tries as well as in Western countries, the beneficial effect of the ARB candesartan for the prevention of new-onset diabetes should prove to be valuable.

To evaluate the efficacy of drugs that are widely used all over the world, clinical trials should be designed to examine patient outcomes for various races in many countries. In the VALUE Trial, the largest percentage of the randomly assigned patients was from the United States and European countries, whereas only 3.5% of the patients in the VALUE Trial were from Asian countries.[8,20] The event rates of cardiovascular disease and the severity of obesity in Asian countries such as Japan (mean BMI in the CASE-J Trial: 24.6 kg/m^2) differ from those in Western countries (mean BMI in the VALUE Trial: 28.6 kg/m^2).[20] As far as we know, there is no published evidence about the efficacy of ARBs in mildly obese populations. The outcome of the CASE-J Trial provides useful information about Asian populations that have similar genetic predispositions and lifestyles as the Japanese population.

Perspectives

The CASE-J Trial indicates that, with strict BP control, there is no significant difference between candesartan-based and amlodipine-based regimens in terms of the primary cardiovascular end point in high-risk hypertensive patients. Nevertheless, the ARB candesartan is more effective than the CCB amlodipine for the prevention of new-onset diabetes.

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Disclosures

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CNP infusion attenuates cardiac dysfunction and inflammation in myocarditis

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Abstract

Myocarditis is an acute inflammatory disease of the myocardium for which there is currently no specific therapy. We investigated the therapeutic potential of C-type natriuretic peptide (CNP) in acute experimental autoimmune myocarditis. One week after injection of porcine myosin into male Lewis rats, CNP (0.05 μg/kg/min) was continuously administered for 2 weeks. CNP infusion significantly increased maximum dP/dt, decreased left ventricular end-diastolic pressure, and improved fractional shortening compared with vehicle administration. In vehicle-treated hearts, severe necrosis and marked infiltration of CD68-positive inflammatory cells were observed. Myocardial and serum levels of monocyte chemoattractant protein-1 were elevated in myocarditis. However, these changes were attenuated by CNP infusion. In addition, treatment with CNP significantly increased myocardial capillary density. Guanylyl cyclase-B, a receptor for CNP, was expressed in myocarditic heart, and cyclic guanosine monophosphate was elevated by CNP infusion. In conclusion, CNP infusion attenuated cardiac function in acute myocarditis through anti-inflammatory and angiogenic effects.

Keywords: Angiogenesis; Inflammation; MCP-1; Myocarditis; Natriuretic peptides

Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation. Acute myocarditis is associated with rapidly progressive heart failure, arrhythmias, and sudden death [1]. Immunomodulatory therapies such as immunoglobulin and interferon are regarded as promising for myocarditis [2,3]; however, the efficacy of those treatments still remains controversial [3,4]. Other treatment options are restricted to supportive care for heart failure or arrhythmias. The lack of specific treatment and the potential severity of the illness underlie the importance of novel and effective therapeutic strategies for myocarditis.

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There are three main natriuretic peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), all of which signal through natriuretic receptors and cyclic guanosine monophosphate (cGMP) signaling pathways. ANP and BNP are predominantly secreted from cardiac myocytes. They have anti-hypertrophic effects on cardiac myocytes in an autocrine manner and also have inhibitory effects on collagen synthesis of cardiac fibroblasts in a paracrine manner, and thus have suppressive effects on cardiac remodeling. Cardioprotective effects of ANP and BNP have already been demonstrated, and they are used clinically for the treatment of heart failure. On the other hand, CNP, originally identified in the porcine brain [5], is predominantly

expressed in vascular endothelial cells and plays a role in the local regulation of vascular tone and remodeling [6]. However, the potential therapeutic effects of CNP in heart disease are not well understood.

Recently, it has been shown that CNP is also synthesized in cardiac fibroblasts and inhibits collagen synthesis of cardiac fibroblasts more potently than ANP and BNP [7]. In addition, CNP proved to have more potent anti-hypertrophic effects than ANP in cultured cardiac myocytes [8]. More recently, infusion of CNP has been shown to improve cardiac function after myocardial infarction through anti-fibrotic and anti-hypertrophic effects [9]. These findings indicate the therapeutic potential of CNP in heart disease. However, it remains unknown whether CNP infusion improves acute myocarditis leading to severe heart failure. In the present study, cardiac myosin purified from pig hearts was injected into rats, and autoimmune myocarditis was induced [10].

Thus, the purposes of this study were (1) to investigate whether infusion of CNP improves cardiac function in a rat model of acute myocarditis, and (2) to investigate the mechanisms responsible for the effect of CNP on the myocarditic heart.

Materials and methods

Model of acute myocarditis. We produced a rat model of acute myocarditis by injecting pig cardiac myosin. In brief, purified myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [11]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl, mixed with an equal volume of complete Freund's adjuvant containing 11 mg/ml of Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg) and 0.2 ml of the antigen-adjuvant emulsion was injected into the foot pads.

CNP preparation and treatment. CNP was diluted in PBS with 5% glucose and administered via an ALZET mini-osmotic pump (DURECT Corporation, Cupertino, CA, USA) inserted subcutaneously, which discharged CNP at a rate of 0.05 μg/kg/min for the duration of 14 days beginning 1 week after myosin injection.

Experimental groups. Rats with sham operation or those with acute myocarditis were treated with vehicle or CNP. Fifty-four male 10-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were randomly placed into four groups and received the following treatments: (1) sham rats given vehicle ($n = 12$), (2) sham rats given CNP ($n = 12$), (3) myocarditis rats given vehicle ($n = 15$), and (4) myocarditis rats given CNP ($n = 15$).

