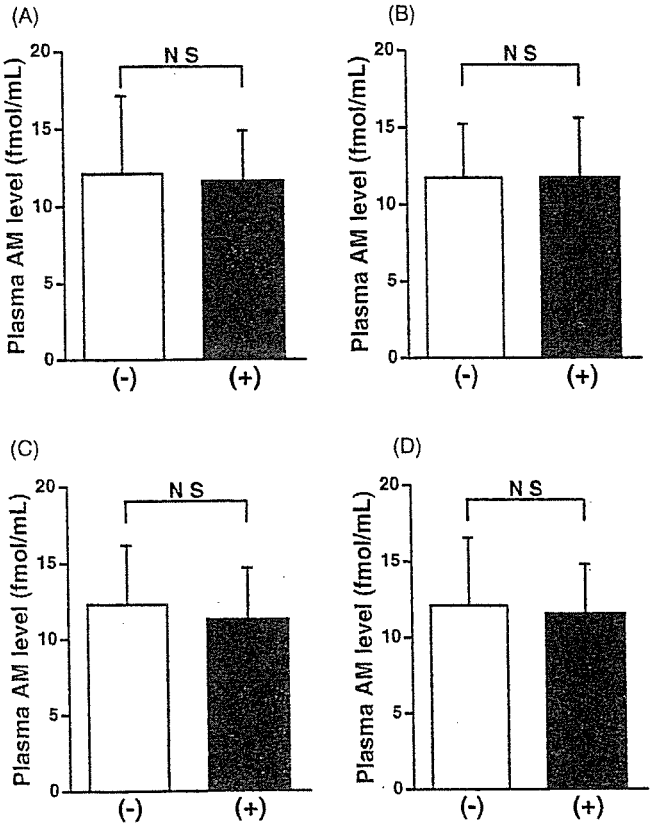

Fig. 1. Comparison of plasma AM concentration between the two groups with and without each cardiovascular risk factor, i.e., hypertension (A), diabetes mellitus (B), hyperlipidemia (C), or smoking (D). Values are given as mean ± S.D.

no significant difference in its plasma concentration among the four groups (Table 2). Although the associations of AM levels with several parameters linked to the control of hypertension, diabetes, and hyperlipidemia were examined, the plasma AM was not significantly correlated with any of blood pressure, plasma glucose, hemoglobin A1c, and serum lipid levels (Table 3). On the other hand, the plasma

Table 2
Relation between number of cardiovascular risk factors and AM concentration

Number of risk factors	*N*	Plasma AM level (fmol/mL)
1	17	11.7 ± 3.2
2	42	11.9 ± 3.4
3	36	11.3 ± 3.4
4	19	11.4 ± 2.8

The cardiovascular risk factors are hypertension, diabetes mellitus, hyperlipidemia, and smoking habit. Values are mean ± S.D.

Table 3
Correlation of AM concentration with clinical and laboratory parameters

	r	*P*
Age	0.09	0.350
Systolic blood pressure	0.06	0.553
Diastolic blood pressure	−0.01	0.923
Fasting plasma glucose	0.07	0.448
Hemoglobin A1c	−0.07	0.471
Total cholesterol	−0.12	0.207
Triglycerides	−0.14	0.135
HDL cholesterol	−0.01	0.909
CRP	0.42	<0.0001
IL-6	0.52	<0.0001

concentration of AM was strongly correlated with inflammatory parameters such as CRP ($r = 0.42$, $P < 0.0001$) and IL-6 ($r = 0.52$, $P < 0.0001$) (Fig. 2).

Next, we investigated the contribution of two cardiovascular diseases to plasma AM concentration in the present study subjects. The patients with IHD had a significantly higher concentration of plasma AM than those without IHD (13.2 ± 4.3 fmol/mL versus 10.6 ± 2.6 fmol/mL, $P < 0.001$) (Fig. 3A). The AM level in subjects with PAD was also increased significantly compared with those without PAD (12.7 ± 4.3 fmol/mL versus 11.2 ± 3.1 fmol/mL, $P < 0.05$) (Fig. 3B). We further analyzed the plasma levels of this peptide by dividing all subjects into three groups; subjects with

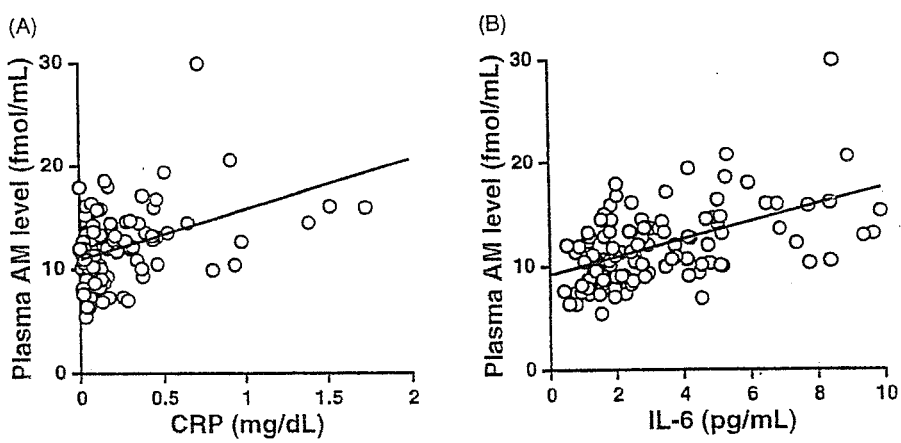

Fig. 2. Correlation of plasma AM concentration with CRP (A) and IL-6 (B) in all subjects. The AM level was significantly correlated with CRP ($r = 0.42$, $P < 0.001$) and with IL-6 ($r = 0.52$, $P < 0.001$).

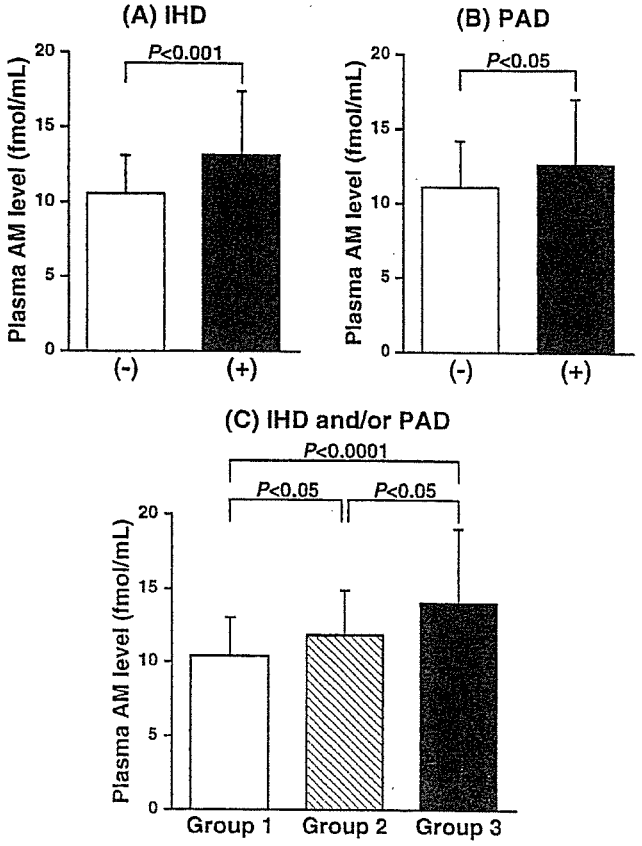

Fig. 3. (A and B) Comparison of plasma AM concentration between the two groups with and without IHD (A) or PAD (B). (C) Comparison of plasma AM concentration among the three groups with and without IHD and/or PAD. Group 1 ($n = 44$), patients with neither IHD nor PAD; group 2 ($n = 46$), patients with IHD alone or PAD alone; group 3 ($n = 24$), patients with both IHD and PAD. Values are given as mean ± S.D.

neither IHD nor PAD (group 1, $n = 44$), with IHD alone or PAD alone (group 2, $n = 46$), and with both IHD and PAD (group 3, $n = 24$). The plasma AM concentration was significantly higher in group 2 (11.8 ± 3.1 fmol/mL) than in group 1 (10.4 ± 2.6 fmol/mL), and its concentration was further increased in group 3 (14.0 ± 5.0 fmol/mL) than in group 2 (Fig. 3C). That is, the patients who had both complications of IHD and PAD had the highest level of plasma AM in the present study subjects.

Since plasma concentrations of CRP and IL-6 were closely related to that of AM in the present study, these plasma levels were also compared between the two groups with and without IHD or PAD. Both CRP and IL-6 levels in subjects with IHD were higher than in those without IHD (Table 4). However, no significant differences were observed in these plasma levels between patients with and without PAD. To assess which factor among AM, CRP, and IL-6 is the most sensitive marker for the presence of IHD and PAD in the present patients with atherosclerotic risks, multiple logistic regression analysis was performed. As shown in Table 5, only plasma level of AM among three parameters was an independent predictor for the presence of both IHD and PAD. Furthermore, when subjects were divided into two groups by the median value of plasma AM levels, having a higher level of AM (≥11 fmol/mL) was a significant predictor for IHD ($\chi^2 = 11.809$, $P = 0.0006$) and PAD ($\chi^2 = 4.254$, $P = 0.039$), independently of the absence or presence of atherosclerotic risks such as hypertension, diabetes mellitus, hyperlipidemia, and smoking. In fact, compared with patients with lower plasma AM (<11 fmol/mL), those with its higher levels had a significantly higher incidence of IHD (odds ratio 3.250, $P = 0.002$) and PAD (odds ratio 2.177, $P = 0.048$).

Table 5
Independent predictors for the presence of IHD and PAD by multiple logistic regression analysis

	χ^2	d.f.	P
IHD			
AM	4.893	1	0.027
CRP	1.466	1	0.226
IL-6	1.276	1	0.259
PAD			
AM	5.528	1	0.019
CRP	0.838	1	0.360
IL-6	0.351	1	0.554

d.f.: degrees of freedom. χ^2 and P values, adjusted for age, sex, and the absence or presence of each risk factor (hypertension, diabetes mellitus, hyperlipidemia, or smoking), are given.

