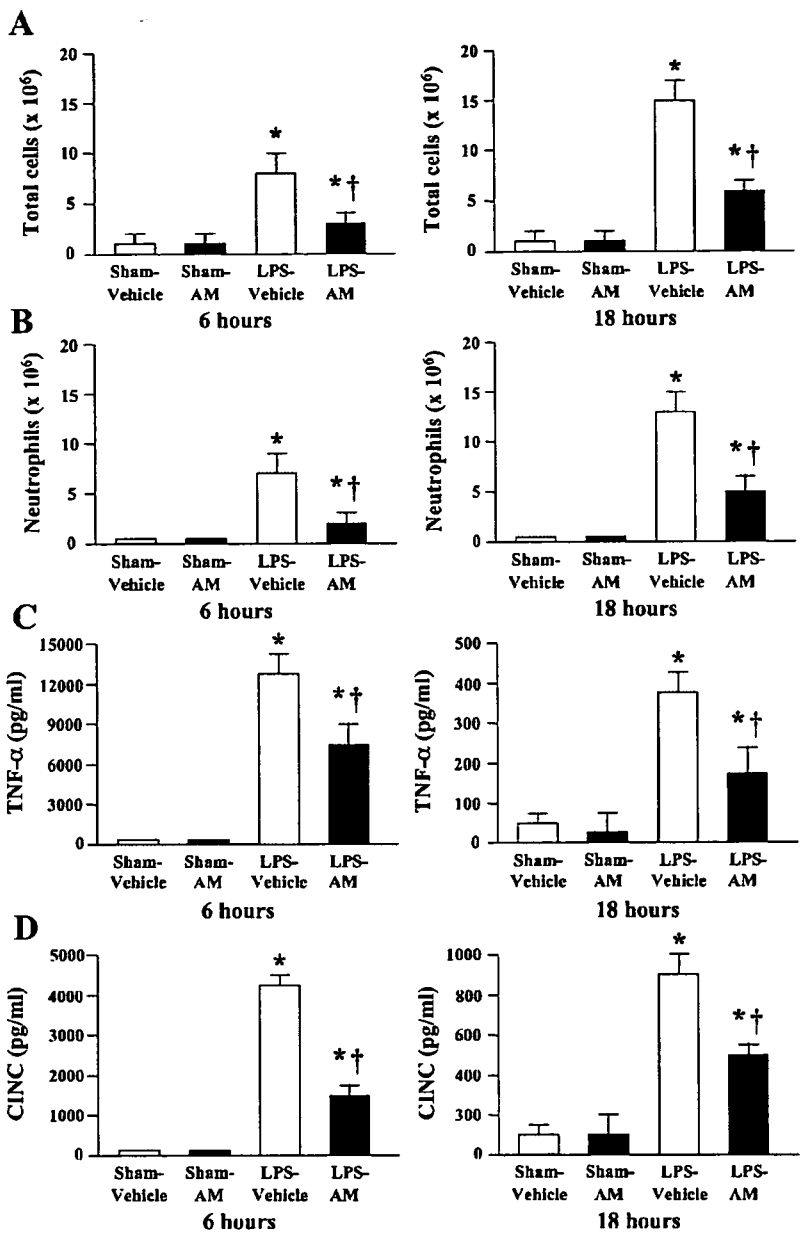

Fig. 4. Effects of AM infusion on numbers of total cells (*A*) and neutrophils (*B*) and levels of tumor necrosis factor (TNF)-α (C) and cytokine-induced neutrophil chemoattractant (CINC; D) in BAL fluid (BALF) at 6 and 18 h after LPS instillation. Numbers of total cells and neutrophils were significantly increased at 6 and 18 h after LPS instillation. However, numbers of these cells in LPS-AM group were significantly lower than those in LPS-Vehicle group. AM infusion significantly decreased BALF TNF-α and CINC levels. Data are means ± SE. *$P < 0.05$ vs. Sham-Vehicle; †$P < 0.05$ vs. LPS-Vehicle.

DISCUSSION

In the present study, we demonstrated that AM infusion *1*) ameliorated LPS-induced histological changes and attenuated the increase in lung weight after LPS instillation, *2*) decreased the numbers of total cells and neutrophils and the levels of TNF-α and CINC in BALF, *3*) reduced the levels of total protein and albumin in BALF, and *4*) inhibited apoptosis of alveolar wall cells.

In the present study, intratracheal instillation of LPS was used to produce a model of ARDS in rats. Acute lung injury was histologically confirmed in rats subjected to LPS instillation. LPS instillation also increased the lung wet/dry weight ratio, an index of acute lung injury. AM infusion significantly attenuated these abnormalities, suggesting that AM ameliorates LPS-induced acute lung injury in rats. We also demonstrated that the circulating level of AM was significantly increased after intratracheal instillation of LPS, which is consistent with previous observations that AM expression is increased in animals and humans with acute lung injury (1, 46). In the present study, AM infusion caused a significant additional increase in the circulating level of AM in rats subjected to LPS instillation. Thus supplementation of AM may produce beneficial actions at pharmacological levels. However, the underlying mechanisms still remain unclear. Considering the variety of protective effects of AM, the present study investigated the effects of AM on lung inflammation, permeability, and cell apoptosis, all of which are responsible for acute lung injury.

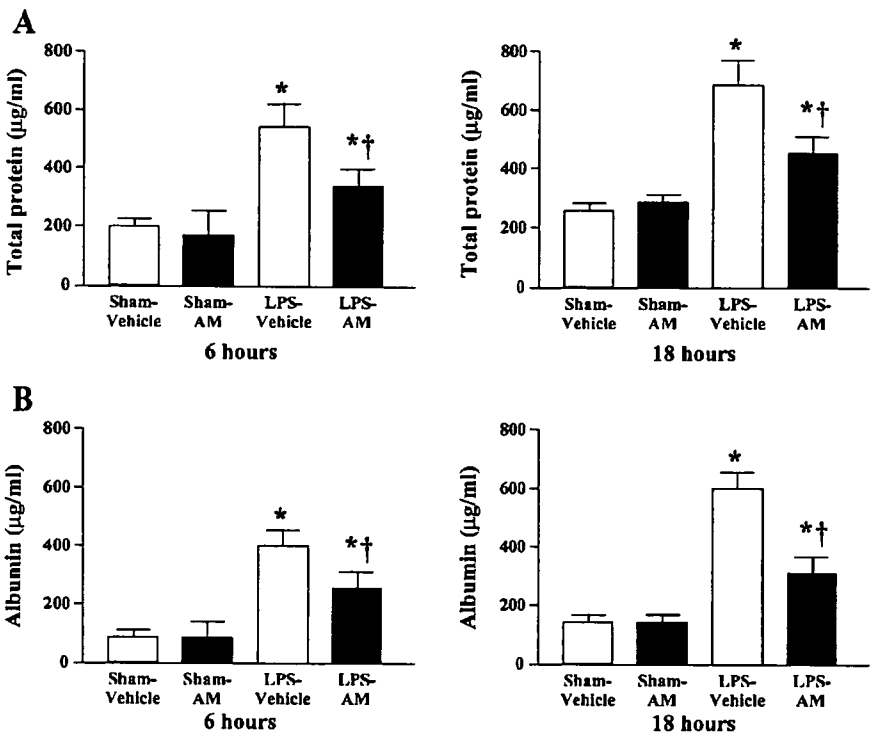

Fig. 5. Effects of adrenomedullin (AM) infusion on BALF total protein (*A*) and albumin (*B*) at 6 and 18 h after LPS instillation. AM infusion significantly reduced BALF total protein and albumin levels. Data are means ± SE. $*P < 0.05$ vs. Sham-Vehicle; $\dagger P < 0.05$ vs. LPS-Vehicle.

LPS is known to induce severe lung inflammation through the migration and activation of inflammatory cells. In particular, neutrophils are considered to be responsible (54). The present study also showed that LPS instillation markedly increased the number of neutrophils in BALF. However, AM infusion significantly attenuated the increase in neutrophils. These findings suggest that AM infusion ameliorates LPS-induced lung inflammation at least in part through inhibition of neutrophil infiltration. Several investigations have identified that several cytokines play pivotal roles in the initiation and development of inflammation (44, 47, 57). LPS has been reported to induce the production of several cytokines in vivo

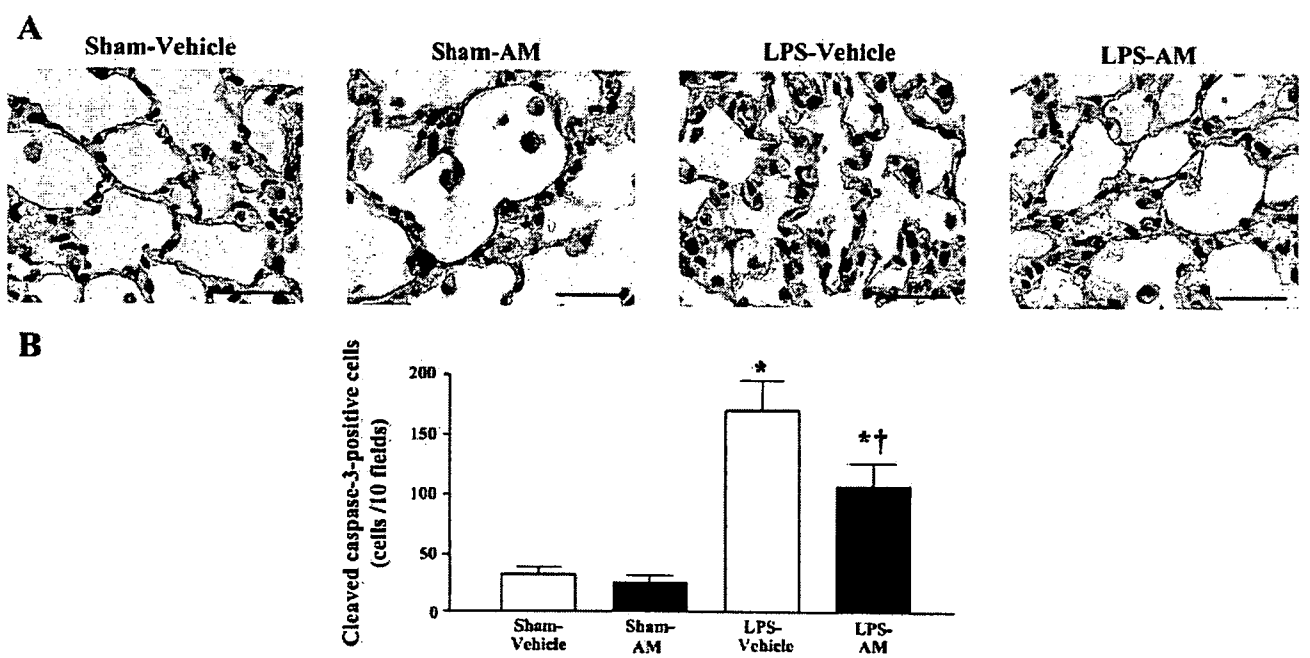

Fig. 6. *A*: immunohistochemical demonstration of cleaved caspase-3 antigen, a marker for cell apoptosis, in lungs at 6 h after LPS instillation. Scale bars, 20 μm. *B*: semiquantitative analysis of cleaved caspase-3-positive alveolar wall cells. Number of cleaved caspase-3-positive alveolar wall cells was significantly decreased in LPS-AM group compared with LPS-Vehicle group. Data are means ± SE. $*P < 0.05$ vs. Sham-Vehicle; $\dagger P < 0.05$ vs. LPS-Vehicle.