Echocardiography. Echocardiography was performed at day 21 post-myosin injection. Rats were anesthetized with sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). Left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), anterior wall thickness (AWT), and posterior wall thickness (PWT) were measured, and taken as an average of three beats. Fractional shortening (%FS) was calculated as follows;

$$\%FS = (LVDd - LVDs)/LVDd \times 100$$

Hemodynamic study. Hemodynamic measurements were taken at day 21 post-myosin injection. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery (Millar Instruments, Houston, TX, USA). Heart rate was also monitored with electrocardiogram. As indexes of hemodynamics, heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dP/dt, and minimum dP/dt were used. Anesthesia was maintained with sodium pentobarbital, and the above mentioned indexes were recorded simultaneously during spontaneous ventilation after an equilibration period of a minimum of 20 min.

Histopathology. The heart was excised above the origin of the great vessels, and the heart and body weights were recorded. A midventricular portion of the heart was fixed with formalin and embedded in paraffin, and 4-μm sections were cut and stained with either hematoxylin and eosin (H&E) or Masson's trichrome stain, or subjected to immunohistochemical staining. H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) for the characterization of myocardial injury and inflammation, without knowledge of the experimental groups, on the following scale: (0) no or questionable presence, (1) limited focal distribution, (2 and 3) intermediate severity, and (4) coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue.

Immunohistochemistry. Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Sections were incubated with DakoCytomation protein block, then with anti-von Willebrand factor (vWF) (DakoCytomation, Glostrup, Denmark), CD68 (DakoCytomation), or monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences, San Jose, CA, USA) antibodies, followed by sequential incubations with HRP-linked rabbit anti-mouse IgG (DakoCytomation). The reaction products were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide. Sections were counterstained with hematoxylin. The numbers of vWF-stained capillaries and CD68-stained cells were determined in ten randomly selected fields (vWF; 400×, CD68; 200×).

Enzyme-linked immunosorbent assay (ELISA). Serum MCP-1 level on day 21 post-myosin injection was measured using a Rat MCP-1 ELISA Kit (Biosource International, Camarillo, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of guanylyl cyclase-B (GC-B) mRNA, a receptor for CNP, was examined by RT-PCR. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and myocarditis rats given vehicle ($n = 5$ in each group). Total RNA was extracted from heart with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed (PCR Amplification Kit, Takara, Shiga, Japan). The complementary DNA was amplified by the PCR using specific primers for GC-B or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers for GC-B were as follows [12]: sense primer 5′-AACGGGCG CATTGTGTATATCTGCGGC-3′ and antisense primer 5′-TTATCA CAGGATGGGTCGTCCAAGTCA-3′. For GAPDH, the primers were as follows: sense primer 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and antisense primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

Radioimmunoassay. To investigate whether subcutaneous administration of CNP has a biological activity in heart, we measured myocardial level of cGMP. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and those given CNP ($n = 10$ in each groups). Myocardial level of cGMP was measured with a radioimmunoassay kit (cGMP assay kit; YAMASA Co., Chiba, Japan).

Statistical analysis. Data were presented as means ± SEM. Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $P < 0.05$.

Results

Improvement in cardiac function by CNP treatment

Myocarditis rats given vehicle had two deaths 19 and 21 days after myosin injection, respectively, whereas those treated with CNP showed no mortality. At 3 weeks post-myosin injection, Myocarditis rats given vehicle showed decreased maximum dP/dt and minimum dP/dt, and

increased LVEDP compared with the sham rats (Fig. 1A–C), indicating the presence of acute heart failure in this model. Such parameters subsequently returned to baseline with CNP treatment. On echocardiography, rats with myocarditis showed an increase in LVDd and a significant reduction in %FS (Fig. 1D–F). CNP infusion significantly improved %FS in myocarditis rats. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was reduced by CNP treatment (Table 1). MAP was significantly decreased in myocarditis rats, and the decrease was significantly attenuated by CNP treatment. CNP did not significantly influence cardiac function in sham rats.

Attenuation of inflammatory cell infiltration by CNP treatment

Histological examination showed that myocardial necrosis and tissue granulation as well as inflammation and edema were markedly increased in our model of acute myocarditis (Fig. 2A and B). CNP administration significantly attenuated necrotic changes observed in myocarditis rats. CNP-treated hearts exhibited a consistent tendency for a reduction of tissue granulation, inflammation and edema, on blinded histological grading by a cardiovascular pathologist (H.I-U.) as compared to vehicle-treated hearts. Although, CNP is known to have potent anti-fibrotic activ-