4. Discussion

In the present study, no significant association was found between the plasma concentration of AM and cardiovascular risk factors such as hypertension, diabetes mellitus, hyper-

Table 4
CRP and IL-6 levels in patients with and without IHD or PAD

	IHD		PAD	
	(−)	(+)	(−)	(+)
CRP (mg/dL)	0.16 ± 0.25	0.34 ± 0.54*	0.24 ± 0.35	0.24 ± 0.50
IL-6 (pg/mL)	2.60 ± 1.97	3.93 ± 2.38†	3.08 ± 2.41	3.38 ± 1.99

Values are mean ± S.D.
* $P < 0.05$.
† $P < 0.01$ versus IHD (−).

lipidemia, and smoking habit. In addition, the plasma AM level was not significantly correlated with blood pressure, plasma glucose, or serum lipid levels. Although previous studies reported that plasma AM levels were increased in patients with hypertension and diabetes mellitus [4,10,16,22], the increased AM levels in these patients were also shown to be closely related to the complications and organ damage such as cardiac hypertrophy, renal insufficiency, and microangiopathy [3,10,16,21,34]. Similarly, in the present study subjects, the increase in plasma AM depended on the absence or presence of coronary and peripheral vascular lesions. Taken together, elevated levels of plasma AM in patients with cardiovascular risks are probably associated with organ damage including vascular complications, rather than with underlying disease itself.

It is known that low-grade inflammation is importantly involved in the initiation and progression of atherosclerotic vascular lesions [17]. In fact, increased serum levels of CRP and IL-6, markers of inflammation, have been shown to be related to the development of atherosclerotic lesions in several cardiovascular diseases including IHD and PAD [2,9,15,19,26–29]. Since the plasma AM level in the present subjects was closely correlated with both CRP and IL-6 levels, the increased level of plasma AM in patients with IHD and PAD may have reflected inflammatory response linked to advanced atherosclerosis. Then, why was AM a more sensitive marker for coronary and peripheral arterial complications than CRP and IL-6 in the present study? Although the exact reason remains to be elucidated, there are some possible explanations for the superiority of AM. AM is produced in various organs and tissues, but the main source of circulating AM is the blood vessels (especially vascular endothelial cells) [31], in contrast to the major sites of the production of CRP and IL-6. Therefore, AM may directly reflect vascular inflammation and endothelial injury, compared with other inflammation-related substances. Furthermore, since several studies have shown that ischemic and hypoxic conditions stimulate the production and secretion of AM [20,24,39,40], it is possible that the increase in AM production in patients with IHD and PAD might be induced by not only vascular inflammation but also ischemia in coronary and peripheral atherosclerotic lesions.

Our previous studies showed that AM inhibited migration and proliferation of vascular smooth muscle cells induced by platelet-derived growth factor [7,12]. Chun et al. [1] reported that oxidative stress augmented endothelial secretion of AM and that this peptide played a compensative role for endothelial dysfunction. Shimosawa et al. [30] revealed that AM had a protective action against cardiovascular damage, through the inhibition of oxidative stress production. These findings suggest that AM has anti-atherosclerotic and anti-oxidative effects on vascular wall cells. Therefore, it is possible that plasma AM levels were elevated compensatively in patients with IHD and PAD to exert a self-protective effect in atherosclerotic vascular lesions.

There were some limitations in the present study. The majority of patients on this study had received antihypertensive, hypoglycemic, and/or lipid-lowering medication, and their blood pressure, plasma glucose, and serum lipid levels were adequately controlled. Thus, we cannot exclude the possibility that these treatments might spoil the relationship between atherosclerotic risk factors and plasma AM levels. In addition, it remains unclear whether its plasma levels increase with the progression of atherosclerotic lesions and decrease during treatment. Many longitudinal studies have shown that high levels of CRP and IL-6 are independent predictors of cardiovascular morbidity and mortality [15,18,19,26–29,35–37,41]. Therefore, a prospective study is essential to establish the predictive value and prognostic potential of AM in cardiovascular diseases. Further studies are also necessary to evaluate the association of other atherosclerotic vascular diseases than IHD and PAD, e.g., cerebrovascular disease, carotid atherosclerosis, and renal artery stenosis, with plasma AM levels.

In conclusion, the present study has demonstrated that an increase in plasma AM concentration in patients with some of cardiovascular risk factors is importantly related to the presence of vascular complications such as IHD and PAD. Our findings suggest that plasma AM may be a novel sensitive marker for coronary and peripheral vascular lesions in subjects with atherosclerotic risks.

Acknowledgments

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Protective Effects of Endogenous Adrenomedullin on Cardiac Hypertrophy, Fibrosis, and Renal Damage

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Background—Adrenomedullin (AM) is a novel vasodilating peptide thought to have important effects on cardiovascular function. The aim of this study was to assess the activity of endogenous AM in the cardiovascular system using AM knockout mice.

Methods and Results—Mice heterozygous for an AM-null mutation (AM+/−) and their wild-type littermates were subjected to aortic constriction or angiotensin II (Ang II) infusion. The resultant cardiovascular stress led to increases in heart weight/body weight ratios, left ventricular wall thickness, and perivascular fibrosis, as well as expression of genes encoding angiotensinogen, ACE, transforming growth factor-β, collagen type I, brain natriuretic peptide, and c-*fos*. In addition, renal damage characterized by decreased creatinine clearance with glomerular sclerosis was noted. In all cases, the effects were significantly more pronounced in AM+/− mice. Hearts from adult mice subjected to aortic constriction showed enhanced extracellular signal-regulated kinase (ERK) activation, as did cardiac myocytes from neonates treated acutely with Ang II. Again the effect was more pronounced in AM+/− mice, which showed increases in cardiac myocyte size, protein synthesis, and fibroblast proliferation. ERK activation was suppressed by protein kinase C inhibition to a greater degree in AM+/− myocytes. In addition, treatment of cardiac myocytes with recombinant AM suppressed Ang II–induced ERK activation via a protein kinase A–dependent pathway.

Conclusions—Endogenous AM exerts a protective effect against stress-induced cardiac hypertrophy via protein kinase C– and protein kinase A–dependent regulation of ERK activation. AM may thus represent a useful new tool for the treatment of cardiovascular disease. (*Circulation.* **2004;109:1789-1794.**)

Key Words: peptides ■ angiotensin ■ cardiovascular diseases ■ hypertrophy ■ kidney

Adrenomedullin (AM) is a vasodilator peptide originally isolated from the extract of human pheochromocytoma.[1] It has also been shown to be produced in vascular endothelial and smooth muscle cells and in cardiac myocytes,[2,3] and high levels are found in the heart, lung, kidney, and adrenal medulla.[4] In addition to its ability to reduce vascular tone, it stimulates hormone secretion[5] and cell growth and differentiation.[6,7] AM appears to circulate in plasma and has been implicated in the regulation of cardiovascular function.[8,9] Indeed, plasma and tissue levels of AM are increased in patients with such cardiovascular ailments as hypertension, congestive heart failure, myocardial infarction, and renal dysfunction.[9,10] This led us to hypothesize that AM participates in the pathophysiology of cardiovascular disease, although it is not clear whether its effects are causative or compensatory.

Cardiac hypertrophy, which is recognized in many cardiovascular diseases, is an independent risk factor of cardiac morbidity and mortality[11] and can be induced by mechanical stress and by humoral factors such as angiotensin II (Ang II).[12,13] Several studies have suggested that application of exogenous AM suppresses the development of cardiac hypertrophy and renal damage.[14,15] Moreover, mechanical stretch causes AM production in cardiac myocytes,[16] whereas Ang II stimulation causes its production in cardiac fibroblasts,[17] which suggests that AM serves as a local paracrine or autocrine modulator of cardiac remodeling.

To directly evaluate the function of endogenous AM, we recently generated a strain of AM knockout mice. Homozygous mice (AM−/−) died in utero by embryonic day (E) 13.5,[18] but heterozygotes (AM+/−) survived until adulthood. They did exhibit reduced AM expression and elevated blood pressure, however. The aim of the present study was to use this model to examine the effect of endogenous AM expression on cardiac hypertrophy, fibrosis, and renal damage.

Methods

Generation of AM Knockout Mice

AM gene targeting was performed as described previously.[18] At 12 weeks of age, male mice were used in the experiments. All experiments were performed in accordance with the Declaration of

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Helsinki and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Pressure Overload Produced by Aortic Constriction

Pressure overload was produced by constriction of the abdominal aorta as described previously.[19] Thirty minutes, 2 hours, or 28 days after ligation, the hearts were removed, weighed, and subjected to further analysis.

Ang II Infusion

Ang II was infused for 14 days into mice with a subcutaneously implanted osmotic minipump (Alzet Co). The delivery rate was 3.2 $mg \cdot kg^{-1} \cdot d^{-1}$. To control for the effect of the elevated basal blood pressure on cardiac hypertrophy in AM+/− mice, an antihypertensive drug, hydralazine hydrochloride, was dissolved in the drinking water of some AM+/− mice. This led to a daily intake of 5 $mg \cdot kg^{-1} \cdot d^{-1}$, which was sufficient to eliminate the difference in blood pressure between AM+/− and wild-type mice.

In our knockout mice, proadrenomedullin N-terminal 20 peptide (PAMP), another product of the proadrenomedullin gene, is also deleted. To evaluate the effect of that deletion in the present study, in some mice PAMP was also infused at a rate of 20 ng/h along with Ang II.

Cell Culture of Cardiac Myocytes

Cardiac myocytes were harvested from 1-day-old neonatal mice and rats as described previously.[20] The cells were cultured for 24 hours, after which the medium was replaced with a complete serum-free medium for 24 hours. The cells were then incubated for an additional 24 hours, with or without 1 μmol/L Ang II. After Ang II treatment, myocyte surface area was measured, and protein synthesis was assayed by measuring ^{14}C-phenylalanine incorporation (see below).

For Western blot analysis, cardiac myocytes from AM+/− or wild-type mice were incubated for 30 minutes with or without 1 μmol/L H7 (Seikagaku Co), an inhibitor of protein kinase C (PKC), and then treated with 1 μmol/L Ang II for 8 minutes. Cardiac myocytes from rat were incubated with 300 nmol/L human recombinant AM with or without 1 μmol/L H89 (Seikagaku Co), an inhibitor of protein kinase A (PKA), and then treated with Ang II.

Incorporation of ^{14}C-Phenylalanine

Protein synthesis by cardiac myocytes was assayed by measuring ^{14}C-phenylalanine (Phe) incorporation. After the cells were incubated with 1 μmol/L Ang II and 0.4 μCi/mL ^{14}C-Phe for 24 hours, they were fixed with 10% TCA. Radioactivity incorporated into the TCA-precipitated material was determined by liquid scintillation counting after solubilization in 1 N NaOH.

Cell Culture of Cardiac Fibroblasts

Cardiac fibroblasts isolated from neonates were cultured for 24 hours, and the serum was starved for 72 hours, after which 1 μmol/L Ang II was added for 48 hours and cell proliferation was quantified with a cell counter (Coulter).

Echocardiographic Analysis

Echocardiography was performed with an HP Sonos 100 (Hewlett-Packard Co) with a 12-MHz imaging transducer as described previously.[19,21] Intraventricular septum thickness, left ventricular diastolic diameter, and left ventricular systolic diameter were measured. Ejection fraction (EF) was calculated by the cubed method with the formula $EF=[(LVEDD)^3-(LVESD)^3]/LVEDD^3 \cdot 100$ (%).