and in vitro (3, 8). In fact, in the present study, BALF TNF-α and CINC levels were markedly increased in rats with LPS instillation. TNF-α, a proinflammatory cytokine, participates in several important processes involved in the inflammatory response (23, 48–50, 55). On the other hand, CINC, a member of the CXC chemokine family, plays a pivotal role in neutrophil migration in rats (47, 56). These findings suggest that these cytokines are potentially important mediators of LPS-induced lung inflammation. It should be noted that AM infusion significantly decreased BALF TNF-α and CINC levels. Our results may be supported by earlier in vitro findings that AM reduces LPS-stimulated secretion of TNF-α and CINC from macrophages (17, 58). These findings suggest that AM infusion suppresses LPS-induced lung inflammation through inhibition of cytokine production.

Lung hyperpermeability is also involved in the pathogenesis of ARDS (4, 36). Intratracheal instillation of LPS has been shown to injure pulmonary endothelial and epithelial cell layers and to increase lung permeability, resulting in pulmonary edema (25). Recent studies have shown that a reduction of lung hyperpermeability protects against LPS-induced acute lung injury (35, 43). In the present study, LPS instillation significantly increased BALF total protein and albumin levels, markers for lung permeability. AM infusion attenuated the LPS-induced increases in BALF total protein and albumin levels. Recently, AM has been shown to reduce endothelial hyperpermeability through a cyclic adenosine 3′,5′-monophosphate-dependent mechanism in perfused rabbit lungs (11). Thus the therapeutic effects of AM on acute lung injury may be mediated by a reduction of lung hyperpermeability.

Apoptosis of several cell types, including neutrophils, alveolar epithelial cells, and endothelial cells, is involved in the pathogenesis of acute lung injury in ARDS (9, 24, 26). In fact, in the present study, LPS instillation significantly increased the number of apoptotic alveolar wall cells. AM infusion attenuated the LPS-induced increase in the number of apoptotic alveolar wall cells, although it did not affect inflammatory cell apoptosis. Cell apoptosis and survival in the alveolar wall play an important role in the maintenance of lung homeostasis. Several studies have shown that apoptosis inhibitors attenuate LPS-induced acute lung injury in animals (21, 52). Inhibition of alveolar wall cell apoptosis has been shown to be associated with the attenuation of LPS-induced acute lung injury (21, 30). In addition, AM has been shown to protect against apoptosis in vivo and in vitro (15, 19, 34, 39, 45). These findings suggest that AM infusion ameliorates LPS-induced acute lung injury at least in part through inhibition of alveolar wall cell apoptosis. Further studies are necessary to clarify whether the hemodynamic effect of AM influences LPS-induced acute lung injury.

In conclusion, continuous infusion of AM ameliorated LPS-induced acute lung injury in rats. This beneficial effect of AM may be mediated by inhibition of inflammation, hyperpermeability, and alveolar wall cell apoptosis.

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Original Article

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Midregional proadrenomedullin reflects cardiac dysfunction in haemodialysis patients with cardiovascular disease

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Abstract

Background. Although adrenomedullin is an indicator of cardiac dysfunction in haemodialysis patients, the clinical significance of midregional proadrenomedullin has not been elucidated.

Objectives. We evaluated whether midregional proadrenomedullin reflects cardiac dysfunction, systemic inflammation or blood volume in haemodialysis patients.

Methods. Plasma midregional proadrenomedullin, C-reactive protein and delta body weight (indicating excessive blood volume), and two-dimensional as well as Doppler echocardiographic variables were measured just before haemodialysis in 70 patients with cardiovascular disease.

Results. The median value of midregional proadrenomedullin was 1.93 nmol/l before haemodialysis, and these levels were significantly reduced following haemodialysis. Log [midregional proadrenomedullin] was positively correlated with left ventricular end-systolic volume index, diameter of inferior vena cava, C-reactive protein and delta body weight ($r=0.328$, $r=0.421$, $r=0.356$, $r=0.364$), and negatively with blood pressure, deceleration time of an early diastolic filling wave, pulmonary venous flow velocity ratio and left ventricular ejection fraction ($r=-0.330$, $r=-0.324$, $r=-0.479$, $r=-0.373$). Multivariate regression analysis revealed that pulmonary venous flow velocity ratio, diameter of inferior vena cava and C-reactive protein were independently related factors for midregional proadrenomedullin concentration.

Conclusion. Plasma midregional proadrenomedullin levels increase in association with cardiac dysfunction, systemic inflammatory status and systemic blood volume in haemodialysis patients with concomitant cardiovascular disease.

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Keywords: cardiac dysfunction; excessive blood volume; haemodialysis; midregional proadrenomedullin; systemic inflammatory status

Introduction

Cardiovascular disease [1], excessive blood volume [2] and systemic inflammation [3] are the major causes of mortality in haemodialysis patients. Early diagnosis and treatment of these processes in haemodialysis patients may lead to improved survival. For this purpose, a non-invasive biochemical testing method would be ideal for screening and monitoring cardiac status, blood volume and inflammatory state.

Plasma adrenomedullin levels are elevated in left ventricular (LV) failure [4], myocardial infarction [5] and peripheral arterial occlusive disease [6], and these levels increase according to disease severity. Plasma adrenomedullin levels are additionally increased in haemodialysis patients [7] and these increases may be involved in the regulation of systemic blood pressure [7], and may reflect changes in systemic blood volume [8] in these patients. We also recently reported that adrenomedullin reflects cardiac dysfunction, excessive blood volume and inflammation, thereby proving to be a good predictor of mortality and cardiovascular morbidity in haemodialysis patients with cardiovascular disease [9].

Because adrenomedullin has a short half-life (22 min) in human plasma and is regulated by a proteolytic enzyme [10], the pre-analytical conditions of the plasma samples, particularly the storage temperature, may influence the final test results. Furthermore, several other factors may influence adrenomedullin measurement, including a binding protein that may be present in human plasma, which may have a specific inhibitory effect on the adrenomedullin radioimmunoassay [11]. Also, autocrine or paracrine interactions between adrenomedullin and

its receptors in the vicinity of release may lead to a removal of adrenomedullin from the circulation. Taken together, these pre-analytical factors may lead to an underestimation of the true adrenomedullin values by immunoassays.

Adrenomedullin is derived from a larger precursor peptide (prepro-adrenomedullin; 185 amino acids) by posttranslational processing [12]. During the processing of prepro-adrenomedullin, two peptides flank Adrenomedullin: one midregional part of proadrenomedullin (proadrenomedullin 45–92) and the COOH terminus of the molecule (proadrenomedullin 153–185). We have recently reported the technical characterization of this midregional proadrenomedullin (MR-proADM) sandwich immunoassay [13]. In contrast to mature adrenomedullin, MR-proADM is stable in plasma at room temperature for at least 72 h. MR-proADM probably has no physiological effects and its apparent stability may be attributable to this lack of function because only bioactive substances require careful regulation by proteolysis. The released amounts of MR-proADM may directly reflect levels of adrenomedullin. In addition, circulating MR-proADM is unlikely to be influenced by a binding protein, making it suitable for immunometric analysis. Although we recently reported that MR-proADM is increased in patients with cardiovascular disease [13], a more detailed analysis of the relationship between plasma MR-proADM concentration and cardiac function or cardiovascular disease severity is still lacking.

Therefore, we conducted the present study to investigate whether plasma MR-proADM accurately reflects cardiac dysfunction, removal of fluid volume by ultrafiltration, and systemic inflammatory status in haemodialysis patients admitted for evaluation of cardiovascular disease. We also performed pilot work to assess whether MR-proADM levels are predictive of subsequent mortality.

Methods

Patients

Seventy consecutive haemodialysis patients (51 men, 19 women; mean age, 65 ± 10 years), admitted to the National Cardiovascular Center for evaluation of cardiovascular disease, were enrolled in the present study. Patients having atrial fibrillation, overt pulmonary effusion, or pulmonary congestion were excluded from the present study. All patients underwent regular haemodialysis for 3–4 h three times weekly (Monday, Wednesday and Friday). Written informed consent was obtained from all patients. The procedures were in accordance with the Helsinki Declaration of 1975 (and as revised in 1983). After release from our institute, patients were followed up for an average 14.7 months. All-cause deaths were recorded.

Echocardiographic measurement

A skilled echocardiographer without knowledge of the clinical features of the patients performed the echocardiographic studies using a cardiac ultrasound unit (Sonos 5500; Philips Medical Systems, Andover, MA) just prior to haemodialysis treatment, as previously reported [9]. Left ventricular end-diastolic volume index (LVEDVI), left ventricular end-systolic volume index (LVESVI) and left ventricular ejection fraction (LVEF) were calculated using the modified Simpson method according to the recommendations of the American Society of Echocardiography [14].

To assess left ventricular (LV) diastolic function, LV diastolic filling (LV inflow) was examined using Doppler echocardiography. The LV diastolic filling pattern was obtained with the sample volume at the tips of the mitral valve in the apical four-chamber view and was recorded at the end-expiratory phase during quiet breathing. Peak velocity of early diastolic filling (E) and peak velocity of atrial filling (A) were recorded, and the E/A ratio was calculated. The deceleration time (DcT) was measured as the time between the top of the E wave and the point at which the descending part of the E wave or its asymptote crossed the zero line.