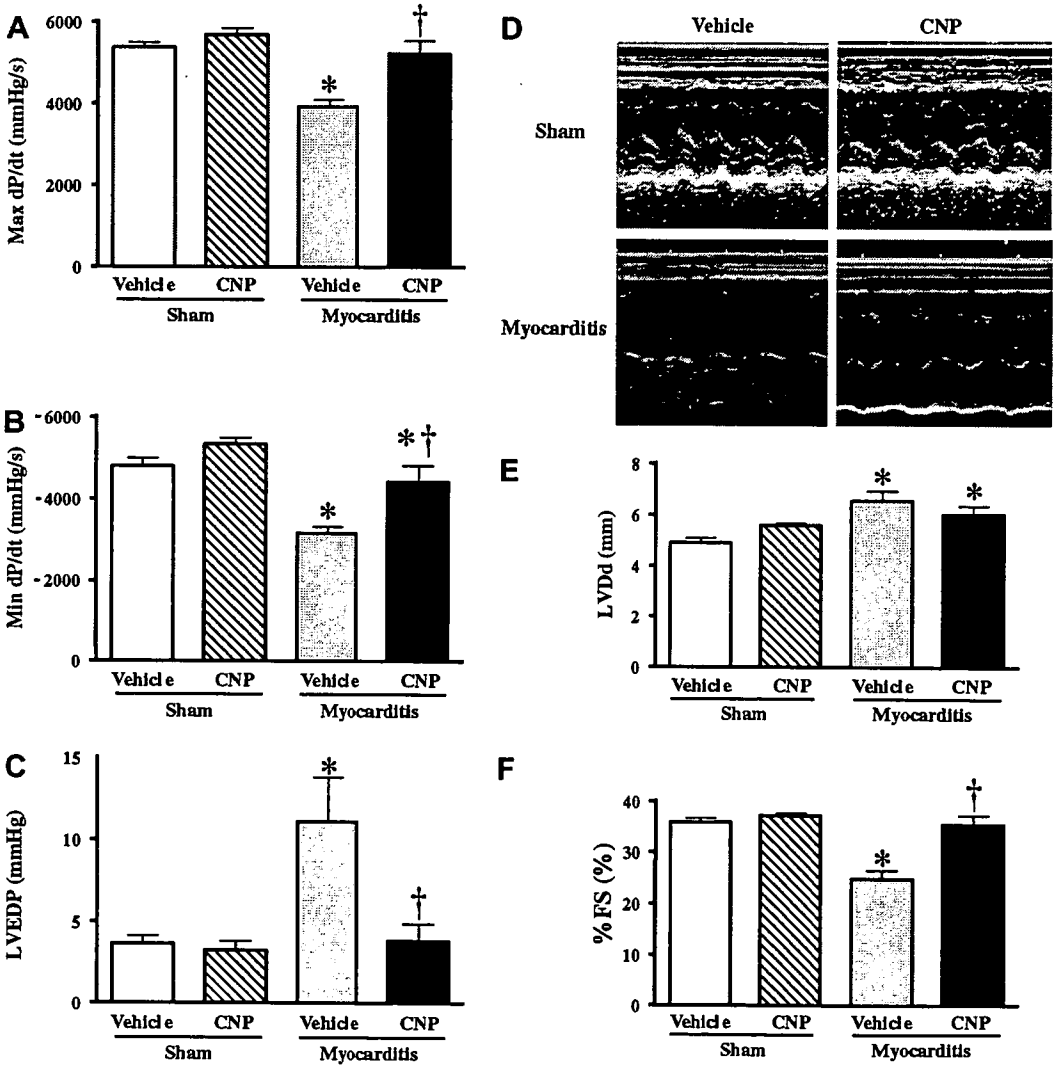

Fig. 1. Effects of CNP administration on hemodynamic parameters in acute myocarditis. (A) Maximum dP/dt (Max dP/dt), (B) minimum dP/dt (Min dP/dt), and (C) left ventricular end-diastolic pressure (LVEDP) were measured in sham rats given vehicle, sham rats given CNP, myocarditis rats given vehicle, and myocarditis rats given CNP. (D) Representative echocardiographic images showing wall thickening and poor myocardial movement in rats with myocarditis and improved cardiac contractility in those treated with CNP. (E,F) CNP administration in myocarditis tended to attenuate the increase in left ventricular diastolic dimension (LVDd) and significantly improved fractional shortening (%FS). Values are means ± SEM. $^{*}P < 0.05$ *vs.* Sham-Vehicle, $^{\dagger}P < 0.05$ *vs.* Myocarditis-Vehicle.

Table 1
Physiological and catheter-based parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 12$)	CNP ($n = 13$)
BW (g)	282 ± 2	282 ± 3	208 ± 4*	224 ± 3*,†
HW/BW (g/kg)	2.86 ± 0.04	2.81 ± 0.03	6.33 ± 0.25*	5.29 ± 0.20*,†
HR (bpm)	428 ± 7	422 ± 5	367 ± 13*	431 ± 13*,†
MAP (mmHg)	111 ± 4	103 ± 4	87 ± 3*	105 ± 5†
LVSP (mm Hg)	124 ± 5	125 ± 4	104 ± 4*	123 ± 6†

BW, body weight; HW/BW, heart weight to body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