Histological Analysis

In the heart, perivascular fibrotic area/vascular area ratios were calculated in the sections of left ventricle. The cross-sectional length of cardiac myocytes was also measured. In the kidney, glomerular sclerosis scores were assigned as follows: 0, no changes; 1, lesions involving <25% of the capillary tuft; 2, lesions involving 25% to 49% of the capillary tuft; 3, lesions involving 50% to 75% of the capillary tuft; 4, lesions involving >75% of the capillary tuft.

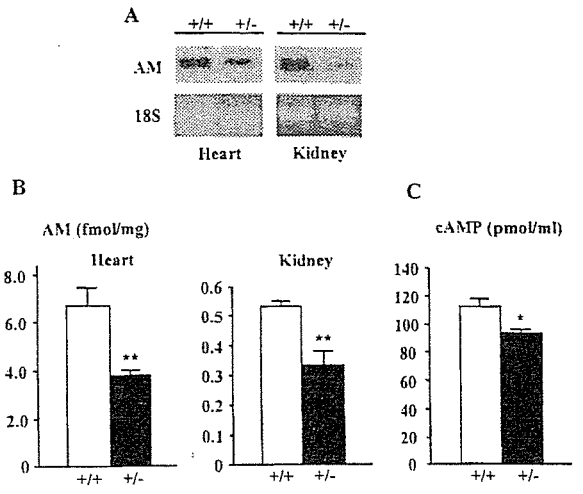

Figure 1. A, Northern blot analysis of cardiac and renal AM mRNA expression in AM knockout heterozygote (AM+/−) and wild-type (AM+/+) mice. B, Levels of AM protein in heart and kidney. C, Plasma cAMP levels. Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively. *P<0.05, **P<0.01.

Statistics

Data are expressed as mean±SEM. Differences within groups were compared with the Fisher protected least significant difference test and Student t test. Values of P<0.05 were considered significant.

Results

Characterization of AM+/−

Targeted null mutation of the *AM1* gene is lethal in utero.[18] AM+/− mice, by contrast, survived until adulthood and were apparently normal, although levels of cardiac and renal AM mRNA and protein were reduced to approximately half that seen in their wild-type (AM+/+) littermates (Figures 1A and 1B). Plasma cAMP levels were also lower in AM+/− (Figure 1C).

Cardiac Hypertrophy Induced by Pressure Overload or Continuous Ang II Infusion

The survival rate after aortic constriction was lower among AM+/− mice (Figure 2A). In particular, the death rate

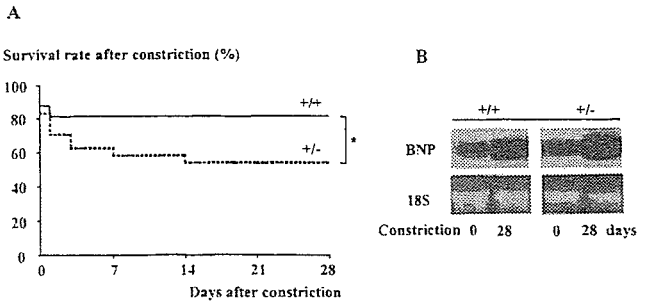

Figure 2. A, Percentages of surviving AM+/− mice (n=35) and AM+/+ mice (n=20) after aortic constriction are plotted. B, Northern blots showing cardiac expression of *BNP* mRNA in hearts after 28 days of aortic constriction. *P<0.05.

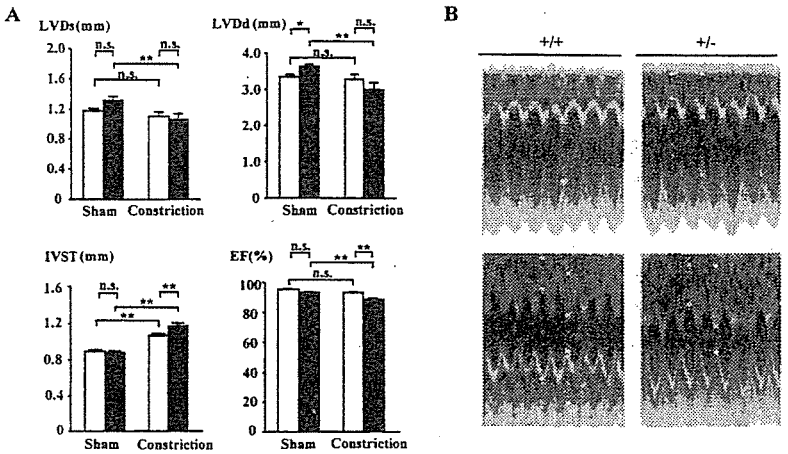

Figure 3. M-mode echocardiograms. A, Analysis of cardiac morphology and function after 28 days of aortic constriction in AM+/− (n=14) and AM+/+ mice (n=18). Fisher protected least significant difference was used for statistical analysis. IVST indicates intraventricular septum thickness (mm); LVDd, left ventricular diastolic diameter (mm); LVDs, left ventricular systolic diameter (mm); and EF, ejection fraction (%). Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively. *P<0.05; **P<0.01. B, Representative transthoracic M-mode echocardiograms after aortic constriction. Top panels show recordings from sham-operated animals, bottom panels from treated animals.

during the first week after the surgery was markedly higher among AM+/− mice (AM+/− 38% versus AM+/+ 18%, P<0.05). An additional 9% of the AM+/− mice died between weeks 2 and 4 after surgery; no wild-type mice died during this period. Accompanying the higher death rate in AM+/− mice were increased levels of *BNP* gene expression, which has been positively correlated with cardiac dysfunction[22] (Figure 2B).

Transthoracic echocardiography was performed to visualize the changes in cardiac morphology and function. After aortic constriction, the ventricular wall was thicker in AM+/− mice (intraventricular septum thickness: AM+/+ 1.067±0.02 mm versus AM+/− 1.164±0.02 mm; P<0.01; Figures 3A and 3B). Although left ventricular diastolic diameter was wider in AM+/− mice in the sham-operated group, the difference was offset by the wall thickening after aortic constriction. Ejection fraction was smaller in AM+/− mice after aortic constriction (Figure 3A). When Ang II was infused continuously for 14 days, the ventricular wall in AM+/− mice was also thickened to a greater degree than in wild-type mice (data not shown).

Histological Analysis of the Heart After Aortic Constriction and Ang II Infusion

Cardiac hypertrophy induced by aortic constriction or Ang II infusion was exacerbated in AM+/− mice (Figure 4A). In addition, cardiac myocytes from AM+/− mice showed greater cross-sectional lengths (Figure 4B). Although heart weight/body weight ratios were increased in both strains, the change was more prominent in AM+/− mice (Figure 4C).

Hydralazine treatment did not affect the cardiac hypertrophy observed in Ang II–treated AM+/− mice (Figure 4D). This suggests that the cardiac hypertrophy seen in Ang II–treated AM+/− mice is a direct effect of AM reduction in the heart rather than an indirect effect of hemodynamic changes. PAMP treatment also failed to affect the cardiac hypertrophy seen in Ang II–treated mice, which indicates that PAMP deletion did not play a significant role in the observed responses (Figure 4D).

Perivascular fibrosis was also more severe in AM+/− mice (Figure 4E). In both banded and Ang II–infused mice, perivascular fibrotic area/vascular area ratios were significantly higher in AM+/− mice (Figure 4F), which indicates that stress-induced proliferation of fibroblasts was enhanced.

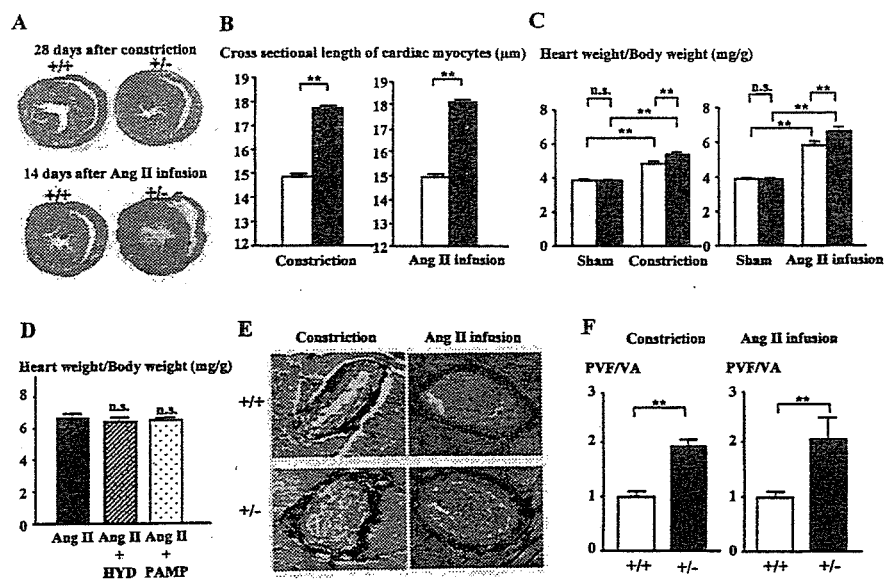

Figure 4. Histological examination of heart. A, Transverse sections of heart at papillary muscle level after 28 days of aortic constriction or 14 days of Ang II infusion. B, Cross-sectional lengths of cardiac myocytes. C, Heart weight/body weight ratios. AM+/− mice showed significantly higher ratios after aortic constriction or Ang II infusion. D, Heart weight/body weight ratios in AM+/− mice after 14 days of Ang II infusion. Both PAMP and hydralazine hydrochloride (HYD) treatment failed to reduce cardiac hypertrophy in Ang II–infused AM+/− mice. E, Accumulation of perivascular collagen (blue stained in Masson trichrome staining) in heart. F, Perivascular fibrotic area (PVF)/vascular area (VA) ratios in mice subjected to aortic constriction or Ang II infusion. B, C, and F, Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively. **P<0.01.