After LV inflow velocities were examined, pulmonary venous flow velocities were obtained from the apical four-chamber view and recorded at end-expiration. The pulsed Doppler sample volume was set at 0.5–1.0 cm into the upper right pulmonary vein. Peak forward flow velocities during ventricular systole (S) and diastole (D) were measured, and the S/D ratio was calculated. Echocardiographic parameters were obtained in 51 patients. LV inflow velocities and pulmonary venous flow velocities were obtained in 50 patients, because in the other patients it proved technically difficult to evaluate these variables.

Blood pressure, excessive blood volume, blood sampling and assay for MR-proADM

Blood pressure was measured with a mercury sphygmomanometer in the supine position after supine rest of 5 min or longer before blood sampling. Excessive blood volume before haemodialysis was defined as the fluid volume removal by ultrafiltration during haemodialysis (=delta body weight). Blood was withdrawn through the shunt before and after haemodialysis to measure MR-proADM, and was transferred into a chilled glass tube containing disodium EDTA and aprotinin. The blood was centrifuged immediately at 4°C, and plasma was frozen and stored at −80°C until assay. Plasma levels of MR-proADM were measured using a specific immunoassay system as previously reported [13]. The personnel responsible for measuring MR-proADM were blinded to the clinical and ultrasound status of the patients. Plasma C-reactive protein (CRP) levels were also measured, but only before haemodialysis by using the standardized methods in an autoanalyser.

Blood sampling and the echocardiographic studies were performed just before haemodialysis because of the need to evaluate the relationship between humoral factors and cardiac function in concurrence with excessive blood volume.

Statistical analysis

Data are expressed as means ± SD or using the median and interquartile range. Statistical comparisons were made with the Wilcoxon rank-tests or Mann–Whitney U-tests between two groups. Significant differences between more than two

groups were evaluated by Kruskal–Wallis tests with subsequent Scheffe's multiple comparison tests. Because MR-proADM data were not normally distributed, log [MR-proADM] was used in the correlations and regression models. Univariate linear and multivariate stepwise regression analyses were used to detect factors related to two-dimensional, Doppler echocardiographic parameters, plasma CRP concentrations or removal fluid volume to log [MR-proADM]. Event-free curves were estimated by the Kaplan–Meier product-limited method and compared with the Mantel (log-rank) test. The prognostic value of MR-proADM was tested by Cox's proportional-hazards regression analysis. Differences were considered to be statistically significant when the P value was <0.05.

Results

Clinical characteristics of the study population and echocardiographic findings just before haemodialysis are listed in Tables 1 and 2, respectively. All patients had a normal cardiac sinus rhythm, but each had cardiovascular disease as shown in Table 1.

The median values and interquartile ranges of MR-proADM before and after haemodialysis in patients with cardiovascular disease are shown in Figure 1. Haemodialysis significantly decreased plasma concentrations of MR-proADM.

In univariate linear regression analysis, log [MR-proADM] was significantly and negatively correlated with systolic blood pressure (SBP), DcT, S/D ratio and LVEF, and positively with LVESVI©, CRP and delta body weight (BW) (Table 3). Multivariate regression analysis revealed that S/D ratio, "diameter of inferior vena cava (IVC)" and CRP were independently related factors for MR-ProAM (Table 3). Log [MR-proADM] tended to be positively correlated with the E/A ratio and LVEDVI, but these did not reach statistical significance.

To evaluate the possible effects of LV diastolic dysfunction on plasma concentrations of MR-proADM, we compared levels among patients with $E/A > 1$ and $E/A \leq 1$, patients with DcT < 180 ms and DcT ≥ 180 ms, and patients with S/D < 1, $1 \leq$ S/D < 2 and S/D ≥ 2. In patients with DcT < 180 ms, plasma MR-proAM concentrations were significantly higher than those with DcT ≥ 180 ms. Furthermore, in patients with $S/D < 1$, plasma MR-proADM concentrations were significantly greater than in patients with $S/D \geq 2$ (Figure 2).

Nine patents died during the 14.7-month follow-up period. Event-free Kaplan–Meier curves comparing MR-proADM cut-off values are shown in Figure 3. We used the median value of MR-proADM (=1.93 nmol/l) as the cut-off in the present study. Seven of 34 patients with MR-proADM levels >1.93 nmol/l died and 2 of 36 with levels of 1.93 nmol/l or lower died during the follow-up period. Patients with greater MR-proADM levels had a greater death rate than those with lower MR-proADM levels (log-rank test, $P = 0.0346$).

Table 1. Clinical characteristics of the study population

Age (years)	64.5 ± 10.3
Sex (Male/female)	51/19
Duration of haemodialysis (years)	6.5 ± 7.9
Cause of renal failure (number of patients)	
Chronic glomerulonephritis	26
Diabetic nephropathy	21
Nephrosclerosis	9
Polycystic kidney disease	4
Renal stone	2
Renal tuberculosis	1
Cardiovascular disease (number of events)	
Coronary artery disease	42
Peripheral arterial occlusive disease	22
Hypertensive heart disease	10
Cerebrovascular attack	9
Aortic aneurysm	6
Aortic valve stenosis	4
Ventricular tachycardia	3
Paroxysmal supraventricular tachycardia	1
Primary pulmonary hypertension	1
Cardiac tumour	1
Heart rate (bpm)	71 ± 12
Systolic blood pressure (mmHg)	145 ± 22
Diastolic blood pressure (mmHg)	70 ± 11
Plasma albumin level (g/dl)	3.6 ± 0.4
Plasma MR-proADM level (nmol/l)	1.93 (1.42, 2.40)
Delta body weight (kg)	1.88 ± 1.12
Plasma C-reactive protein level (mg/dl)	1.13 ± 1.81

Data are expressed as numbers or means ± SD or medians (interquartile range).

Table 2. Echocardiographic findings just before haemodialysis

DcT (msec)	248.7 ± 58.7
E/A ratio	0.90 ± 0.28
S/D ratio	1.49 ± 0.47
LVEDVI (ml/m^2)	59.1 ± 21.7
LVESVI (ml/m^2)	26.2 ± 18.1
LVEF (%)	59.3 ± 13.8
IVC (mm)	16.4 ± 4.0

Data are expressed as means ± SD. Abbreviations: A, peak velocity of atrial filling; E, peak velocity of early diastolic filling; DcT, deceleration time of early diastolic filling; S, peak forward-flow velocity during ventricular systole; D, peak forward-flow velocity during ventricular diastole; LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; LVEF, left ventricular ejection fraction; IVC, diameter of inferior vena cava.

Discussion

In the present study, we showed that (i) the median concentration of MR-proADM was 1.93 nmol/l in a cohort of haemodialysis patients with cardiovascular disease; (ii) plasma MR-proADM concentrations were negatively correlated with blood pressure, DcT, S/D and LVEF, and positively correlated with LVESVI and IVC in these same patients, suggesting that plasma MR-proADM reflects LV diastolic and systolic dysfunction, which were defined as decreases in DcT, S/D and LVEF and increases in LVESVI; (iii) plasma

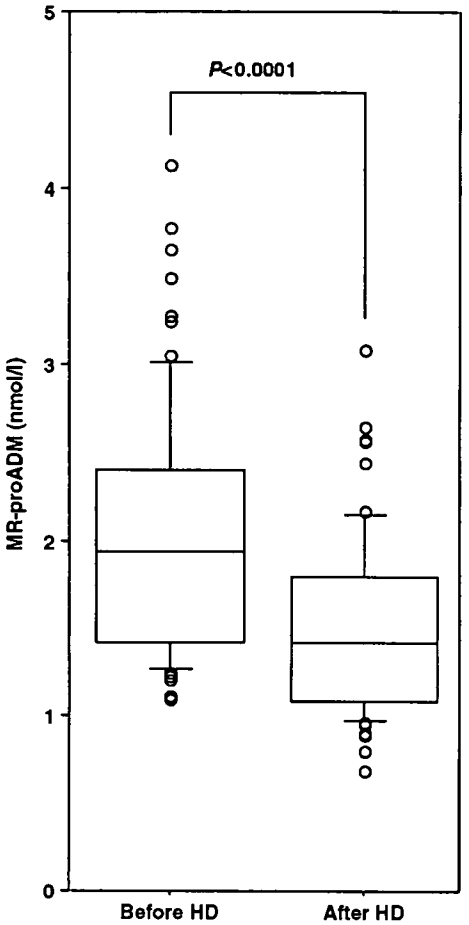

Fig. 1. Plasma midregional proadrenomedullin (MR-proADM) levels before and after haemodialysis (HD) in patients with cardiovascular disease.

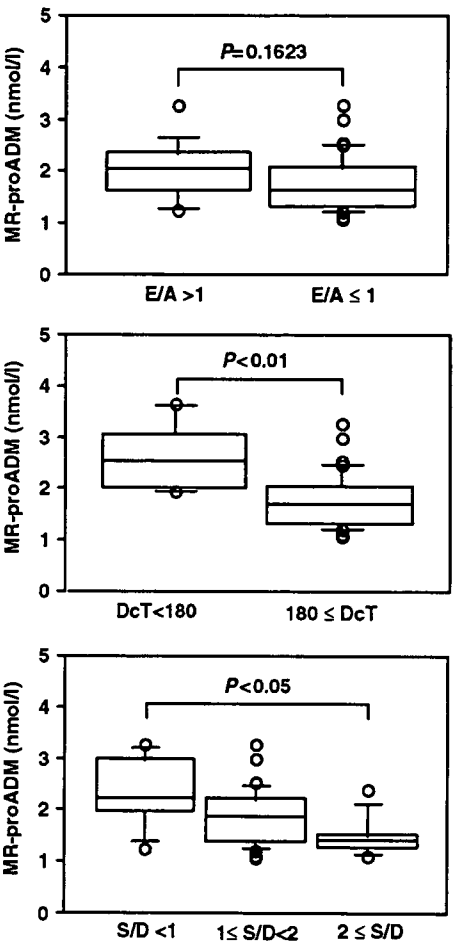

Fig. 2. Midregional proadrenomedullin (MR-proADM) levels in the DcT < 180 ($N=6$) and DcT ≥ 180 groups ($N=44$), in the $E/A > 1$ ($N=12$) and $E/A \leq 1$ groups ($N=38$), and in the $S/D < 1$ ($N=7$), $1 \leq S/D < 2$ ($N=35$) and $S/D \geq 2$ ($N=8$) groups.