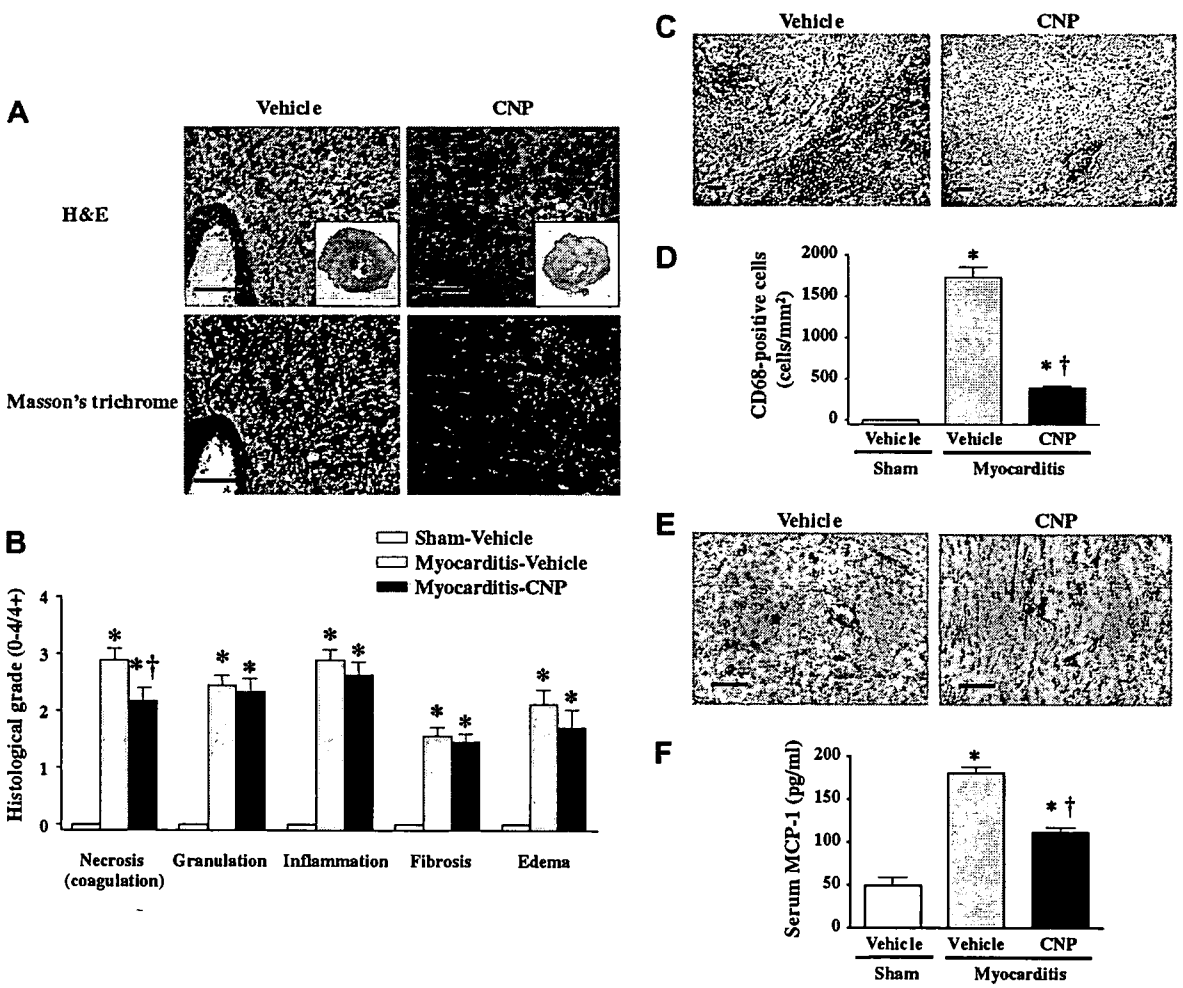

Fig. 2. Histological analysis of the myocardium. (A) Representative myocardial sections showed markedly decreased inflammation and tissue necrosis (H&E) and a comparable degree of early fibrosis (Masson's trichrome) in CNP-treated hearts as compared to myocarditic hearts. Insets are transverse section of the left ventricular section (H&E). (B) Semiquantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were lower in myocarditis rats treated with CNP as compared to untreated rats. Sham rats exhibited no measurable pathological change. Scale bar is 100 μm. (C) Representative myocardial sections immunohistochemically-stained for CD68 demonstrated a marked decrease in CD68-positive cells, including giant cells, in CNP-treated hearts as compared to vehicle-treated hearts. Scale bar is 100 μm. (D) Semi-quantitative counts of CD68-positive cells demonstrate a significant reduction in CNP-treated hearts. (E) Representative MCP-1-stained myocardial sections from rats with acute myocarditis. Scale bar is 100 μm. (F) Serum level of MCP-1 measured by ELISA. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

ity [9], myocardial fibrosis was not significantly attenuated by CNP infusion (Fig. 2B), probably due to the acute nature of this experiment (Table 2).

Notably, marked histiocytic infiltration was demonstrated by the presence of CD68-positive cells, including multinucleated giant cells, in rats with myocarditis, and this

Table 2
Echocardiographic parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 9$)	CNP ($n = 11$)
LVDd (mm)	5.6 ± 0.1	5.6 ± 0.1	6.5 ± 0.4*	6.0 ± 0.3
LVDs (mm)	3.6 ± 0.1	3.5 ± 0.1	4.9 ± 0.4*	3.9 ± 0.2†
%FS (%)	36 ± 1	37 ± 1	25 ± 2*	36 ± 2†
AWT diastole (mm)	1.9 ± 0.1	1.9 ± 0.1	3.1 ± 0.2*	2.8 ± 0.2*
PWT diastole (mm)	1.9 ± 0.1	1.8 ± 0.1	3.5 ± 0.3*	3.6 ± 0.4*

LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; %FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

was significantly attenuated by CNP treatment (Fig. 2C and D). In myocarditis, there was an increase in MCP-1 expression localized to the vascular endothelium and also in myocytes surrounding and adjacent to areas of inflammatory infiltration (Fig. 2E). The hearts in myocarditis rats treated with CNP showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in myocarditis rats, whereas it was significantly decreased in those treated with CNP (Fig. 2F).

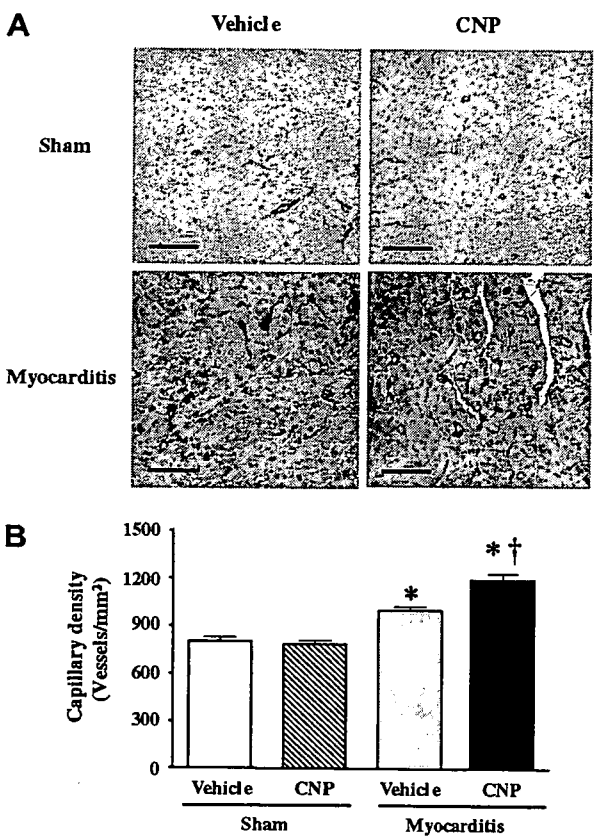

Fig. 3. Angiogenic potential of CNP in acute myocarditis. (A) Representative myocardial sections immunohistochemically-stained for vWF exhibit increased microvasculature in control myocarditic hearts, which was more marked in CNP-treated hearts. (B) Capillary density measured in 10 random representative high powered fields showed a significant increase in rats with acute myocarditis and a further increase in those treated with CNP. Scale bar is 100 μm. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