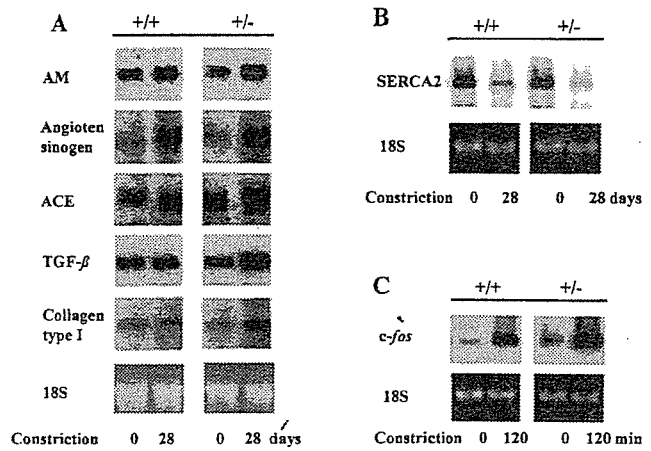

Figure 5. Northern blot analysis of cardiac gene expression. A and B, Aortic constriction for 28 days led to enhanced expression of AM, angiotensinogen, ACE, transforming growth factor (TGF)-β, and collagen type I genes (A) and diminished expression of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA)-2 gene. C, Enhanced expression of immediate-early–response gene c-*fos* induced by acute pressure overload (120 minutes).

Stress-Induced Changes in Cardiac Gene Expression

Hypertrophic responses are thought to involve programmed expression of specific sets of genes.[22] After 28 days of aortic constriction, expression of angiotensinogen, ACE, transforming growth factor-β, and collagen type I was upregulated in the heart, and the effect was more pronounced in AM+/− mice (Figure 5A). At the same time, expression of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA)-2 was downregulated to a greater degree in AM+/− mice (Figure 5B). Thus, pressure overload caused upregulation of the angiotensin system and other growth factors, which in turn led to cardiac hypertrophy and fibrosis and was associated with cardiac dysfunction in AM+/− mice.

We next analyzed the expression of c-*fos*, an early-response gene, the upregulation of which is closely associated with cardiac hypertrophy.[23] As expected, expression of c-*fos* was upregulated to a greater degree in AM+/− mice than in wild-type mice within 120 minutes (Figure 5C).

AM Suppresses Cardiac Myocyte Hypertrophy and Fibroblast Growth

When Ang II was administered to the cardiac myocytes from AM+/− mice, we observed enlargement of cardiac myocytes (Figures 6A and 6B) that was accompanied by increases in protein synthesis (Figure 6C). Ang II stimulation also enhanced proliferation of fibroblasts isolated from AM+/− mice (Figure 6D) and enhanced collagen type I gene expression (Figure 6E).

AM-Induced Changes in Extracellular Signal-Regulated Kinase Activation

The Ras-extracellular signal-regulated kinase (ERK) pathway plays a key role in the induction of early-response genes like c-*fos* during pressure overload.[24] We found ERKs to be activated within 30 minutes after the onset of aortic constriction, and the level of activation was much greater in AM+/− mice (Figure 7A). Treatment with 1 μmol/L Ang II increased ERK phosphorylation in both strains, but the effect was greater in AM+/− mice (Figure 7B). By contrast, the PKC inhibitor H7 suppressed ERK activation in both strains, and the inhibitory effect was more pronounced in AM+/− mice (Figure 7C), which suggests PKC activity is enhanced by Ang II in AM+/− mice.

Using rat cardiac myocytes, we confirmed the ability of Ang II to elicit prompt ERK activation and found the effect to be antagonized by exogenous administration of recombinant AM (Figure 7D). Moreover, the inhibitory effect of AM was attenuated by H89, an inhibitor of PKA (Figure 7D). Taken together, these findings suggest that ERK is activated in Ang II–stimulated cardiac myocytes, that AM attenuates this activation, and that the effect of AM is mediated by both activation of PKA and suppression of PKC.

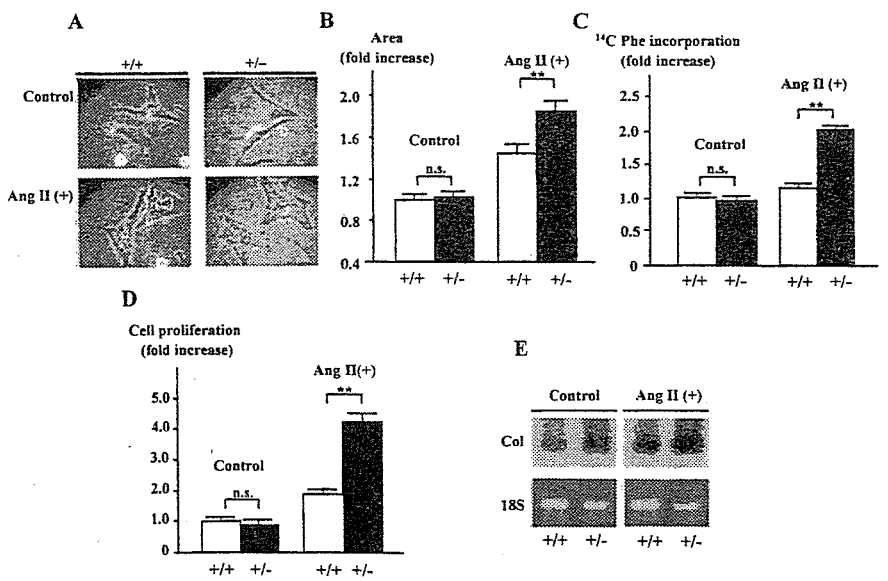

Figure 6. A, Isolated cardiac myocytes from AM+/− and wild-type mice. B, Myocyte cell size is expressed as relative surface area standardized to mean surface area in control cells. C, Protein synthesis is expressed as relative cpm/dish standardized to mean cpm in control cells. D, Proliferation of isolated cardiac fibroblasts. Proliferation of cells from AM+/− mice is expressed relative to that in control cells. E, Northern blot analysis of type I collagen gene expression in cardiac fibroblasts. Data are mean±SEM. $^{**}P<0.01$.

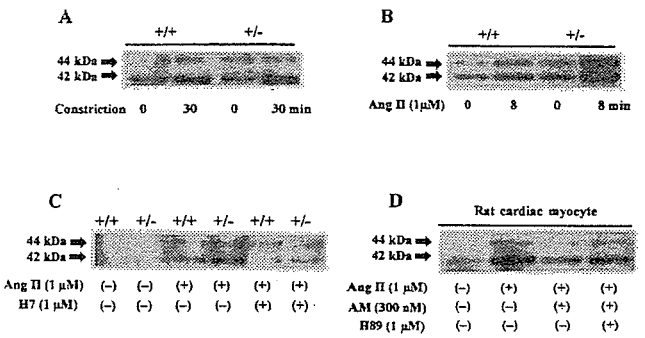

Figure 7. Activation of ERKs. Western blots showing levels of ERK phosphorylation in hearts after aortic constriction for 30 minutes (A) and in cultured cardiac myocytes exposed to 1 μmol/L Ang II for 8 minutes (B). Both aortic constriction and Ang II stimulation elicited ERK activation that was more pronounced in AM+/− mice. C, Western blot analysis of Ang II–induced ERK activation. Ang II increased ERK phosphorylation in both strains, but effect was again greater in AM+/− mice. In AM+/− myocytes, PKC inhibitor H7 (1 μmol/L) suppressed ERK phosphorylation to greater degree than in control cells. D, Western blot analysis of ERK activation in cardiac myocytes isolated from neonatal rat. Exogenous administration of AM attenuated Ang II–induced ERK activation. PKA inhibitor H89 suppressed effect of AM.

Renal Damage Induced by Continuous Ang II Infusion

After continuous infusion of Ang II, creatinine clearance was lower in AM+/− mice (Figure 8D), and morphological examination revealed much greater damage to the kidneys of AM+/− mice (Figure 8A), which had significantly higher glomerular injury scores (Figure 8B). Increased expression of TGF-β was also more prominent in AM+/− mice (Figure 8C).

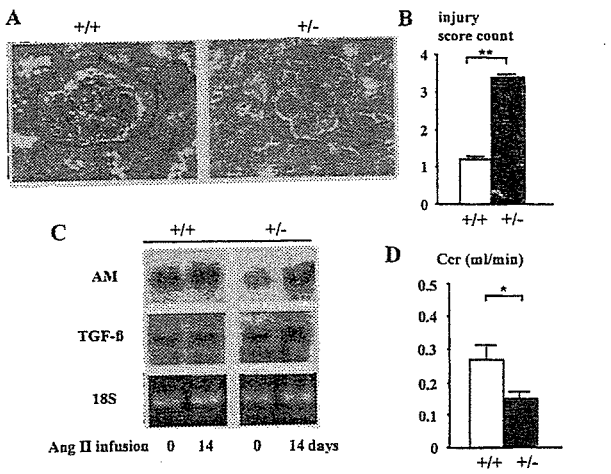

Figure 8. A, Representative histological sections containing glomeruli after Ang II treatment (Masson trichrome staining). B, Glomerular injury scores after Ang II infusion (n=9 in each). C, Renal expression of AM and transforming growth factor-β after Ang II infusion. D, Creatinine clearance (Ccr) after Ang II infusion (n=9 in each). B and D, Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively.

Discussion

Using AM knockout mice, we have been able to make 3 key observations: (1) that reduced expression of endogenous AM exacerbates the cardiac hypertrophy and fibrosis caused by pressure overload (aortic constriction) or Ang II infusion, leading to diminished cardiac function; (2) that reduced AM expression exacerbates the renal damage caused by Ang II infusion; and (3) that AM suppresses development of cardiac hypertrophy via suppression of ERK activation.

In the present study, we found that although the respective heart, kidney, and body weights were similar in AM+/− and wild-type mice, cardiac and renal expression of AM in AM+/− mice was only approximately half that seen in wild-type mice. Moreover, AM+/− mice exhibited wider left ventricular diastolic diameter than wild-type mice, even under basal conditions, which suggests that AM is necessary for proper development of the heart.

The differences between AM+/− and wild-type mice became much more apparent when stress was applied to the cardiovascular system. Compared with wild-type mice, AM+/− mice exhibited lower survival rates, which were accompanied by higher cardiac brain natriuretic peptide levels; higher heart weight/body weight ratios; greater left ventricular wall thickening; and reprogramming of specific cardiac genes. In particular, downregulation of the *SERCA2* gene, which is associated with cardiac dysfunction,[25,26] was more apparent in the hearts of AM+/− mice. Using cultured cardiac myocytes from AM+/− mice, we confirmed directly that these cells show more pronounced hypertrophic responses to stressful stimuli. AM+/− mice also exhibited more extensive perivascular fibrosis, and fibroblasts isolated from AM+/− mice showed faster proliferation and enhanced collagen type I gene expression. Taken together, these results suggest that endogenous AM serves as an autocrine or paracrine factor to exert a number of crucial protective effects when the cardiovascular system comes under stress. Consistent with our findings, Caron et al[27] recently reported hypertrophic ventricular trabeculae in AM−/− mice, whereas Shimozawa et al[28] reported that administration of Ang II with a high-salt diet produced marked perivascular fibrosis in AM+/− mice.