Table 3. Univariate correlation between log (MR-proADM) and age, echocardiographic findings, C-reactive protein levels and delta body weight in haemodialysis patients

	Univariate		Mutivariate	
	R	*P*	Beta-coefficient	*P*
SBP (mmHg)	−0.330	0.0053	NS	
DcT (msec)	−0.324	0.0189	NS	
S/D ratio	−0.479	0.0004	−0.266	0.0368
LVESVI (ml/m^2)	0.328	0.0200	NS	
LVEF (%)	−0.373	0.0077	NS	
IVC (mm)	0.421	0.0015	0.300	0.0153
CRP (mg/dl)	0.356	0.0025	0.410	0.0009
Delta BW (kg)	0.364	0.0020	NS	

For Abbreviations, see Table 2. SBP, systolic blood pressure, CRP, C-reactive protein; BW, body weight.

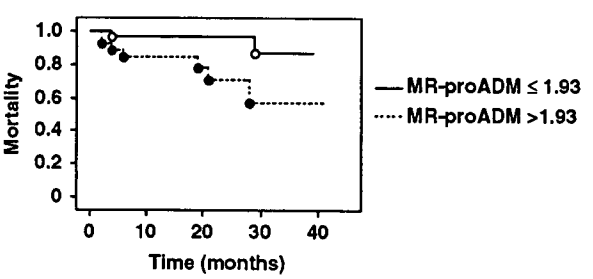

Fig. 3. Kaplan–Meier event-free curves in haemodialysis patients classified according to plasma midregional proadrenomedullin (MR-proADM) level [≤1.93 nmol/l ($N=36$; mortality number: 2) or >1.93 nmol/l ($N=34$; mortality number: 7)] (log-rank test, $P=0.0346$).

MR-proADM increased in association with systemic inflammatory status and/or removal fluid volume during haemodialysis ultrafiltration; (iv) patients with high MR-proADM had a higher mortality rate than those with low MR-proADM during the 14.7-month follow-up period.

We recently reported a MR-proADM mean value of 0.33 nmol/l in healthy individuals [13]. This was subsequently confirmed in other cohorts of healthy

individuals (N.G.M. unpublished data). We did not evaluate MR-proADM concentrations in healthy controls in the present study. Nevertheless, compared with these previous reports, the high median MR-proADM concentration of 1.93 nmol/l in our patients is more than 5-fold higher than in healthy subjects, suggesting that MR-proADM is clearly increased in patients with end-stage renal disease. Because the kidney is the most important clearance organ for circulating peptides such as BNP [15], the correlation coefficient between plasma BNP level and cardiac catheterization data was weakened, despite increased plasma BNP levels in patients with renal dysfunction compared to those with preserved renal function in the intensive care unit. Thus, renal failure resulting in a decrease in peptide clearance may be one possible reason for the increase in plasma MR-proADM concentrations in these patients. In addition, renal failure is well known to cause body fluid retention resulting in volume overload. Volume overload in turn increases shear stress in the arteries of the extremities [16]. Previous reports demonstrated that volume overload by itself increased cardiac biventricular adrenomedullin production [17], and that shear stress also increased adrenomedullin production in vascular endothelial cells [18]. We found a positive correlation between MR-proADM concentration and excessive blood volume in our patients, suggesting that the increased production of adrenomedullin in cardiac ventricles and the vascular endothelium resulted in increased plasma MR-proADM levels in patients with end-stage renal disease.

Plasma MR-proADM concentrations were negatively correlated with systemic blood pressure in our patients. In other studies with haemodialysis patients, plasma adrenomedullin levels have also been reported to be increased and negatively correlated with systemic blood pressure [7]. Given that adrenomedullin has a potent vasodilatory effect, the negative correlation between MR-proADM and systemic blood pressure in the present study suggests that adrenomedullin may be involved in lowering blood pressure in haemodialysis patients. Alternatively, the negative correlation between MR-proADM and systemic blood pressure may be secondary to reduced LV systolic function resulting in low blood pressure in association with an increase in plasma MR-proADM levels.

We previously reported that MR-proADM concentrations are increased in patients with cardiovascular disease [13]. However, there were no data to document the relationship between MR-proADM levels and cardiac function. Here, we report that plasma MR-proADM concentrations related to not only LV systolic dysfunction, which was defined as reduced LVEF, but also to diastolic dysfunction defined as reduced DcT, by the S/D ratio, and by an increased E/A ratio. This suggests that in the present study the plasma MR-proADM concentration may reflect reduced LV systolic and diastolic function. Numerous previous studies showed that plasma adrenomedullin levels were increased in LV failure [4], myocardial infarction [5] and peripheral arterial occlusive disease [6], and these levels increased according to disease severity. Thus, the concomitant cardiovascular disease in our haemodialysis patients may have led to cardiovascular dysfunction, resulting in increased cardiac adrenomedullin production and increased plasma MR-proADM level.

Increasing levels of CRP have been associated with increased risk of death in patients undergoing long-term haemodialysis [3]. In the present study, there was a positive correlation between plasma MR-proAM and plasma CRP concentrations. Previous reports demonstrated that tumour necrosis factor alpha, which is one of the most common inflammation-related cytokines, increased basal secretion of adrenomedullin in a cultured monocyte/macrophage cell line [19]. Furthermore, plasma levels of MR-proADM were markedly increased in patients with sepsis, and these increases may be helpful in individual risk assessment in septic patients [20], where MR-proADM showed an especially strong association with the APACHE II score. Thus, plasma MR-proADM concentrations may provide an indication of chronic inflammatory status in haemodialysis patients that is independent of other cardiac disease conditions.

Study limitations

Although the results of our survival analysis were limited to a small sample pilot population, elevated plasma MR-proADM concentrations predicted a poorer prognosis in our patients. Since cardiovascular disease [1], excessive blood volume [2] and systemic inflammation [3] are the major causes of mortality in haemodialysis patients, our findings that plasma MR-proADM reflected LV systolic and diastolic dysfunction and excessive blood volume are in concordance with these observations. If these findings are confirmed in a much larger sample study, plasma MR-proADM concentrations in haemodialysis patients may provide a tool for physicians to evaluate the severity of LV dysfunction, excessive blood volume and systemic inflammatory condition, and to manage these problems in haemodialysis patients with cardiovascular diseases. We performed echocardiographical studies to non-invasively evaluate LV diastolic and systolic function and remodelling. This was done instead of left-sided and right-sided catheterizations, which are more precise methods for these evaluations. The echocardiographical studies were performed just before dialysis. Because predialysis volume overload may affect both echocardiographical parameters and plasma levels of MR-proADM, individual differences in delta body weight may have altered the relationship between these parameters and MR-proADM in our patients. Concomitant cardiovascular disease was disproportionately distributed in the present study in that coronary artery disease was more frequent than the other cardiovascular diseases. Furthermore,

we did not evaluate possible changes in plasma MR-proADM levels before and after specific management of concomitant cardiovascular diseases or before or after reduction of excessive blood volume during the interval between haemodialysis therapies. Therefore, further investigations should be conducted that will examine these additional factors.

In conclusion, the present results suggest that plasma MR-proADM concentrations may increase in association with cardiac dysfunction, excessive blood volume and systemic inflammation. These levels may provide a possible index of these conditions in haemodialysis patients with concomitant cardiovascular disease. A large prospective population-based study will be necessary to confirm these preliminary observations.

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Conflict of interest statement. None declared.

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Possible role of adrenomedullin in the regulation of Fontan circulation: Mature form of plasma adrenomedullin is extracted in the lung in patients with Fontan procedure

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Abstract

Objective: We investigated the pathophysiological significance of molecular forms of adrenomedullin (AM) in patients after the Fontan procedure.
Methods: Plasma concentrations of mature AM (AM-m), an active form, glycine-extended AM (AM-Gly), an inactive form, and total AM (AM-T: AM-m+AM-Gly) were measured by specific immunoradiometric assay in the femoral vein, pulmonary artery and femoral artery of 29 consecutive patients after the Fontan procedure. The eleven patients who had history of Kawasaki disease and have normal coronary and hemodynamics served as control.
Results: Patients who underwent Fontan procedure had significantly higher venous concentrations of AM-T, AM-Gly, and AM-m than age-matched normal controls (AM-T, 12.0±3.3 vs. 9.6±2.0; AM-Gly, 10.4±3.0 vs. 8.5±1.6; AM-m, 1.6±0.7 vs. 1.0±0.6 pmol/l, each $p<0.05$). In patients with Fontan procedure, there were no differences in plasma AM-T, AM-Gly or AM-m levels between the femoral vein and pulmonary artery, however, there was a significant step-down in the AM-m levels, but not in plasma AM-T or AM-Gly levels, between the pulmonary artery and femoral artery (1.3±0.6 to 1.0±0.6, $p<0.05$). The venous concentrations of AM-m correlated negatively with systemic blood flow (cardiac output) ($r=-0.46$, $p<0.05$).
Conclusions: Results suggest that in Fontan circulation plasma AM-m is increased in parallel with those of AM-T and AM-Gly and that AM-m is extracted in the lung. Extracted AM-m may be involved in the regulation of pulmonary arterial tonus, although further studies are necessary to elucidate the exact role of AM in Fontan circulation.

Keywords: Adrenomedullin; Mature form of adrenomedullin; Fontan procedure

1. Introduction

The patients who underwent the Fontan procedure have a unique circulation, so-called the Fontan circulation, which is lacking in the right heart pump. In the Fontan circulation, central venous pressure is elevated and cardiac output is reduced. Fontan circulation is not simple systemic pump failure; because systemic ventricular filling pressure is not high and systemic ventricular volume is not increased. Fontan circulation has reduced cardiac output and high pulmonary vascular tone for lack of right heart pump.

Adrenomedullin (AM) is a hypotensive 52-amino-acid peptide that was originally discovered in acid extracts from human

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pheochromocytoma [1]. AM mRNA is reported to be highly expressed in the adrenal gland, lung, kidney, heart and vascular walls [1–3]. In addition, AM binding sites are highly expressed in rat lung [4]. Recent studies have demonstrated that AM receptor mRNA is abundantly expressed in human lung tissue [5]. These results suggest an importance of AM in the pulmonary arterial tonus. Indeed, AM has been shown to preferentially dilate the pulmonary vessels and increase pulmonary blood flow [6,7]. In addition, high plasma AM levels have been reported in rats with experimentally induced pulmonary hypertension and in patients with primary and secondary pulmonary hypertension [8–10]. These findings suggest the possibility that circulating AM may participate in the control of pulmonary vascular tone in pathological condition.