Effect of CNP on angiogenesis

To determine the angiogenic effect of CNP treatment in the myocardium, immunohistochemical analysis of vWF was performed. Capillary density in the heart was increased in myocarditis, particularly in areas directly adjacent to tissue necrosis (Fig. 3). Notably, capillary density was increased over that in acute myocarditis alone. The clustering of relatively small vessels seen in CNP-treated myocarditic hearts was indicative of recent endothelial regeneration or angiogenesis. On the other hand, CNP did not significantly influence the capillary density in the sham rats.

Expression of GC-B and cGMP in myocardium

RT-PCR demonstrated that GC-B mRNA was expressed in myocarditic heart (Fig. 4A). Myocardial level

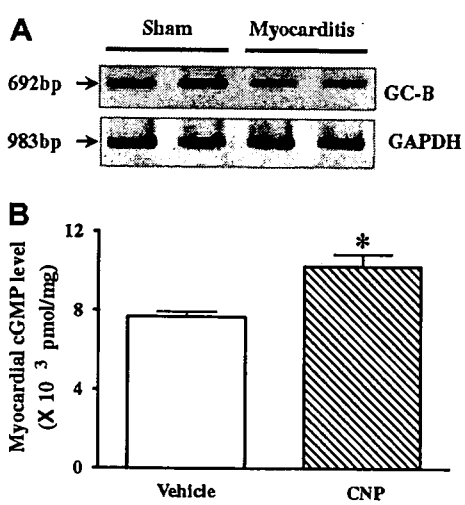

Fig. 4. Expression of GC-B and cGMP in the myocardium. (A) RT-PCR analysis of GC-B mRNA expression in myocarditic heart. (B) Myocardial level of cGMP measured by radioimmunoassay. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle.

of cGMP was significantly elevated by the subcutaneous infusion of CNP (Fig. 4B). These results suggest that subcutaneous infusion of CNP (0.05 μg/kg/min) has biological effects on myocaditic heart.

Discussion

In this study, we focused on the therapeutic potential of CNP in the acute phase of autoimmune myocarditis. We showed that CNP treatment 1 week following myosin injection but prior to the development of myocarditis (1) preserved cardiac function after acute myocarditis, (2) significantly decreased tissue necrosis, inflammatory cell infiltration and MCP-1 expression in the heart and serum, and led to a tendency for reduced overall inflammation, granulation and edema, and (3) stimulated angiogenesis in myocarditic hearts beyond the baseline increase seen in myocarditis.

The rat model of myosin-induced experimental autoimmune myocarditis closely resembles human giant cell myocarditis [11]. This disease model is triphasic, consisting of antigen priming phase from days 0–14, an autoimmune response phase from days 14–21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [13]. In our experiments, CNP was administered 1 week following myosin injection, corresponding to an early time point in the disease process. In the present study, CNP treatment significantly improved cardiac function as determined by increased maximum dP/dt and %FS as well as decreased LVEDP in rats with acute myocarditis. Importantly, earlier studies have shown that the vasodilator effect of CNP is much less potent than that of ANP [5,9,14,15]. ANP and BNP cause vasodilatation and hypotension, thus limiting their use as treatment for patients with severe heart failure. Because the effects of CNP on blood pressure and HR were very small, CNP treatment is considered as a safer alternative for the treatment of those patients [16]. Indeed, administration of CNP did not decrease arterial pressure, but sustained its biological activity.

Our data showed a significant decrease in inflammatory cell infiltration and a consistent tendency for decreased overall inflammation and edema by CNP treatment. In addition, CNP infusion decreased MCP-1 expression in the heart and serum. A previous study has demonstrated that CNP reduces macrophage infiltration by inhibition of MCP-1 expression [17]. These findings suggest that attenuation of inflammatory cell infiltration by CNP may be regulated, at least in part, by suppression of MCP-1 expression.

Recently, it was shown that CNP has anti-fibrotic properties in pulmonary fibrosis and myocardial infarction, through a cGMP-dependent pathway [9,18]. However, since the present experiments were carried out in the acute phase of myocarditis, the anti-fibrotic effect of CNP in the myocarditic heart was not clear. Further studies are necessary to examine the anti-fibrotic effects of CNP in the chronic phase of myocarditis.

We demonstrated that CNP induces endothelial regeneration beyond the increase seen in myocarditis. In rabbit balloon injury, infectious vein graft disease and hindlimb ischemia models, CNP overexpression stimulated reendothelialization via a cGMP-dependent pathway [19]. Endothelial dysfunction including microvascular constriction and microaneurysm formation has previously been reported in myocarditis [20], as well as chronic impairment of endothelial-dependent vasorelaxation of coronary resistance vessels in myocarditis [21]. Thus, the endothelial regenerative effects of CNP are likely to be beneficial in preventing myocardial injury and dysfunction in acute myocarditis. In this study, capillary density in normal heart was not increased by CNP infusion. In inflammatory tissue, it is speculated that CNP does not have an effect on initiation of angiogenesis, but promote angiogenesis at the phase of forming mature blood vessels. However, a further examination is necessary to elucidate the mechanisms of angiogenic effects.