The findings of the present study are also suggestive of the mechanism by which AM exerts its antihypertrophic effects. Because ERKs are known to play a key role in the development of cardiac hypertrophy,[29] we examined the extent to which AM-mediated intracellular signaling affected transduction in the ERK. In the hearts of adult mice subjected to pressure overload, as well as in neonatal cardiac myocytes isolated from mice that received acute infusion of Ang II, activation of ERKs was more pronounced in AM+/− mice. AM was originally identified as a peptide that increases cAMP levels in platelets.[1] Subsequent experiments in mesangial cells yielded results similar to those reported herein[30,31]: AM increased cAMP levels, which led to a PKA-dependent decrease in cell proliferation that was associated with decreased ERK activity. It was also shown that delivery of the *AM* gene attenuates cardiac hypertrophy via production of cAMP[32,33] and that AM cAMP-dependently inhibited DNA

the present study, PKC inhibition suppressed ERK activation to a greater degree in AM+/− cardiac myocytes. Thus, AM appears to inhibit the development of cardiac hypertrophy, at least in part, by suppressing ERK activation via activation of PKA and inhibition of PKC.

In conclusion, using AM knockout mice, we showed that endogenous AM plays an important role in guarding against cardiovascular damage. We also provide evidences of the potential utility of AM in the treatment of cardiovascular and renal diseases.

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Plasma adrenomedullin concentration is increased in patients with peripheral arterial occlusive disease associated with vascular inflammation

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Abstract

Adrenomedullin (AM), a potent vasodepressor, is known to have anti-atherosclerotic and anti-inflammatory effects. However, there is no information about its level in severe atherosclerotic diseases, such as peripheral arterial occlusive disease (PAOD). The present study investigated the plasma concentration of AM and several inflammatory parameters in 72 patients with and without PAOD. The plasma AM concentration in patients with PAOD was significantly higher than in those without PAOD. Its concentration had significant correlations with ankle–brachial index and Fontaine's stage. The plasma AM level also correlated with high sensitive C-reactive protein and interleukin-6. As an additional study, plasma levels of two forms of AM drawn from the femoral artery and saphenous vein were measured in 27 other subjects. Both mature and intermediate forms of plasma AM in the femoral artery and saphenous vein were higher in patients with PAOD than in those without PAOD. A significant step-up of the mature form of AM from the femoral artery to the saphenous vein was observed. Our findings indicate that the plasma AM concentration was elevated in patients with PAOD in proportion to the severity of the disease and associated with vascular inflammation. An increased production of AM in PAOD may play a protective role against advanced atherosclerosis with an inflammatory signature.

Keywords: Atherosclerosis; Inflammation; Peptide; Vascular disease; Human

1. Introduction

Adrenomedullin (AM) is a potent vasodilator and natriuretic peptide that was originally isolated from human pheochromocytoma by monitoring an increase in cyclic adenosine monophosphate [1]. Subsequent studies have revealed that AM is widely distributed in various organs and tissues including the cardiovascular system (myocardium, vascular endothelium, and vascular smooth muscle) [2–4]. Plasma AM levels are elevated in various pathological states such as essential hypertension, acute myocardial infarction, congestive heart failure, chronic renal failure, diabetes mellitus, and sepsis [5–12]. In in vitro studies, AM production is strongly stimulated by inflammatory cytokines such as interleukin (IL), tumor necrosis factor (TNF), and lipopolysaccharide [2,13]. In contrast, several in vitro and in vivo studies demonstrate that AM has anti-inflammatory activities and suppresses inflammation-induced organ damages [14–17]. AM also appears to exert an anti-atherosclerotic effect, because our previous studies showed that AM inhibits migration and proliferation of vascular smooth muscle cells [18,19]. These findings suggest that AM may play a protective role against some kinds of inflammatory and atherosclerotic diseases.

Peripheral arterial occlusive disease (PAOD) usually occurs in male patients over 40 years old, especially in individuals with hypertension, diabetes mellitus, hyperlipidemia, and cigarette smoking [20,21]. Patients with PAOD have not only clinical discomfort, e.g., intermittent claudication and pain in their lower extremities, but also a high incidence of other atherosclerotic lesions and cardiovascular events [22]. Furthermore, it is now recognized that low-grade inflammation contributes importantly to the initiation and progression of vascular atherosclerotic lesions [23], and

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in fact elevated levels of C-reactive protein (CRP) are shown to be related to the development of PAOD [22,24]. However, AM production and its pathophysiological significance in patients with PAOD, advanced atherosclerosis with an inflammatory signature, remain to be elucidated. Thus, we conducted the present study to examine the plasma levels of AM in PAOD and evaluate the association of plasma AM with clinical severity and inflammatory parameters.

2. Methods

2.1. Study 1

A total of 72 subjects (35 with PAOD and 37 without PAOD) with atherosclerotic risk factors and/or disease who were admitted to our hospital were enrolled in this study. Diagnosis of PAOD was performed based on clinical symptoms, low ankle–brachial index, and/or findings of magnetic resonance angiography. The subjects were classified into five groups according to Fontaine's classification (stage 0: no symptom; stage 1: feeling of cold in the lower leg; stage 2: intermittent claudication; stage 3: leg pain at rest; stage 4: ulcers caused by ischemia). However, individuals with Fontaine's stage 4 were excluded from Study 1, because local inflammation of the leg strongly affected serum levels of several inflammatory parameters. Patients with congestive heart failure or chronic renal failure were also excluded from the study. Hypertension was defined as a systolic blood pressure of ≥ 140 mm Hg and/or a diastolic blood pressure of ≥ 90 mm Hg by repeated measurements or when subjects had already been treated with antihypertensive drugs. Diabetes mellitus was diagnosed according to the American Diabetes Association criteria (a fasting plasma glucose of ≥ 126 mg/dl and/or a plasma glucose level at 2 h after 75 g oral glucose load of ≥ 200 mg/dl), or when medication was taken for treatment of hyperglycemia. Diagnosis of hyperlipidemia required a serum cholesterol level ≥ 220 mg/dl and/or a serum triglyceride level ≥ 150 mg/dl or the use of lipid-lowering drugs. Blood samples were taken from the antecubital vein at rest in the supine position. Blood was immediately transferred into ice-chilled glass tubes containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) and centrifuged for 10 min at 4 °C. Plasma samples were frozen and stored at −80 °C until assayed. All subjects gave their informed consent to participate in the present study.

Table 1
Clinical characteristics of patients with and without PAOD in Study 1

	No PAOD ($n=37$)	PAOD ($n=35$)	P
Age (years)	65 ± 11	69 ± 8	NS
Male gender (%)	62	74	NS
Hypertension (%)	76	80	NS
Diabetes mellitus (%)	51	43	NS
Hyperlipidemia (%)	70	57	NS
Smokers (current or past, %)	57	77	NS
History of ischemic heart disease (%)	32	43	NS
Systolic blood pressure (mm Hg)	136 ± 22	139 ± 21	NS
Diastolic blood pressure (mm Hg)	73 ± 12	71 ± 13	NS
Fasting plasma glucose (mg/dl)	107 ± 32	103 ± 26	NS
Hemoglobin A_{1c} (%)	6.6 ± 2.2	6.1 ± 1.4	NS
Total cholesterol (mg/dl)	202 ± 34	193 ± 29	NS
Triglycerides (mg/dl)	130 ± 64	112 ± 49	NS
HDL cholesterol (mg/dl)	44 ± 13	46 ± 13	NS
Serum creatinine (mg/dl)	0.8 ± 0.3	0.9 ± 0.3	NS
Fontaine's stage	0.0 ± 0.0	1.7 ± 0.8	<0.0001
Ankle–brachial index	1.04 ± 0.10	0.62 ± 0.19	<0.0001

NS, not significant. Values are given as mean ± S.D. or percentage.

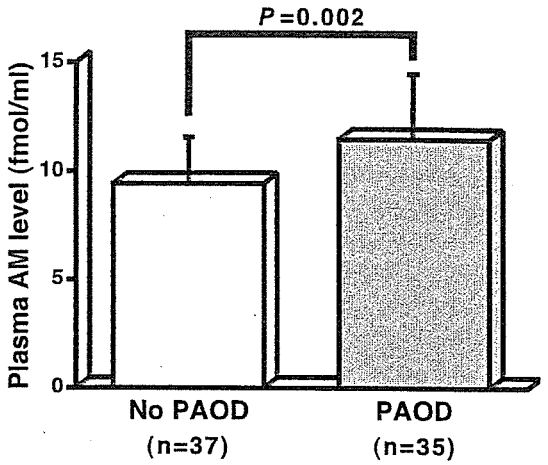

Fig. 1. Comparison of plasma AM concentrations between the two groups with and without PAOD in Study 1. Values are given as mean ± S.D.

2.2. Study 2

Twenty-seven inpatients (10 with PAOD and 17 without PAOD) were enrolled in this study. Diagnosis of PAOD and

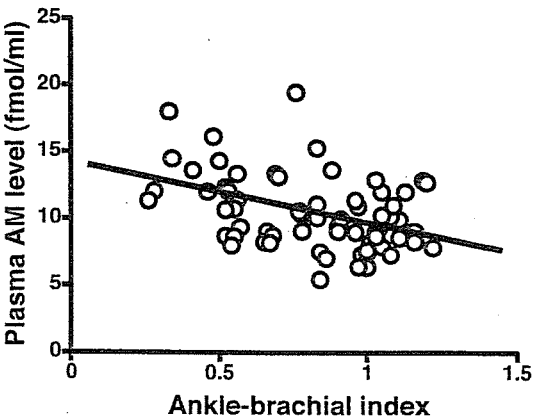

Fig. 2. Correlation between plasma AM concentration and ankle–brachial index in all subjects of Study 1 ($r=-0.39$, $P=0.001$).

Table 2
Association of plasma AM concentration with clinical parameters in Study 1

	R	P
Age	0.06	NS
Systolic blood pressure	0.07	NS
Diastolic blood pressure	0.03	NS
Fasting plasma glucose	−0.09	NS
Hemoglobin A_{1c}	−0.18	NS
Total cholesterol	0.12	NS
Triglycerides	−0.06	NS
HDL cholesterol	−0.06	NS
Serum creatinine	0.38	<0.01
Fontaine's stage	0.32	<0.01
Ankle–brachial index	−0.39	<0.01

NS, not significant.

Fontaine's classification were performed as in Study 1. Blood samples were taken from the femoral artery and the saphenous vein in respective subjects. In patients with PAOD, blood was drawn from the leg with the lower ankle–brachial index. The collected samples were dealt with using the same method in Study 1. Informed consent was obtained from the participants prior to their participation in the study.