The human AM precursor consists of 185 amino acids, including a putative signal peptide [11]. AM is produced from the AM precursor by a two-step enzymatic reaction. First the AM precursor is converted into C-terminally glycine-extended AM (AM-Gly), a 53-amino-acid peptide that represents an intermediate, inactive form of AM. Subsequently, inactive AM-Gly is converted into the mature, active form of AM (AM-m), a 52-amino-acid peptide with a C-terminal amide structure, by enzymatic amidation. Kitamura et al. previously reported that both AM-m and AM-Gly circulate in human blood [12]. However, the ratio of these two molecular forms of plasma AM, as well as their production and clearance sites and their pathophysiological significance in patients after the Fontan procedure, remains unknown.

The present study aimed to explore the production and clearance sites and the pathophysiological significance of these two molecular forms of AM in patients with the Fontan procedure. Our hypothesis is that plasma AM-m is increased in the Fontan circulation and plays a role in this condition. To investigate this hypothesis, we measured plasma AM-m and total AM (AM-T; AM-T=AM-m+AM-Gly) concentrations in blood samples obtained from the femoral vein, pulmonary artery and femoral artery of 29 consecutive patients after the Fontan procedure, using a specific immunoradiometric assay [13,14].

2. Methods

This protocol was approved by the institutional ethical committee.

2.1. Patients

We studied 29 consecutive patients after the Fontan procedure (15 females and 14 males aged 1.4 to 22.6 years; mean age of 9.7±5.9 years) who underwent follow-up cardiac catheterization under local anesthesia. Their diagnoses were all functionally univentricular hearts. The techniques of the Fontan procedure were as follows: atrio-pulmonary connection in four patients, total cavo-pulmonary connection with intra-atrial rerouting in six patients, total cavo-pulmonary connection with intra-atrial grafting in nine patients, total cavo-pulmonary connection with-extra-cardiac grafting in ten patients. Mean age at the Fontan procedure was 3.8±2.4 years, mean age at the examination was 9.7±5.9 years, and mean period between the Fontan procedure and the examination was 5.8±4.9 years. We selected as age- and gender-matched control subjects 11 patients with a history of Kawasaki disease without coronary artery dilation (5 females and 6 males aged 0.9 to 17.3 years; mean age 7.8±5.5 years) who had normal hemodynamics and cardiac function. They had no other abnormality. Cardiac catheterization was performed, mean 2016 days (61 days to 5732 days, median 1938 days) after activity of Kawasaki disease was relieved. No inflammatory response was observed in any patients. In our institution, we have been doing cardiac catheterization only in patients with Kawasaki disease who had coronary artery dilatation in acute phase, because some of them develop coronary artery stenosis. As regard to the blood sampling, informed consent was obtained from the participants and/or their parents prior to initiation of the study.

2.2. Cardiac catheterization

All patients underwent right- and left-sided heart catheterization. The procedure included: (1) measurement of mean central venous pressure, mean pulmonary arterial pressure, mean pulmonary capillary wedge pressure, systemic ventricular end-diastolic pressure, and mean systemic arterial pressure, (2) measurement of oxygen saturation in pulmonary artery, systemic ventricle, systemic artery, and the determination of mixed vein saturation by using superior and inferior vena cava, (3) calculation of pulmonary blood flow, systemic blood flow (cardiac output) by using the oxymetric principle of Fick [15], and pulmonary vascular resistance, and (4) volummetry of systemic ventricular end-diastolic volume and systemic ventricular end-systolic volume by using Shimpson's method, and calculation of systemic ventricular ejection fraction.

Table 1
Haemodynamic variables in patients after the Fontan procedure and controls

Variable	Fontan	Control	*p* Value
CVP (mm Hg)	12.0 (2.2)	3.5 (1.9)	<0.0001
mPAP (mm Hg)	11.6 (2.7)	13.4 (1.6)	<0.05
PCWP (mm Hg)	7.5 (2.5)	7.2 (2.6)	NS
SVEDP (mm Hg)	9.0 (3.7)	10.2 (3.8)	NS
mSAP (mm Hg)	85.3 (9.8)	82.8 (6.9)	NS
MVsat (%)	64.3 (6.9)	74.9 (2.7)	<0.0001
SA sat (%)	93.7 (3.7)	97.7 (1.2)	<0.005
Hb (mg/dl)	13.9 (2.0)	13.2 (2.1)	NS
Qs (l/min/m^2)	2.8 (0.7)	3.8 (0.9)	<0.001
Rp (units·m^2)	1.6 (0.5)	1.6 (0.5)	NS
SVEDV (percent of normal)	88.7 (27.0)	81.8 (15.5)	NS
SVEF	0.50 (0.10)	0.62 (0.06)	<0.005
ANP (pg/ml)	39.9 (47.8)	18.5 (12.5)	NS

Values are means (SD) unless otherwise stated.
ANP, atrial natriuretic peptide; CVP, central venous pressure; Hb, haemogrobin; mPAP, mean pulmonary arterial pressure; mSAP, mean systemic arterial pressure; MVsat, oxygen saturation in mixed vein; NS, not significant; PCWP, pulmonary capillary wedge pressure; Qs, systemic blood flow; Rp, pulmonary vascular resistance; SAsat, oxygen saturation in systemic artery; SVEDP, systemic ventricular end-diastolic pressure; SVEDV, systemic ventricular end-diastolic volume; SVEF, systemic ventricular ejection fraction.

2.3. Blood sampling

Blood samples were obtained via catheters from the femoral vein, pulmonary artery and femoral artery of patients after the Fontan procedure and controls. The blood was transferred immediately into a chilled glass tube containing disodium EDTA (1 mg/ml) and aprotinin (500 U/ml) for the measurement of plasma concentrations of AM-m, AM-T (=AM-m+AM-Gly) and atrial natriuretic peptide. The blood was centrifuged immediately at 4 °C, and the plasma was frozen and stored at −80 °C until assayed.

2.4. Assays

Both AM-m and AM-T were measured using recently developed specific immunoradiometric assay kits (AM mature RIA and AM total RIA; Shionogi Co., Osaka, Japan) [13,14].

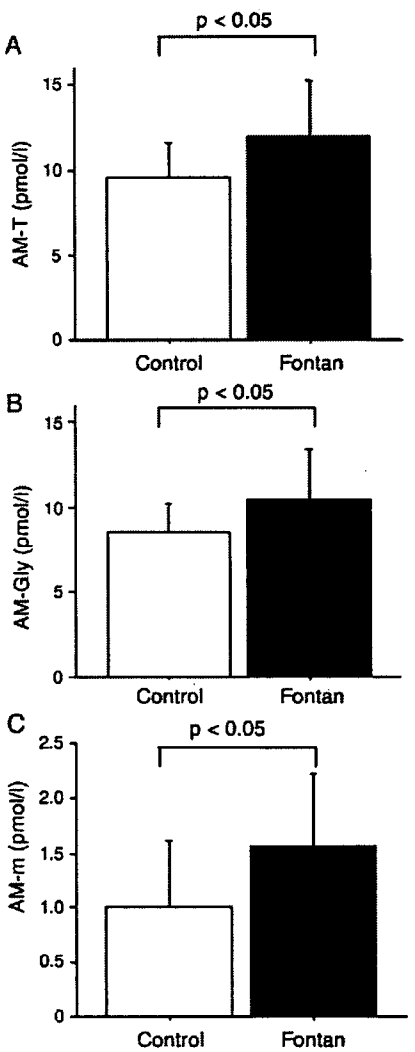

Fig. 1. Plasma concentrations of total adrenomedullin (AM-T) (A), glycine-extended adrenomedullin (AM-Gly) (B), and mature adrenomedullin (AM-m) in femoral vein in normal controls (control) and patients with the Fontan procedure (Fontan). Values are means±SD.

Table 2
Plasma concentrations of total adrenomedullin (AM-T), glycine-extended adrenomedullin (AM-Gly) and mature adrenomedullin (AM-m) in the femoral vein, pulmonary artery and femoral artery in patients after the Fontan procedure

Vessel	Plasma concentration (pmol/l)		
	AM-T	AM-Gly	AM-m
Femoral vein	11.7(3.4)	10.3(3.1)	1.4(0.5)
Pulmonary artery	11.5(4.2)	10.0(3.6)	1.3(0.6)
Femoral artery	11.7(5.1)	10.7(4.9)	1.0(0.6)*,†

Data are expressed as means(SD). Significance of differences: *$p<0.05$ compared with femoral vein; †$p<0.05$ compared with pulmonary artery.

These assay systems use two monoclonal antibodies against human AM, one recognizing a ring structure of human AM (both kits) and the other recognizing either the C-terminal sequence (AM-m kit) or AM-(25–36) (AM-T kit). The assay measures human AM-m or AM-T by sandwiching it between the two antibodies without the extraction of plasma. The minimum quantity of human AM-m or AM-T detectable using these assays is 0.5 pmol/l (both kits). The plasma AM-Gly concentration was calculated using the following formula: AM-Gly=AM-T−AM-m. Plasma concentrations of atrial natriuretic peptide were measured using the Shiono RIA ANP assay kit (Shionogi Co.), as reported previously [16].

2.5. Statistical analysis

All data are expressed as means±standard deviation. Student's unpaired *t*-test was used to evaluate differences between the controls and the patients with the Fontan procedure. The comparisons of plasma AM concentrations between the femoral vein, pulmonary artery and femoral artery were done by nested ANOVA followed by Scheffé's test. The correlation coefficients were calculated by linear regression analysis. Multiple regression analysis was applied to analyze dependence between variables. A value of $p<0.05$ was considered statistically significant.

Table 3
Correlation coefficients between AM levels and hemodynamic variables

Variables	AM-T(FV)		AM-m(FV)		AM-Gly(FV)	
	r	*p* Value	*r*	*p* Value	*r*	*p* Value
CVP (mm Hg)	−0.248	NS	0.051	NS	−0.286	NS
mPAP (mm Hg)	−0.028	NS	0.215	NS	−0.08	NS
PCWP (mm Hg)	−0.147	NS	0.162	NS	−0.2	NS
SVEDP (mm Hg)	−0.334	NS	0.004	NS	−0.372	NS
mSAP (mm Hg)	−0.101	NS	−0.155	NS	−0.077	NS
MVsat (%)	**−0.53**	**<0.005**	−0.201	NS	**−0.54**	**<0.005**
SA sat (%)	−0.165	NS	0.128	NS	−0.21	NS
Hb (mg/dl)	−0.093	NS	0.107	NS	−0.125	NS
Qp (l/min/m^2)	−0.133	NS	−0.343	NS	−0.071	NS
Qs (l/min/m^2)	−0.291	NS	**−0.46**	**<0.05**	−0.129	NS
Rp (units·m^2)	0.261	NS	0.344	NS	0.212	NS
SVEDV (percent of normal)	0.062	NS	−0.104	NS	0.093	NS
SVEF	−0.093	NS	−0.276	NS	−0.038	NS
ANP (pg/ml)	0.027	NS	0.292	NS	−0.034	NS

NS, not significant, Other abbreviations as in Table 1.