Considering the importance of natriuretic peptides, such as ANP and BNP, in the diagnosis and treatment of cardiovascular diseases, there is currently much interest in the role of CNP. Since, CNP has marked cardioprotective effects including anti-inflammatory and angiogenic effects, and has less vasodilator effects, which enable the use of this peptide in patients with hypotension, this molecule may have great potential for the treatment of patients with acute myocarditis.

In summary, administration of CNP ameliorated cardiac dysfunction in a rat model of acute myocarditis. The beneficial effects may be due, at least in part, to anti-inflammatory and angiogenic effects. This work expands the beneficial effects of CNP to acute myocarditis, and increases our understanding of the role of natriuretic peptides in severe heart failure.

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Plasma adrenomedullin as an independent predictor of future cardiovascular events in high-risk patients: Comparison with C-reactive protein and adiponectin

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ABSTRACT

This study investigated the predictive power of plasma adrenomedullin (AM) for future cardiovascular (CV) events. In 121 patients with multiple CV risk factors and/or disease, plasma concentrations of AM, high sensitive C-reactive protein (hs-CRP), and adiponectin were measured. During follow-up periods (mean, 3.5 years) after the baseline assessment, 28 patients newly experienced CV events such as stroke/transient ischemic attack, acute coronary syndrome, and congestive heart failure. The plasma level of AM, but not hs-CRP or adiponectin, was significantly higher in patients who had CV events than in event-free subjects. When the patients were divided into three groups by tertiles of basal levels of AM (<10.1, 10.1–13.1, and ≥13.1 fmol/mL), cumulative event-free rates by the Kaplan–Meier method were decreased according to the increase in basal AM levels (83.2%, 68.6%, and 52.8% in the lowest, middle, and highest tertiles of AM, respectively; log-rank test, $P = 0.033$). By univariate Cox regression analysis, previous coronary artery disease, creatinine clearance, and plasma AM and hs-CRP levels were significantly associated with CV events during follow-up. Among these possible predictors, high plasma AM ($P = 0.004$) and low creatinine clearance ($P = 0.043$) were independent determinants for morbidity in multivariate analysis. These findings indicate that plasma AM is a powerful independent predictor of future CV events in high-risk patients, suggesting its predictive value is superior to that of hs-CRP or adiponectin.

1. Introduction

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma [14]. Subsequent studies have revealed that AM is widely distributed in various organs and tissues including the cardiovascular (CV) system [6,38,39]. Plasma levels of AM are elevated in various CV disorders, such as essential hypertension [8,17,24], chronic renal failure [8,24], coronary artery disease [15,22,41], congestive heart failure [11,25], ischemic

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stroke [7], and peripheral artery disease [40,41], and the degree of increase in AM levels is shown to be in proportion to the clinical severity of the disease [8,15,17,22,24,25,40]. These previous findings suggest that plasma AM may be a biochemical marker reflecting the presence and severity of CV complications in patients with CV risk factors. However, it remains unclear whether plasma AM levels have a predictive value for the occurrence of future CV events in such patients.

It is currently recognized that low-grade inflammation and insulin resistance contribute importantly to the initiation and progression of CV lesions [19,20]. In fact, many studies have shown that a mild increase in C-reactive protein (CRP), a sensitive inflammatory marker, is an independent predictor of future CV events [1,31–34,36]. It has also been shown that decreased blood levels of adiponectin, an adipocytokine with insulin sensitizing, anti-inflammatory, and anti-atherogenic properties, are a novel predictive factor for atherosclerotic CV disease [5,9,16,37,47]. In the present study, we aimed to determine whether an elevated level of plasma AM is a significant predictor of future CV events in high-risk patients, comparing its predictive power with those of CRP and adiponectin.

2. Methods

2.1. Study subjects

A total of 121 patients with two or more CV risk factors and/or diseases were enrolled in the present study. All subjects were inpatients who were admitted to the National Cardiovascular Center, Suita, Japan, for examination and treatment of hypertension, diabetes mellitus, and CV diseases including stable coronary artery disease. Patients with acute coronary syndrome (i.e., acute myocardial infarction and unstable angina pectoris) or congestive heart failure were excluded from the study. Hypertension was defined as a systolic blood pressure of ≥140 mmHg and/or a diastolic blood pressure of ≥90 mmHg 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 total cholesterol level of ≥220 mg/dL and/or a serum triglyceride level of ≥150 mg/dL or the use of lipid-lowering drugs. Coronary artery disease was diagnosed by electrocardiographic, radioisotope cardiographic, and coronary angiographic criteria. All subjects gave their informed consent to participate in the present study. All procedures of the present study were carried out in accordance with institutional and national ethical guidelines for human studies.

2.2. Biochemical measurement

Peripheral blood samples were obtained at rest in the supine position. Blood for AM measurement 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. Human AM concentration was measured by immunoradiometric assay using a specific kit (AM RIA SHIONOGI, Shionogi Pharmaceutical Co. Ltd., Osaka, Japan), as described previously [27].

Plasma adiponectin was determined by a sandwich ELISA system (Adiponectin ELISA Kit, Otsuka Pharmaceutical Co. Ltd.), as previously reported [9,10]. High sensitive CRP (hs-CRP) was measured by nephelometry (SRL Inc., Tokyo, Japan). Fasting plasma glucose, hemoglobin A1c, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, and serum creatinine were determined by standard laboratory measurements. Creatinine clearance was calculated from the Cockcroft–Gault formula [3].

2.3. Follow-up

After the initial assessment, all patients periodically visited our hospital for the treatment of risk factors (hypertension, diabetes mellitus, and/or hyperlipidemia) and CV diseases. CV events as clinical endpoints were stroke and transient ischemic attack confirmed by clinical symptoms, computed tomography, magnetic resonance angiography, and/or cerebrovascular angiography findings, acute coronary syndrome confirmed by electrocardiographic changes, coronary angiography, and/or myocardial scintigraphy findings, and congestive heart failure requiring hospitalization. Congestive heart failure was defined as clinical symptoms and signs (dyspnea, pulmonary rale, and/or leg edema), hypoxemia, and findings of chest radiography (pulmonary congestion and/or pleural effusion). Diagnosis of heart failure and need for admission were determined by clinical physicians who were blind to the basal level of AM, hs-CRP, or adiponectin. For patients who experienced multiple episodes, the analysis included only the first event. For patients without any CV event mentioned above, the date of censor was that of the last contact with the subject. The mean follow-up period was 42.0 months (0.3–81.3 months).