2.3. Measurement of AM

Plasma concentration of AM (total AM) was measured by immunoradiometric assay using a specific kit (AM RIA SHIONOGI, Shionogi Pharmaceutical, Osaka, Japan), as described previously [25]. Plasma mature AM concentration was also measured using an immunoradiometric assay kit (AM mature RIA SHIONOGI, Shionogi Pharmaceutical) [26]. Plasma concentration of glycine (Gly)-extended AM, an intermediate inactive form, was calculated by subtracting the plasma level of mature AM from that of total AM.

2.4. Inflammatory and other laboratory parameters

Plasma levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were measured using commercially available enzyme-linked immunosorbent assay kits (Quantikine HS, R&D Systems, Minneapolis, MN, USA). High sensitive C-reactive protein (hs-CRP) was measured by nephelometry (SRL, Tokyo, Japan). Fasting plasma glucose, hemoglobin A_{1c}, total cholesterol, triglycerides, HDL cholesterol, and serum creatinine were determined by standard laboratory measurements.

2.5. Statistical analysis

Values were expressed as mean ± S.D. Unpaired Student's *t*-test was used for comparison between the two groups (No PAOD and PAOD). The significance of differences in plasma AM levels between the femoral artery and saphenous vein was evaluated using the paired *t*-test. Relations between plasma AM concentration and various parameters were assessed using univariate linear regression analyses and Pearson's correlation coefficient. Stepwise multiple regression analysis was applied to identify independent determinants of plasma AM. For potential indepen-

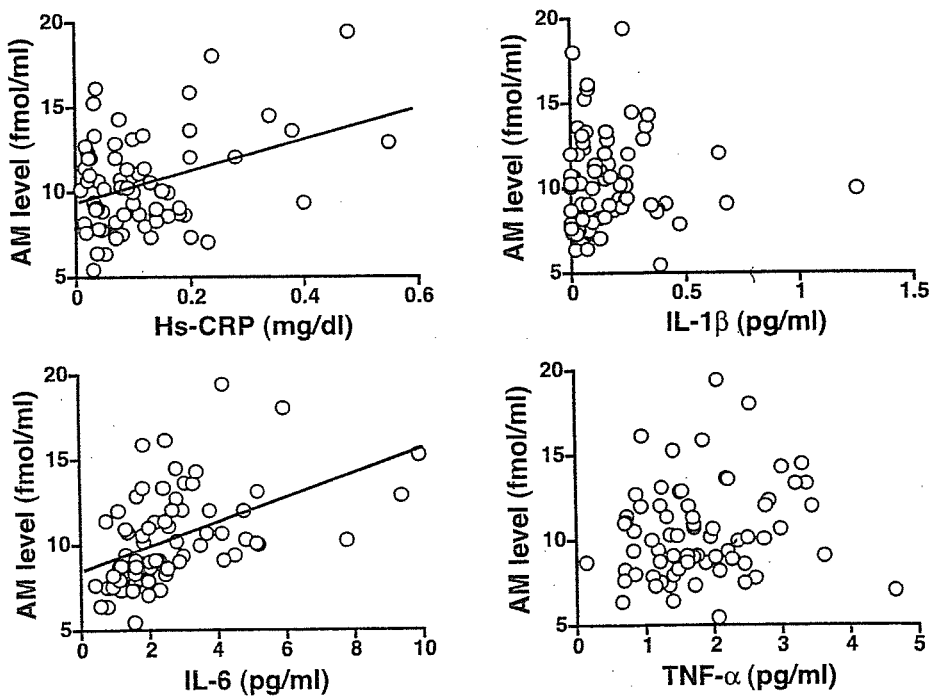

Fig. 3. Correlation between plasma AM concentration and several inflammatory parameters in all subjects of Study 1. The AM level was significantly correlated with hs-CRP ($r=0.36$, $P=0.002$) and with IL-6 ($r=0.47$, $P<0.0001$).

Table 3
Clinical characteristics of patients with and without PAOD in Study 2

	No PAOD ($n=17$)	PAOD ($n=10$)	P
Age (years)	65 ± 11	72 ± 6	NS
Male gender (%)	71	80	NS
Systolic blood pressure (mm Hg)	134 ± 22	143 ± 25	NS
Diastolic blood pressure (mm Hg)	75 ± 12	69 ± 14	NS
Fasting plasma glucose (mg/dl)	107 ± 32	103 ± 26	NS
Hemoglobin A_{1c} (%)	6.0 ± 1.4	6.6 ± 1.2	NS
Total cholesterol (mg/dl)	182 ± 32	172 ± 18	NS
Triglycerides (mg/dl)	99 ± 48	109 ± 53	NS
HDL cholesterol (mg/dl)	47 ± 16	38 ± 11	NS
Serum creatinine (mg/dl)	1.0 ± 1.2	1.1 ± 0.6	NS
Fontaine's stage	0.0 ± 0.0	2.2 ± 1.0	<0.0001
Ankle–brachial index	1.14 ± 0.09	0.64 ± 0.19	<0.0001

NS, not significant. Values are given as mean ± S.D. or percentage.

dent variables, ankle–brachial index, Fontaine's stage, serum creatinine, hs-CRP, and IL-6 were analyzed. A value of $P<0.05$ was accepted as statistically significant.

3. Results

3.1. Study 1

There were no significant differences in clinical characteristics including blood pressure, glucose and lipid levels, and renal function between patients with and without PAOD except significant findings of PAOD itself (Table 1). Therefore, the two groups had the same risk factors for atherosclerosis. The plasma concentration of AM in patients with PAOD (11.4 ± 3.0 fmol/ml) was significantly higher than in those without PAOD (9.4 ± 2.1 fmol/ml) (Fig. 1).

The association of plasma AM levels with clinical parameters was examined in all subjects ($n=72$). The AM concentration was negatively correlated with ankle–brachial index (Fig. 2) and positively with Fontaine's stage (Table 2). Its concentration showed a positive correlation with serum creatinine, but had no significant correlation with the other clinical parameters.

Next, we examined the relation between plasma AM concentration and several inflammatory parameters in all 72 subjects. As shown in Fig. 3, the plasma AM level was significantly correlated with hs-CRP ($r=0.36$, $P<0.01$) and with IL-6 ($r=0.47$, $P<0.0001$), but not with IL-1β or TNF-α. To investigate the independent relations of clinical and inflammatory parameters with AM levels, stepwise multiple regression analysis was performed. As a result, ankle–brachial index ($\beta=-0.32$) and IL-6 ($\beta=0.45$) were independent determinants of plasma AM concentration in the overall subject group ($r^2=0.31$, $P<0.0001$).

3.2. Study 2

There were no significant differences in age, gender, blood pressure, glucose and lipid levels, and renal function between the two groups (Table 3). In the femoral artery, both plasma levels of mature AM and immature AM (Gly-extended AM) were significantly higher in patients with PAOD than in those without PAOD (Table 4). In the saphenous vein, similarly, both mature and immature AM levels were higher in patients with PAOD. The plasma concentration of mature AM, but not immature AM, was significantly increased in the saphenous vein than in the femoral artery in each group. Therefore, a significant step-up of the mature active form of AM from the femoral artery to the saphenous vein was found. However, there was no significant difference in the extent of the step-up of mature AM between the two groups.

4. Discussion

The present study demonstrated that the plasma AM concentration was increased in patients with PAOD, and the increase was related to the clinical severity of the disease. Several studies have shown that plasma AM levels were elevated in various cardiovascular diseases, e.g., hypertension, chronic heart failure, acute myocardial infarction, and shock [5–9,12]. However, little has been elucidated about the relation between plasma AM and atherosclerotic lesions. Recently, Shinomiya et al. [27] showed in patients with chronic ischemic stroke that an increased plasma AM level was associated with carotid atherosclerosis, independent of the blood pressure level. Our present study also showed that the plasma concentration of AM in patients with PAOD was significantly higher than in those with similar atherosclerotic risks including hypertension but without PAOD. These

Table 4
Plasma levels of two forms of AM in FA and SV in Study 2 patients

	No PAOD ($n=17$)			PAOD ($n=10$)		
	FA	SV	Δ(SV − FA)	FA	SV	Δ(SV − FA)
Mature AM (fmol/ml)	1.4 ± 0.3	1.9 ± 0.5†	0.5 ± 0.4	1.7 ± 0.4*	2.3 ± 0.7*†	0.6 ± 0.5
Gly-extended AM (fmol/ml)	12.2 ± 2.1	12.2 ± 1.8	0.0 ± 1.4	15.8 ± 6.6*	16.2 ± 6.2*	0.3 ± 1.6

FA, femoral artery; SV, saphenous vein. Values are given as mean ± S.D.
* $P<0.05$ vs. No PAOD.
† $P<0.01$ vs. FA of the same group.

findings clearly indicate that atherosclerosis itself, apart from hemodynamic factors, elevates plasma AM levels in cardiovascular diseases.

Currently, it is well recognized that vascular inflammation plays an important role in the development of atherosclerosis [23]. In fact, CRP has been shown to be one of the independent predictors for the progression of atherosclerotic lesions in several cardiovascular diseases including PAOD [22–24]. Since the plasma AM level in this study had a significant positive correlation with hs-CRP, the increased level of plasma AM in patients with PAOD may have reflected inflammatory response linked to advanced atherosclerosis.

Previous studies have shown that inflammation is a powerful inductive factor of AM production. Patients with sepsis, a severe systemic inflammatory disorder, have very high levels of plasma AM [11,12]. Ueda et al. [12] reported that the plasma AM concentration in patients with septic shock was significantly higher than in those with other severe diseases (e.g. traumatic shock and burns). Many kinds of cytokines are released in inflammatory conditions and inflammation-related cytokines such as IL, TNF, and interferon have been shown to stimulate AM production and secretion from vascular and cardiac cells [2,13,28]. In a clinical study, Fujioka [29] showed that an increased plasma concentration of AM after major surgery was associated with an increase in serum IL-6 level. The plasma AM level in our study subjects was also well correlated with IL-6. Taken together, production and secretion of AM in atherosclerotic diseases, at least in part, are probably up-regulated by several cytokines based on pathological conditions with an inflammatory signature.

In contrast to the stimulation of AM production by inflammatory cytokines, AM has been reported to suppress the secretion of IL-6, TNF-α, and cytokine-induced neutrophil chemoattractant, a member of IL-8 family, from macrophages [14,15]. AM also inhibits migration and proliferation of vascular smooth muscle cells induced by platelet-derived growth factor [18,19]. Chun et al. [30] reported that oxidative stress augmented endothelial secretion of AM and that this peptide played a compensative role for endothelial dysfunction. These findings suggest that AM has an anti-atherosclerotic effect through such mechanisms as anti-inflammatory, anti-chemotactic, and anti-proliferative actions on vascular wall cells. Therefore, it is possible that plasma AM levels were elevated compensatively in patients with PAOD to exert a self-protective effect in atherosclerotic vascular lesions.