3. Results

3.1. Hemodynamic characteristics

Hemodynamic variables in patients after the Fontan procedure and controls are presented in Table 1. These patients after the Fontan procedure are characterized by higher mean central venous pressure, and a lower mean pulmonary arterial pressure, oxygen saturation in mixed vein, oxygen saturation in systemic artery, systemic blood flow and systemic ventricular ejection fraction. Thus, these patients had characteristics of Fontan hemodynamics.

3.2. Plasma AM concentrations

The venous plasma concentrations of AM-m, AM-Gly and AM-T in the control subjects and in the patients after the Fontan procedure are shown in Fig. 1. Venous plasma AM-m, AM-Gly, and AM-T levels in the patients with the Fontan procedure were higher than those in the age-matched controls; the extent of the increase was similar for all forms (all $p<0.05$). There was a good relationship between the levels of AM-m and AM-T ($r=0.54$, $p<0.005$). There were no differences in the concentrations of AM-m between the femoral vein and pulmonary artery (Table 2). However, the concentrations of AM-m were significantly ($p<0.05$) lowered in the femoral artery compared with the pulmonary artery and femoral vein (Table 2). In contrast, there were no differences in plasma AM-T and AM-Gly levels between any of the sample sites (Table 2).

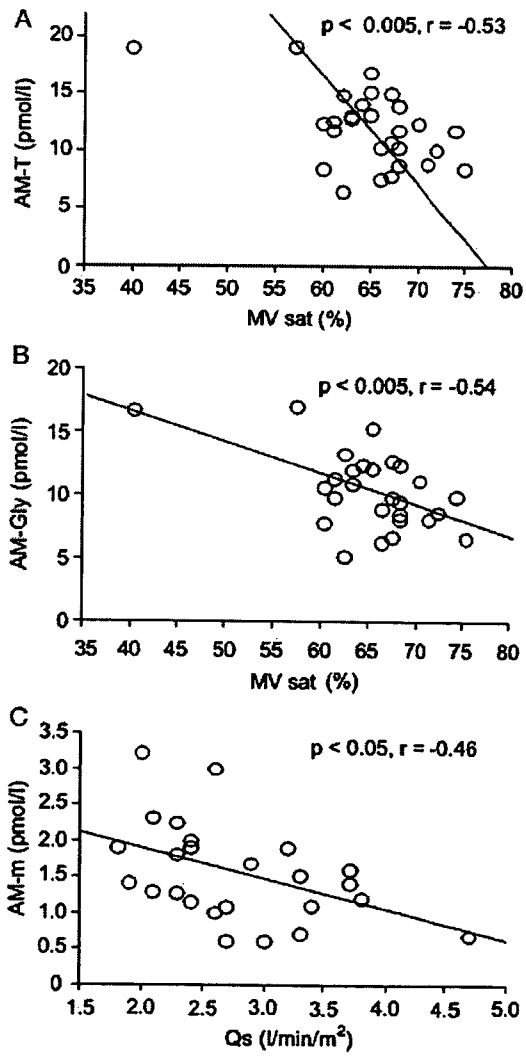

Fig. 2. Relationships between plasma concentrations of total adrenomedullin (AM-T) and oxygen saturation in mixed vein (MVsat) (A), glycine-extended adrenomedullin (AM-Gly) and oxygen saturation in mixed vein (MVsat) (B), mature adrenomedullin (AM-m) and systemic blood flow (Qs) (C) in femoral vein in patients after the Fontan procedure.

Table 4
Multiple regression analysis

Variable	AM-T (FV)	AM-Gly (FV)	AM-m (FV)
	R squad	*R* squad	*R* squad
	0.328	0.357	0.301
	p Value	*p* Value	*p* Value
CVP	NS	NS	NS
mSAP	NS	NS	NS
SVEDP	NS	NS	NS
MVsat	<0.05	<0.05	NS
Qs	NS	NS	<0.05

NS, not significant, Other abbreviations as in Table 1.

3.3. Relationships of plasma AM-m, AM-Gly and AM-T levels with hemodynamic variables

Correlations between the venous concentrations of AM and the hemodynamic variables are shown in Table 3 and Fig. 2. Plasma AM-T and AM-Gly levels negatively correlated with oxygen saturation of mixed vein (AM-T: $r=-0.53$, $p<0.005$; AM-Gly: $r=-0.54$, $p<0.005$), whereas plasma AM-m levels correlated negatively with systemic blood flow ($r=-0.46$, $p<0.05$) (Fig. 2). Multiple regression analysis also showed that plasma AM-T and AM-Gly correlated MVsat, whereas AM-m was correlated with Qs (Table 4).

4. Discussion

According to the study of Kitamura et al. [12], plasma AM levels reported previously were levels of AM-T, being the sum of AM-m and AM-Gly, because the radioimmunoassay system used polyclonal antibodies, which could not distinguish the structures with or without a C-terminal amide. For this reason, in order to measure the molecular forms of plasma AM, Kitamura et al. used two kinds of radioimmunoassay systems after the extraction of large amounts of plasma [12]. A one-step direct immunoradiometric assay system for the measurement of AM-m and AM-T using different monoclonal antibodies has been developed recently [13,14], which enables us to measure AM-m and AM-T specifically in a small sample without the prior extraction of plasma. The immunoradiometric assay for AM-m has no cross-reactivity with AM-Gly or other inactive metabolites of AM, and the immunoradiometric assay for AM-T

recognizes both AM-m and AM-Gly. In the present study, using this immunoradiometric assay for AM-T, we showed that plasma AM-T levels were increased in patients with the Fontan procedure compared with normal controls. We also showed that plasma AM-m and AM-Gly levels were increased significantly in patients with the Fontan procedure compared with normal controls. In addition, plasma AM-Gly and AM-T levels showed similar negative correlations with oxygen saturation of mixed vein, whereas plasma AM-m levels showed negative correlations with systemic blood flow. These findings indicate that the production of AM-m and AM-Gly is regulated similarly under pathological conditions such as the Fontan circulation.

AM-m represented approximately 13% of AM-T in the present study, suggesting that the major circulating form of AM is AM-Gly. This finding is consistent with a previous report [12]. The low AM-m/AM-T ratio in plasma may be explained by a low level of secretion of AM-m from tissues, and/or by a longer half-life of AM-Gly. Cultured endothelial cells and vascular smooth muscle cells produce large amounts of AM-m and small amounts of AM-Gly [2,3,17]. However, as AM acts as an autocrine and/or paracrine factor, AM-m produced in the tissues may be consumed almost entirely via receptor binding, and only small amounts of AM-m may be released into the circulation. In contrast, AM-Gly, an inactive form, cannot bind to the receptors, and therefore most of the AM-Gly produced may be released into the circulation. Furthermore, the low biological activity of AM-Gly suggests that the half-life of AM-Gly is longer than that of AM-m. This expected longer half-life of AM-Gly may, at least in part, be responsible for the low AM-m/AM-T ratio in human plasma.

In the present study, the plasma AM-m levels were markedly lower in the femoral artery than in the pulmonary artery, although there were no differences in plasma AM-Gly and AM-T levels between the femoral artery and pulmonary artery. An autoradiographic study showed that an intravenous injection of ^{125}I-AM was strongly taken up by the lung, suggesting that the lung has abundant binding sites for AM [18]. In addition, a previous study reported that AM binding sites were highly concentrated in the lung [4]. Furthermore, a recent study revealed that AM receptor mRNA is highly expressed in human lung tissue [5]. Indeed, several studies have shown that plasma AM was partially metabolized in the pulmonary circulation of patients with primary and secondary pulmonary hypertension and ischemic heart disease [10,19,20]. The present study demonstrated that this observed extraction of plasma AM in the lung is due to the extraction of AM-m. A study by Hirayama et al. showed that plasma AM-m levels were lower in the pulmonary capillary wedge portion than in the pulmonary artery portion in patients with ischemic heart disease [17], which is in good agreement with the present study. As regards the action of AM, previous studies have demonstrated that AM preferentially and strongly dilates pulmonary vessels over systemic vessels in animals [6,7,21,22]. Furthermore, a recent study reported that the pulmonary vasodilatory activity of AM is much more potent than that of acetylcholine or ATP on a molar basis in patients with pulmonary hypertension [23]. Many neurohumoral factors including nitric oxide, endothelin, norepinephrine, atrial natriuretic peptide, brain natriuretic peptide and renin–angiotensin are reported to be activated in Fontan circulation [24] and it is possible that these neurohumoral factors may modulate the pulmonary arterial tonus in this condition. The balance between vasoconstricting factors and vasodilating factors are important determinant for pulmonary vascular resistance. Indeed, a recent study demonstrated that imbalance between increased ET-1 and relatively decreased AM contributed to dominant effects of ET-1, leading pulmonary vasoconstriction after the Fontan procedure [25]. In addition, endothelin antagonist significantly reduced pulmonary artery resistance in Fontan circulation [26]. Moreover, inhalation of nitric oxide significantly attenuated pulmonary artery resistance in this condition [27]. Thus, many neurohumoral factors modulate the pulmonary artery resistance in Fontan circulation and AM also may be involved in the regulation of pulmonary vascular resistance in this condition.

In conclusion, the major molecular form of circulating AM is AM-Gly in normal subjects and in patients with the Fontan procedure, and plasma AM-m levels increase in parallel with those of AM-Gly in the Fontan circulation. AM-m is produced in the peripheral circulation, and the main site for clearance of circulating AM-m is the lung. Extracted AM-m may be involved in the regulation of pulmonary arterial tonus, although further studies are necessary to elucidate the exact role of AM in Fontan circulation.