2.4. Statistical analysis

Statistical analysis was performed using StatView Version 5 Software (Abacus Concepts Inc., Berkeley, CA). Values were expressed as mean ± S.D. An unpaired Student's t-test was used for comparison between the two groups. The significance of differences among the three groups was evaluated by an unpaired ANOVA with subsequent Scheffe's multiple comparison test. Event-free curves were derived by means of the Kaplan–Meier method and were compared by log-rank test. The predictive value for CV events was tested by univariate Cox proportional hazards regression analysis. Then, a multivariate analysis using stepwise regression model was applied to identify independent predictors and their prognostic power. A value of $P < 0.05$ was accepted as statistically significant.

3. Results

Baseline clinical characteristics of total study subjects are shown in Table 1. The present subjects had a high percentage

Table 1 – Clinical characteristics of total subjects (n = 121)

Variable	
Age (years)	67.6 ± 9.5
Sex (men) (%)	68.6
Body mass index (kg/m^2)	23.6 ± 4.4
Hypertension (%)	84.3
Diabetes mellitus (%)	44.6
Hyperlipidemia (%)	57.0
Smokers (current or past) (%)	76.0
Previous coronary artery disease (%)	48.8
Systolic blood pressure (mmHg)	136 ± 18
Diastolic blood pressure (mmHg)	73 ± 11
Heart rate (beats/min)	65 ± 8
Fasting plasma glucose (mg/dL)	106 ± 31
Hemoglobin A1c (%)	6.2 ± 1.6
Total cholesterol (mg/dL)	191 ± 30
Triglycerides (mg/dL)	114 ± 51
HDL cholesterol (mg/dL)	45.1 ± 13.4
Creatinine clearance (mL/min)	78.6 ± 35.5

Values are mean ± S.D. or percentage.

of CV risk factors such as hypertension, diabetes mellitus, hyperlipidemia, and smoking habit, although their blood pressure, plasma glucose, and serum lipid levels were controlled by adequate treatments. In addition, 59 patients (48.8%) had a history of coronary artery disease.

During follow-up periods after the baseline assessment, 28 patients newly experienced major CV events. There were six subjects with cerebral infarction, one with cerebral hemorrhage, five with transient ischemic attack, six with unstable angina pectoris, one with acute myocardial infarction, and nine with congestive heart failure. The plasma AM level was significantly higher in patients who had CV events than in event-free subjects (Table 2). There was no significant difference in hs-CRP or adiponectin level between the two groups.

Table 2 – Association of basal AM, hs-CRP, and adiponectin levels with the following CV events

Variable	CV event		P
	(–) (n = 93)	(+) (n = 28)	
AM (fmol/mL)	11.6 ± 3.3	14.6 ± 6.3	<0.001
Hs-CRP (mg/dL)	0.23 ± 0.30	0.31 ± 0.65	0.359
Adiponectin (μg/mL)	5.8 ± 4.7	7.2 ± 5.6	0.214

Values are mean ± S.D.

All subjects were divided into three groups according to tertiles of basal AM levels (<10.1, 10.1–13.1, and ≥13.1 fmol/mL). Mean plasma levels of basal AM in the lowest, middle, and highest tertile groups were 8.3 ± 1.1, 11.5 ± 1.0, and 16.9 ± 4.1 fmol/mL, respectively (Table 3). Age, sex, body mass index, prevalence of hypertension, diabetes mellitus, and hyperlipidemia, smoking habit, blood pressure, heart rate, and glucose and lipid parameters did not differ among the three groups. The group in the highest tertile of AM had a significantly higher rate of past history of coronary artery disease, and lower creatinine clearance compared with the other two groups. Hs-CRP and adiponectin levels were also elevated in the highest tertile than in the lowest and/or middle tertiles. CV event-free Kaplan–Meier curves in the three groups are presented in Fig. 1. Cumulative event-free rates in the lowest, middle, and highest tertiles of AM were 83.2%, 68.6%, and 52.8%, respectively. These curves showed that higher basal levels of plasma AM were significantly associated with higher rate of CV events during follow-up (log-rank test, P = 0.033).

Table 3 – Clinical characteristics of three groups divided by tertiles of basal AM levels

Variable	Lowest tertile (n = 40)	Middle tertile (n = 40)	Highest tertile (n = 41)
Age (years)	66.6 ± 8.4	66.8 ± 10.6	69.3 ± 9.3
Sex (men) (%)	75.0	60.0	70.7
Body mass index (kg/m^2)	24.3 ± 3.9	24.3 ± 5.6	22.1 ± 3.0
Hypertension (%)	80.0	77.5	95.1
Diabetes mellitus (%)	37.5	55.0	41.5
Hyperlipidemia (%)	67.5	55.0	48.8
Smokers (current or past) (%)	77.5	67.5	82.9
Previous coronary artery disease (%)	35.0	40.0	70.7*,‾
Systolic blood pressure (mmHg)	133 ± 14	135 ± 22	139 ± 16
Diastolic blood pressure (mmHg)	74 ± 9	73 ± 12	71 ± 11
Heart rate (beats/min)	65 ± 9	64 ± 8	65 ± 8
Fasting plasma glucose (mg/dL)	109 ± 30	103 ± 27	107 ± 35
Hemoglobin A1c (%)	6.4 ± 2.1	6.2 ± 1.3	6.0 ± 1.2
Total cholesterol (mg/dL)	191 ± 31	199 ± 29	183 ± 29
Triglycerides (mg/dL)	125 ± 59	109 ± 51	108 ± 39
HDL cholesterol (mg/dL)	44.6 ± 12.6	47.7 ± 14.7	43.0 ± 12.6
Creatinine clearance (mL/min)	87.4 ± 26.1	85.4 ± 39.1	63.3 ± 35.4*,‾
AM (fmol/mL)	8.3 ± 1.1	11.5 ± 1.0*	16.9 ± 4.1*,‾
Hs-CRP (mg/dL)	0.11 ± 0.14	0.17 ± 0.23	0.47 ± 0.60*,‾
Adiponectin (μg/mL)	4.7 ± 3.5	6.4 ± 4.4	7.5 ± 6.2*