In the present study (Study 2), we showed that the plasma concentration of mature AM in the saphenous vein was significantly higher than in the femoral artery. Therefore, a significant step-up of the mature active form of AM from the femoral artery to the saphenous vein was observed. In contrast, there was no significant step-up of the Gly-extended AM level, an immature and inactive form. These observations are consistent with the previous findings shown by Hirayama et al. [31] that mature AM, but not immature AM, was released from the vasculature of the lower extremities. In the present study, there was no significant difference in the extent of the step-up of mature AM from the femoral artery to the saphenous vein between the two groups with and without PAOD, although the respective plasma levels of mature AM in the femoral artery and saphenous vein were significantly higher in patients with PAOD than in those without the disease. This result suggests that the increased plasma AM in PAOD might result from not local but systemic vascular reaction, because patients with PAOD usually have systemic advanced atherosclerosis.

In conclusion, the plasma AM concentration was elevated in patients with PAOD in proportion to the severity of the disease. In addition, the plasma level of this peptide was correlated with hs-CRP, one of the most reliable markers of inflammation, and with IL-6, an inflammatory cytokine. On the basis of these results and the findings that AM has anti-inflammatory and anti-atherosclerotic effects, an increased production of AM in PAOD may function protectively against advanced atherosclerosis with an inflammatory signature.

Acknowledgements

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ORIGINAL ARTICLE

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Adrenomedullin Contributes to Vascular Hyporeactivity in Cirrhotic Rats with Ascites Via a Release of Nitric Oxide

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Kojima H, Sakurai S, Uemura M, Satoh H, Nakashima T, Minamino N, Kangawa K, Matsuo H, Fukui H. Adrenomedullin contributes to vascular hyporeactivity in cirrhotic rats with ascites via a release of nitric oxide. Scand J Gastroenterol 2004;39:686–693.

Background: Plasma levels of adrenomedullin, a potent vasodilator peptide, are increased in cirrhotic patients, whereas its role in vascular hyporeactivity in cirrhosis has not been clarified. **Methods:** Adrenomedullin expression was evaluated by radioimmunoassay and reverse-transcription polymerase chain reaction. Vascular reactivity to phenylephrine, α_1-adrenoceptor agonist, was investigated in the aortic rings from control rats and CCl_4-induced cirrhotic rats with ascites in the presence of the neutralizing antibody against adrenomedullin, human adrenomedullin and/or N^G-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor. **Results:** Plasma adrenomedullin levels were significantly higher in cirrhotic rats than in controls (16.3 ± 2.9 versus 7.4 ± 1.7 fmol/mL, $P < 0.05$) and correlated negatively with systemic arterial pressure ($r = -0.62$, $P < 0.05$). Gene expression of adrenomedullin in various organs (liver, kidney, lung) and vessels (portal vein, aorta) was enhanced in cirrhotic rats compared with controls. Neutralizing antibody against adrenomedullin ameliorated the blunted contractile response to phenylephrine in cirrhotic aorta (Rmax: 1.5 ± 0.1 versus 1.0 ± 0.1 g/mg tissue, $P < 0.05$), whereas contraction remained unchanged in control aorta (Rmax: 1.9 ± 0.2 versus 1.9 ± 0.2 g/mg tissue). Intravenous infusion of human adrenomedullin induced a reduction of mean arterial pressure together with an increase of serum nitrate levels, which was abolished by neutralizing antibody against adrenomedullin. Human adrenomedullin caused a blunted contractile response to phenylephrine in both control and cirrhotic aortas, which was not observed in the presence of N^G-nitro-L-arginine methyl ester. **Conclusions:** These findings indicate that the overproduction of adrenomedullin may contribute to vascular hyporeactivity in cirrhosis via a release of nitric oxide.

Key words: Adrenomedullin; liver cirrhosis; nitric oxide; vascular hyporeactivity

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Peripheral arterial vasodilatation is the most outstanding hemodynamic alteration in human and experimental liver cirrhosis and occurs despite the stimulation of endogenous vasoconstrictive systems such as the renin-angiotensin-aldosterone system, the sympathetic nervous system, and antidiuretic hormone (1–3). In addition to peripheral arterial vasodilatation, the tonus of larger arteries is also changed in patients with cirrhosis, as shown as an increase of the arterial compliance which is assessed as stroke volume relative to pulse pressure (4, 5) There is increasing evidence that arterial vasodilatation contributes to the formation of ascites and portal hypertension through the hyperdynamic circulation characterized by arterial hypotension, reduced peripheral vascular resistance, and high cardiac output (1–3, 6, 7). However, pharmacological treatment of these complications is limited by an apparent hyporeactivity to vasoconstrictors in liver cirrhosis (6–10). The mechanisms responsible for this vascular hyporeactivity are important in understanding the pathogenesis of the complications of cirrhosis and in development of better pharmacological interventions. Although several mechanisms including the increased levels of endogenous vasodilators, receptor down-regulation, and post-receptor signaling defects in the vascular smooth muscle cells have been suggested for the vascular hyporeactivity in cirrhosis, the precise mechanism is controversial and has not yet been fully clarified (8–12).

Adrenomedullin (AM) is a hypotensive peptide found in human pheochromocytoma and causes a potent vasodilatation via release of nitric oxide (NO) from vascular endothelial cells together with an increase in intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) in vascular smooth muscle cells (13–16). This peptide is overproduced through stimulation by various agents including endotoxin, cytokines, vasoactive substances, and/or shear stress (17–19) which are enhanced in liver cirrhosis (20, 21). In several studies it has been demonstrated that plasma AM levels are increased

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together with the progression of cirrhosis (22–25). However, the origin of increased plasma AM in cirrhosis has not been fully clarified and whether AM contributes to the vascular hyporeactivity in cirrhosis remains to be established. In this study, we investigated the expression of AM in various organs and vessels of control and cirrhotic rats. Moreover, the reactivity of the thoracic aorta of control rats and cirrhotic animals with ascites to phenylephrine (α_1-adrenoceptor agonist) was evaluated using an exogenous AM and/or neutralizing antibody against AM. The aim of this study was to assess whether the vascular hyporeactivity to α_1-adrenoceptor agonist in liver cirrhosis is related to the increased AM production.

Materials and Methods

Chemicals

Human AM and neutralizing monoclonal antibody against AM were supplied by Diagnostic Science Division, Shionogi & Co., Ltd. (Settsu, Japan). This antibody belonged to the immunogloblin G_1 subclass and equally cross-reacted with rat AM (1–50), but not with calcitonin gene-related peptide (CGRP) or amylin (26). KCl, acethylcholine, N^G-nitro-L-arginine methyl ester (L-NAME), indomethacin and phenylephrine were purchased from Sigma Chemical (St. Louis, Mo., USA).

Animal preparation

The investigation was performed in male Sprague-Dawley rats. Liver cirrhosis was induced by weekly intragastric administration of carbon tetrachloride and phenobarbital (35 mg/dL) in the drinking water (27). Control rats were treated with phenobarbital alone. All animals received humane care and all experiments were performed in accordance with the criteria of the Committee for the Care and Use of Laboratory Animals in Nara Medical University.

Analytical procedures

Plasma and tissue concentrations of AM were measured in control rats and cirrhotic animals with or without ascites ($n = 8$, respectively). Blood samples were collected in chilled tubes containing EDTA-2Na (1 mg/mL) and aprotinin (500 KIU/mL), and centrifuged at 4 °C. The liver, kidney and lung were homogenized for 1 min in 10 volumes of 1 M acetic acid and immediately heated at 100 °C for 10 min. The homogenates were centrifuged at 15,000 *g* for 10 min at 4 °C. Plasma and supernatants were frozen until analyzed. AM level was measured by radioimmunoassay (28). Protein concentrations were determined by the Bradford method (29).

Gene expression of AM

Gene expression of AM was evaluated by semiquantitative reverse-transcription polymerase chain reaction (30). Total RNA was extracted from the pool of various organs and vessels of the animals used for the measurement of AM concentrations with RNA zol B (TEL-TEST, Inc., Friendswood, Tex., USA). Chloroform extraction, isopropanol precipitation and 75% ethanol washing were subsequently performed according to the manufacturer's instructions. Total RNA (3 μg) was primed with 0.5 μg oligo-dT_{12-18} primer and converted to cDNA by murine reverse transcriptase. Aliquots (3 μL) of synthesized cDNA were added to 45 μL of 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 μM dGTP, 220 μM dATP, 220 μM dTTP, 220 μM dCTP and 22 U/mL recombinant Taq DNA polymerase (PCR Supermix, Life Technologies, Tokyo, Japan) with 10 pmol specific primers (31). Their sequences were 5′-GAAGCTGGTTTC-CATCGCCC- 3′ (sense 3–22), 5′-TGCCACCCGCACCTA-TAACC- 3′ (antisense 551–570). Samples were amplified by 35 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min and elongation at 72 °C for 1 min. The final incubation was performed at 72 °C for 7 min. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard.

Isolated aortic ring studies

On the day of the experiment, thoracic aorta was removed from the control rats and cirrhotic animals with ascites and cut into 3-mm rings. The rings were suspended between two triangular-shaped stainless steel stirrups in a 20-mL jacketed organ chamber containing modified Krebs-Henseleit solution (118 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 11.1 mM glucose, 27.2 mM $NaHCO_3$, 0.03 mM Na_2 ethylenediaminetetraacetic acid, 1.8 mM $CaCl_2$) at 37 °C and bubbled with 95% O_2 and 5% CO_2. The lower stirrup was anchored and the upper stirrup was attached to a force-displacement transducer (TB-652T; Nihon Kohden, Tokyo, Japan) to record the isometric force. All aortic rings were stretched to generate a resting tension of 2 g, which was optimal for contractions with phenylephrine, α-adrenergic receptor agonist, in aortic rings. After 1 h of equilibration, KCl (final concentration 30 mM) was added to the tissue bath to confirm tissue viability. The presence of functional endothelium was determined by the addition of acethylcholine (10 μM). All rings were rinsed and allowed to equilibrate for one more hour in the presence of indomethacin (10 μM) to prevent the influence of endogenous prostanoids. The aortic rings of control and cirrhotic rats were incubated with vehicle, human AM (100 nM) or the neutralizing antibody against AM (1 mg/L) ($n = 6$, respectively). Another control and cirrhotic aortas were incubated with L-NAME (30 μM) with or without human AM (100 nM) ($n = 6$, respectively). The cumulative dose–response curves to phenylephrine (1 nM to 10 μM) were then investigated. On completion, the aortic rings were dried and weighed. The force of contraction was expressed as gram of contraction per milligram of dried tissue. The following parameters were used: the maximal contraction to phenylephrine (Rmax) to compare the contractility and the logarithm of the effective molar concentration of phenylephrine causing 50% of maximal contraction (EC_{50}) to compare