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The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity

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Adrenomedullin (AM) is a peptide involved both in the pathogenesis of cardiovascular diseases and in circulatory homeostasis. The high-affinity AM receptor is composed of receptor activity–modifying protein 2 or 3 (RAMP2 or -3) and the GPCR calcitonin receptor–like receptor. Testing our hypothesis that RAMP2 is a key determinant of the effects of AM on the vasculature, we generated and analyzed mice lacking RAMP2. Similar to *AM*$^{-/-}$ embryos, *RAMP2*$^{-/-}$ embryos died in utero at midgestation due to vascular fragility that led to severe edema and hemorrhage. Vascular ECs in *RAMP2*$^{-/-}$ embryos were severely deformed and detached from the basement membrane. In addition, the abnormally thin arterial walls of these mice had a severe disruption of their typically multilayer structure. Expression of tight junction, adherence junction, and basement membrane molecules by ECs was diminished in *RAMP2*$^{-/-}$ embryos, leading to paracellular leakage and likely contributing to the severe edema observed. In adult *RAMP2*$^{+/-}$ mice, reduced RAMP2 expression led to vascular hyperpermeability and impaired neovascularization. Conversely, ECs overexpressing RAMP2 had enhanced capillary formation, firmer tight junctions, and reduced vascular permeability. Our findings in human cells and in mice demonstrate that RAMP2 is a key determinant of the effects of AM on the vasculature and is essential for angiogenesis and vascular integrity.

Introduction

Many vasoactive substances play critical roles in the maintenance of cardiovascular homeostasis; moreover, imbalances among these substances have been implicated in the pathogenesis of various cardiovascular diseases. Among these, adrenomedullin (AM) was originally identified as a vasodilating peptide isolated from human pheochromocytoma (1) and, based on its structural homology and similar vasodilatory effects, has been classified as a member of the peptide family that also includes calcitonin gene-related peptide (CGRP). Although produced by a variety of tissues and cell types, AM is primarily secreted by vascular cells and functions as a local autocrine or paracrine (2) mediator, as well as a circulating hormone (3). Apart from its vasodilatory effect, AM also exerts diuretic (4) and cardiotonic (5) effects and is involved in the regulation of hormone release (6, 7), inflammation (8), and oxidative stress (9, 10) as well as the proliferation, migration, and differentiation of various cell types (11–13). Thus, AM is now recognized to be a pleiotropic vasoactive molecule. To better understand the in vivo functions of AM, we established and analyzed genetically engineered AM-deficient mice (14–25). Homozygous AM KO (*AM*$^{-/-}$) mice die in utero at around midgestation from systemic hemorrhage and edema resulting from the fragility of their vasculature (14). In addition to mediating vascularization during development, we found that AM also enhances revascularization in adult tissues subjected to ischemia (25). The potential clinical applications of AM implied by these findings have attracted much attention, with particular attention being paid to AM's ability to stimulate vascular regeneration in ischemic tissue (26, 27).

AM signaling is regulated by a unique control system (28–31). The AM receptor is a 7-transmembrane domain GPCR named calcitonin receptor-like receptor (CRLR). CRLR associates with an accessory protein, receptor activity-modifying protein (RAMP), which is composed of about 160 amino acids and includes a single membrane-spanning domain. So far, 3 RAMP subtypes have been identified. By interacting with RAMP1, CRLR acquires a high affinity for CGRP, whereas by interacting with either RAMP2 or RAMP3, CRLR acquires a high affinity for AM. This novel system enables CRLR to transduce the signals of multiple ligands, although the precise mechanism remains largely unknown.

We hypothesized that not only the receptor-ligand specificity, but also the diversity, of AM's biological activities reflects its novel regulation by RAMPs. To test this idea, we generated RAMP2 KO mice, which were then used to analyze the physiological functions of the AM-RAMP2 system.

Results

Generation of RAMP2-null mice. We initially analyzed the expression of *AM* and its related genes during midgestational development (E11.5–E14.5),

Nonstandard abbreviations used: AGM, aorta-gonad-mesonephros; AM, adrenomedullin; CDN5, claudin 5; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein; RAMP2O/E, RAMP2-overexpressing (cell); ZO-1, zona occludens-1.

Conflict of interest: The authors have declared that no conflict of interest exists.

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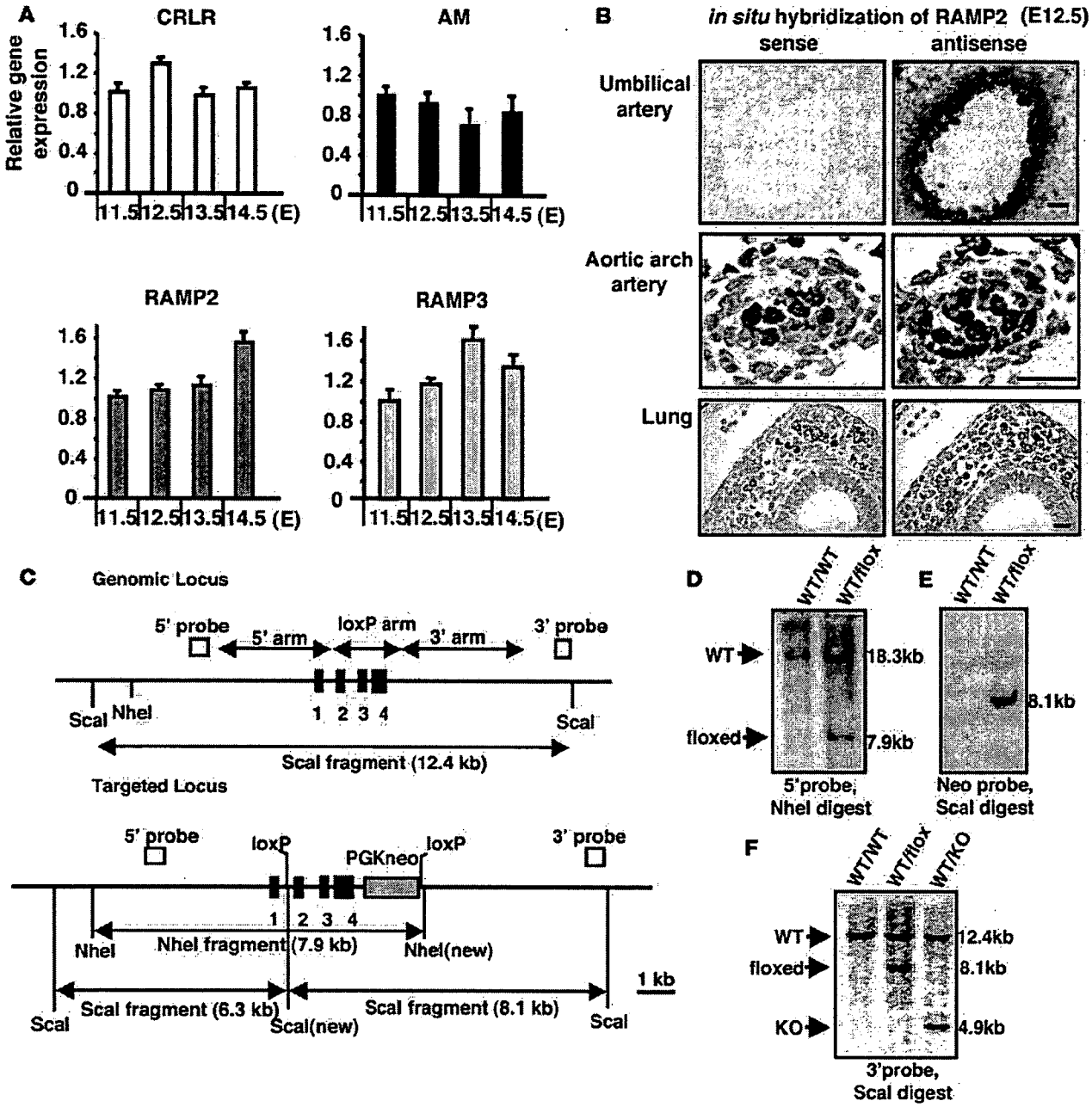


Figure 1

RAMP2 expression during development and generation of RAMP2 knockout mice. **(A)** Real-time PCR analysis of gene expression during E11.5–E14.5 in WT embryos. Expression is shown relative to that at E11.5. $n = 5$ per time point. AM, CRLR and RAMPs were expressed during midgestation. **(B)** In situ hybridization of *RAMP2* in WT embryos. Sections of umbilical artery, aortic arch, and lung from E12.5 WT embryos were used. Intense RAMP2 expression was detected in the vascular ECs. Scale bars: 20 μm. **(C)** Targeted disruption of mouse *RAMP2*. The genomic locus and predicted targeted locus are shown. Boxes denote exons 1–4 of RAMP2; ScaI and NheI restriction sites and loxP sites are indicated. Probes for Southern blot analysis are shown. **(D–F)** Southern blot analysis of mouse genomic DNA. **(D)** DNA was digested with NheI and probed with the 5′ probe. The 7.9-kb and 18.3-kb fragments denote floxed and WT alleles, respectively. **(E)** DNA was digested with ScaI and probed with the Neo probe. The 8.1-kb fragment denotes flox. **(F)** DNA was digested with ScaI and probed with the 3′ probe. The 8.1-kb ScaI fragment denotes flox; the 12.4-kb fragment denotes WT. The 4.9-kb fragment denotes the KO allele, generated by deletion of the loxP site using Cre recombinase.

the stage at which *AM*$^{-/-}$ embryos typically die. We found that in WT mice, AM, CRLR, RAMP2, and RAMP3 all continued to be expressed at midgestation (Figure 1A), which is consistent with previous findings (32, 33). Using in situ hybridization, we detected AM expression in the vascular system (data not shown) and found that, among the RAMPs, only RAMP2 was specifically expressed in the vasculature at that stage (Figure 1B). We therefore speculated that it is RAMP2 that determines AM's function during vascular development and pro-

Table 1
Genotype of embryos from *RAMP2*$^{+/-}$ male and female mouse intercrosses

Stage	Incidence (*n*)			Total
	RAMP2$^{+/+}$	*RAMP2*$^{+/-}$	*RAMP2*$^{-/-}$	
E11.5	5	11	6	22
E12.5	31	58	29	118
E13.5	22	50	24[A]	96
E14.5	13	26	13[B]	52

[A]Of these, 3 embryos were dead. [B]Of these, 12 embryos were dead.

ceeded to generate RAMP2-specific KO mice to directly assess the functions of the AM-RAMP2 system in vivo.

The targeting strategy and analysis of homologous recombination are shown in Figure 1, C–F. RAMP2 heterozygous KO mice (*RAMP2*$^{+/-}$) were apparently normal, although the number of live births was markedly diminished when *RAMP2*$^{+/-}$ mice were intercrossed. No RAMP2 homozygous KO (*RAMP2*$^{-/-}$) newborns were obtained, and analysis of the embryos from timed *RAMP2*$^{+/-}$ intercrosses showed that the *RAMP2*$^{-/-}$ genotype was lethal at midgestation. The mortality rate among *RAMP2*$^{-/-}$ embryos was 13% at E13.5, 92% at E14.5, and 100% at E15.5 (Table 1). The most lethal stage (E13.5–E14.5) was nearly identical to that of the *AM*$^{-/-}$ genotype.