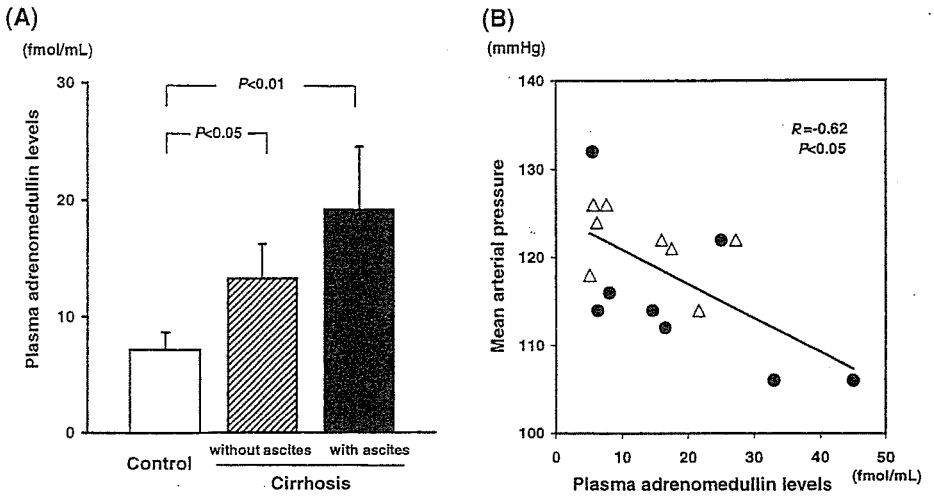

Fig. 1. Plasma adrenomedullin levels in control and cirrhotic rats (A) and its relation to mean arterial pressure (B). Plasma adrenomedullin levels were higher in cirrhotic rats compared with controls and were highest in cirrhotic animals with ascites. In cirrhotic rats, plasma adrenomedullin levels correlated negatively with mean arterial pressure. Δ: cirrhotic rats without ascites; ● : cirrhotic rats with ascites. Values are presented as mean ± $s_{\bar{x}}$ (standard error of the mean) of 8 samples.

the reactivity. These parameters were calculated with a non-linear regression method using computerized curve-fitting software (StatView, Abacus Concept Inc., Berkeley, Calif., USA).

Hemodynamic studies

Hemodynamic studies were performed after the animals had been anesthetized with ketamine (100 mg/kg i.m.), which most closely approximated the conscious state in terms of hemodynamics (32). The left femoral artery and femoral vein were cannulated with PE-50 catheters and were used for the measurement of systemic arterial pressure and drug infusion, respectively. 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 with neutralizing antibody (500 μg/kg) and were thereafter infused with human AM (0.3 nmol kg^{-1} min^{-1} for 10 min), because this dose of AM reduced systemic pressure to the same level as that in cirrhotic rats (data not shown). Mean arterial pressure was continu-

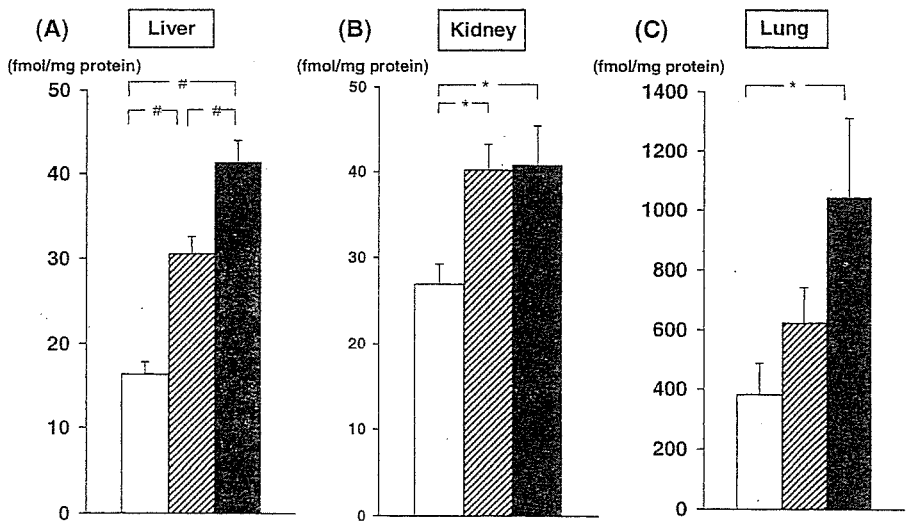

Fig. 2. Tissue concentrations of adrenomedullin in the liver (A), kidney (B) and lung (C) from control rats (□) and cirrhotic animals without (▨) or with ascites (■). Tissue concentrations of adrenomedullin widely varied from organ to organ and were highest in the lung. In every organ, cirrhotic rats with ascites showed higher adrenomedullin concentrations compared with controls. Cirrhotic rats without ascites showed higher hepatic and renal adrenomedullin levels compared with controls, as well. Values are presented as mean ± $s_{\bar{x}}$ (standard error of the mean) of 8 samples. $^{\#}P < 0.01$; $^{*}P < 0.05$.

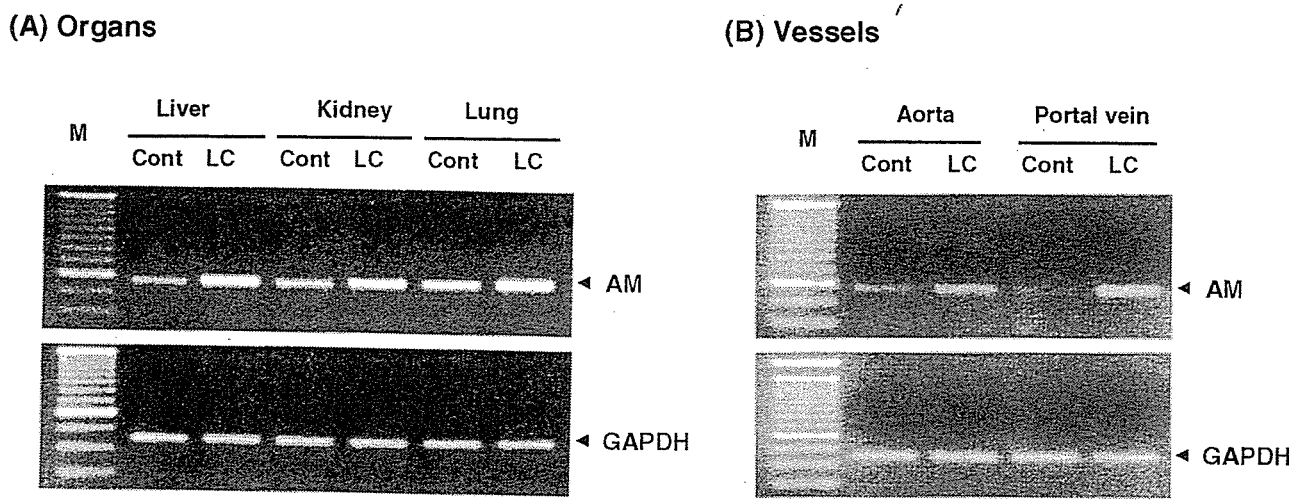

Fig. 3. Gene expression of adrenomedullin (AM) in various organs (A) and vessels (B) from control rats and cirrhotic animals with ascites. Total RNA was extracted from the pool of various organs and vessels from the same animals as the measurement of AM concentrations. Gene expression was analyzed using the reverse-transcription polymerase chain reaction method. Upper panel: adrenomodullin, Lower panel: glyceraldehyde-3-phosphate dehydrogenase as an internal control. Cont = control rats; LC = cirrhotic rats with ascites.

ously monitored. Serum levels of stable metabolite of NO, NOx ($NO_2^- + NO_3^-$), were determined before and after the infusion according to an established method (22).

Statistics

Data were shown as the mean $\pm s_{\bar{x}}$ (standard error of the mean). Statistical comparisons were performed using appropriate methods (one-way analysis of variance (ANOVA) followed by the Scheffe F test for Figs. 1A, 2 and 5B; simple regression analysis for Fig. 1B; one-way ANOVA and the unpaired Student's t test for Figs. 4, 5A and 6). Results were considered statistically significant at $P < 0.05$.

Results

Plasma and tissue concentrations of AM

Plasma AM levels in the cirrhotic rats were higher compared with those in controls (16.3 ± 2.9 versus 7.4 ± 1.7

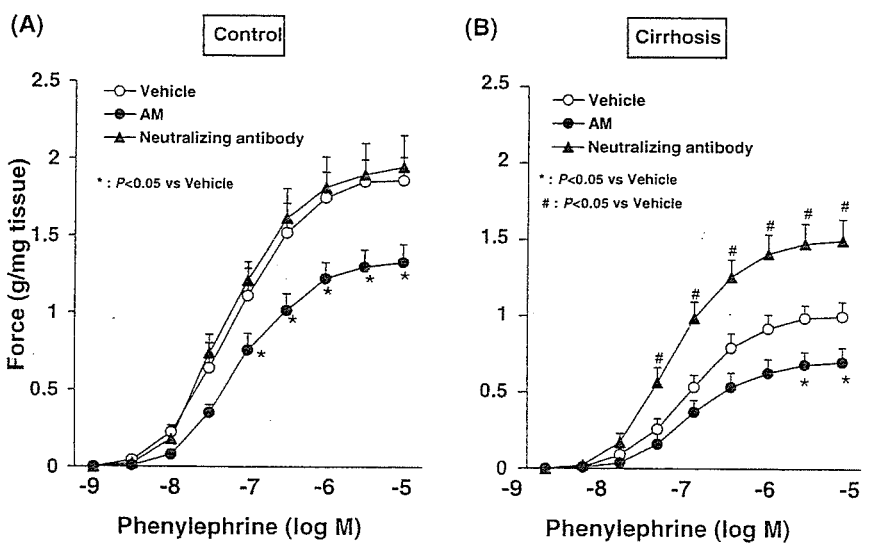

Fig. 4. Effects of adrenomedullin (AM) and neutralizing antibody against AM in phenylephrine-induced contraction of aortic rings. The contraction of thoracic aortas to phenylephrine, α_1-adrenoceptor agonist, was evaluated in the presence of vehicle, human AM or neutralizing antibody against AM. The contractile response to phenylephrine was blunted in cirrhotic aortas compared with control aortas. AM treatment blunted the contractile response in both control and cirrhotic aortas. Neutralizing antibody ameliorated the blunted contractile response in cirrhotic aorta, whereas contraction remained unchanged in control aorta. Values are presented as mean $\pm s_{\bar{x}}$ (standard error of the mean) of 6 aortic rings.