Real-time PCR analyses (Figure 2A) showed there to be no expression of RAMP2 in *RAMP2*$^{-/-}$ embryos, confirming that the *RAMP2* gene was successfully destroyed. By contrast, expression of RAMP3 did not differ in *RAMP2*$^{-/-}$ and WT mice, which indicates that no functional redundancy exists between RAMP2 and RAMP3 during development. Moreover, the expression of AM was upregulated by more than 5-fold in *RAMP2*$^{-/-}$ mice, presumably as a compensatory response to the absence of a functional AM receptor.

Macroscopic and histological observation of RAMP2$^{-/-}$ *embryos.* At E13.5, well-developed vitelline arteries were detected on the yolk sacs of WT embryos, whereas *RAMP2*$^{-/-}$ embryos had only poorly developed vitelline arteries (Figure 2, B–D). Histological examination revealed that the vitelline arteries from *RAMP2*$^{-/-}$ embryos were smaller than those from WT embryos and appeared disorganized (Figure 2, E–H). That these phenotypes resembled those of *AM*$^{-/-}$ embryos (14) showed that deletion of RAMP2 was sufficient to reproduce the major phenotypes of the vascular abnormality seen in *AM*$^{-/-}$ mice. In *RAMP2*$^{-/-}$ embryos, moreover, some of the ECs in the vitelline (Figure 2, I and J) and umbilical (Figure 2, K and L) arteries appeared apoptotic.

As for the embryos, the most apparent finding in *RAMP2*$^{-/-}$ mice was severe systemic edema (Figure 2, M and N). They also showed accumulation of pericardial effusion suggestive of cardiac failure (Figure 2, O–R), and some had bleeding that was observable under the skin (Figure 2S) and within organs (Figure 2U). These phenotypes were also observed in *AM*$^{-/-}$ embryos (14), although *RAMP2*$^{-/-}$ mice showed systemic edema much more severe than that in *AM*$^{-/-}$ mice.

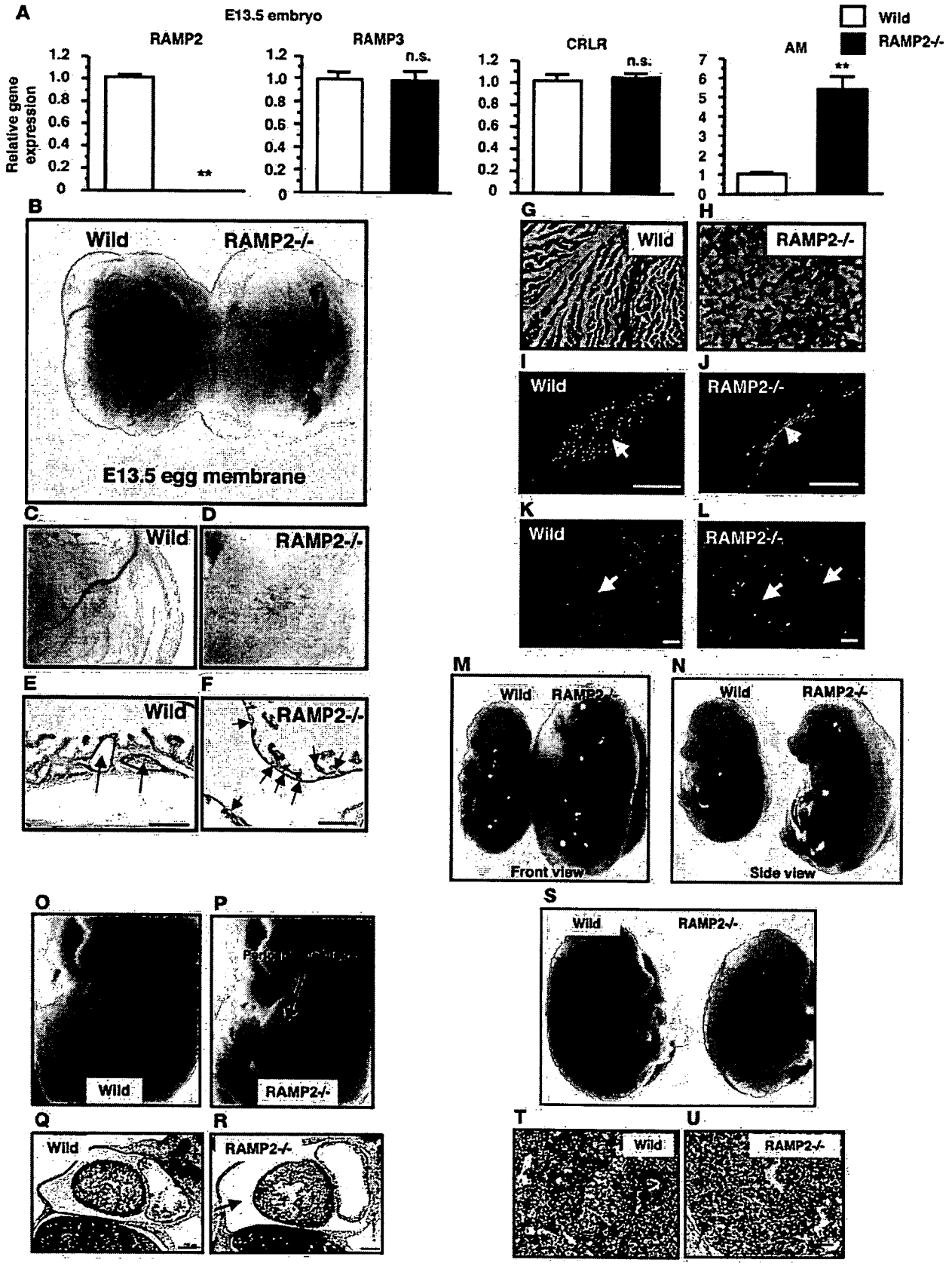
Vascular abnormalities and gene expression in RAMP2$^{-/-}$ *mice.* To determine whether the developmental abnormalities described above were the cause of the vascular fragility seen in *RAMP2*$^{-/-}$ embryos, we analyzed in detail the vascular structure at E12.5. Electron microscopic observation of the ECs of the vitelline arteries in *RAMP2*$^{-/-}$ embryos revealed deformity and detachment from the basement membrane (Figure 3B). Similar EC detachment was also detected in the liver (Figure 3D). In addition, there was abnormal thinning of the arterial walls in *RAMP2*$^{-/-}$ embryos (WT, 1.75 ± 0.12 μm; *RAMP2*$^{-/-}$, 1.36 ± 0.05 μm; $P < 0.05$, $n = 5$ per group; Figure 3F), and the multiple layers of smooth muscle cells and basement membrane that normally comprise arterial walls were severely disrupted in *RAMP2*$^{-/-}$ embryos (Figure 3H). We also found that expression of molecules involved in cell adhesion was altered in arteries from *RAMP2*$^{-/-}$ mice. In particular, expression of vascular endothelial cadherin (VE-cadherin), claudin 5 (CDN5), and type IV collagen was diminished compared with WT mice (Figure 3I). These molecules are all important for the composition of tight junctions, adherence junctions, and the basement membrane of vascular ECs, and abnormalities involving them lead to paracellular leakage from the vascular lumen, which likely explains the severe edema seen in *RAMP2*$^{-/-}$ mice.

Mechanisms underlying the angiogenesis and vascular stability mediated by the AM-RAMP2 system. To analyze the mechanisms underlying the angiogenesis and vascular stability mediated by RAMP2, we next generated EC lines that stably overexpressed RAMP2 (RAMP2O/E cells), utilizing EAhy926 ECs (Figure 4, A and B; see Methods for details). EAhy926 ECs are known to form capillary-like tubes on Matrigel (34, 35). We observed that RAMP2O/E cells showed much greater capillary formation than did control cells in Matrigel assays (Figure 4, C–E), clearly demonstrating that upregulation of the AM-RAMP2 system enhances angiogenesis.

We then assessed endothelial barrier function by assaying vascular permeability in vitro. Cells were seeded onto semipermeable membranes in permeability chambers, after which the passage of FITC-dextran through confluent EC monolayers was monitored. Monolayers of RAMP2O/E cells were significantly less permeable than those of control cells, which suggests that upregulation of the AM-RAMP2 system also enhances vascular barrier function and reduces permeability (Figure 4F). We hypothesized that the reduced permeability reflected the firmer structure of the tight junctions formed by RAMP2O/E cells. To test this idea, we treated RAMP2O/E and control cells with H_2O_2 (0.5 mM), which leads to formation of intercellular gaps and reduced tight junction formation between ECs. Subsequent immunostaining of the tight junction marker zona occludens-1 (ZO-1) revealed that the structure was better preserved in RAMP2O/E than control cells (Figure 4, G–K). In addition, MTT and TUNEL assays revealed that RAMP2O/E cells showed significantly better viability after H_2O_2-induced damage than did control cells (data not shown).

We also found that expression of eNOS, VEGF, and CDN5 was upregulated in the RAMP2O/E cells and that treatment with the PI3K inhibitor LY294002 or a PKA inhibitor blocked those effects (Figure 4L). Thus, signaling via a PI3K- and PKA-dependent pathway appears to play a key role in AM-RAMP2 mediated angiogenesis and vascular stability. By contrast, RAMP3O/E cells did not show either enhanced angiogenesis or changes in permeability (data not shown). Apparently, the vascular functions of AM are exclusively regulated by RAMP2.

Reduced responses to angiogenic stimuli in adult RAMP2$^{+/-}$ *mice.* Unlike their homozygous KO littermates, *RAMP2*$^{+/-}$ mice survived until adulthood and were fertile, though aortic expression of RAMP2 was reduced to about half that seen in WT mice (Figure 5A), and they had higher BP than did WT mice (systolic BP, WT, 102.8 ± 2.2 mmHg; *RAMP2*$^{+/-}$, 112.9 ± 2.2 mmHg; $P < 0.01$, $n = 9$ per group). With acute infusion of AM (10 nmol/kg), *RAMP2*$^{+/-}$ mice showed a smaller BP response than WT mice (maximum percent change in systolic BP,


A
E13.5 embryo
RAMP2
RAMP3
CRLR
AM
Wild
RAMP2-/-
Relative gene expression
n.s.
**
B
Wild
RAMP2-/-
E13.5 egg membrane
G
H
I
J
K
L
C
D
E
F
M
N
Front view
Side view
O
P
Q
R
S
T
U