Values are mean ± S.D. or percentage.
* P < 0.05 vs. lowest tertile.
‾ P < 0.05 vs. middle tertile.

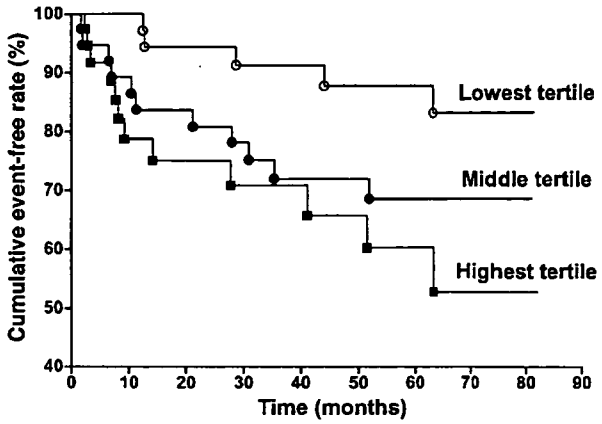

Fig. 1 – CV event-free curves obtained with the Kaplan–Meier method in the three groups divided by tertiles of basal AM levels. Cumulative event-free rates in the lowest, middle, and highest tertiles were 83.2%, 68.6%, and 52.8%, respectively (log-rank test, P = 0.033). Lowest tertile, basal AM <10.1 fmol/mL (n = 40); middle tertile, basal AM ≥10.1 and <13.1 fmol/mL (n = 40); highest tertile, basal AM ≥13.1 fmol/mL (n = 41).

Cox regression analysis was performed to examine the predictive power of plasma AM for future CV events, comparing with those of hs-CRP and adiponectin. In the univariate analysis, past history of coronary artery disease, creatinine clearance, and plasma hs-CRP in addition to plasma AM were significantly related to the incidence of CV events during the follow-up periods (Table 4). Among these possible predictive factors, high plasma AM and low creatinine clearance were independent predictors of CV events in the multivariate analysis, and the predictive value of AM for morbidity was most significant (+10% per 1-fmol/mL increase in AM, P = 0.004). Furthermore, even when the multivariate regression was reanalyzed after excluding subjects with previous coronary artery disease, the predictive value of AM for CV events was still significant, independently of creatinine clearance and other variables (hazard ratio 1.20 (per 1 fmol/mL increase), 95% confidence interval 1.06–1.35, P = 0.004).

4. Discussion

Plasma AM levels are known to be elevated in various pathological states, including several CV diseases [7,8,11, 15,17,22,24,25,40,41]. In addition, some studies showed that AM level was a predictor of survival in patients with acute myocardial infarction and chronic heart failure [12,23,29,30]. However, there have been no reports examining whether plasma AM can predict the occurrence of CV events in subjects with CV risk factors. Thus, the present study has demonstrated for the first time that an increased level of plasma AM becomes a significant predictor of future CV events in high-risk patients, independently of a variety of influencing factors.

In this study, we compared the predictive power of AM with those of hs-CRP and adiponectin. Our findings showed that neither hs-CRP nor adiponectin was an independent

Table 4 – Predictors of future CV events by univariate and multivariate Cox regression analysis

	Hazard ratio (95% CI)	P
Univariate analysis		
Age, 10 years	1.34 (0.88–2.04)	0.174
Sex, male	1.13 (0.50–2.56)	0.772
Body mass index, 1 kg/m^2	0.97 (0.88–1.07)	0.523
Hypertension, yes	2.02 (0.61–6.71)	0.249
Diabetes mellitus, yes	1.90 (0.89–4.06)	0.097
Hyperlipidemia, yes	1.00 (0.47–2.11)	0.999
Smoking (current or past), yes	1.65 (0.63–4.33)	0.313
Previous coronary artery disease, yes	2.90 (1.31–6.43)	0.009
Systolic blood pressure, 10 mmHg	1.03 (0.83–1.27)	0.799
Diastolic blood pressure, 10 mmHg	0.90 (0.65–1.24)	0.509
Heart rate, 5 beats/min	1.02 (0.83–1.27)	0.828
Fasting plasma glucose, 10 mg/dL	1.07 (0.97–1.19)	0.196
Hemoglobin A1c, 1%	1.14 (0.96–1.36)	0.126
Total cholesterol, 10 mg/dL	0.90 (0.80–1.02)	0.102
Triglycerides, 10 mg/dL	1.00 (0.94–1.07)	0.960
HDL cholesterol, 5 mg/dL	1.02 (0.90–1.16)	0.769
Creatinine clearance, 10 mL/min	0.80 (0.70–0.93)	0.003
AM, 1 fmol/mL	1.13 (1.06–1.19)	<0.001
Hs-CRP, 0.1 mg/dL	1.08 (1.00–1.18)	0.047
Adiponectin, 1 μg/mL	1.08 (0.99–1.16)	0.054
Multivariate analysis		
Creatinine clearance, 10 mL/min	0.87 (0.76–0.99)	0.043
AM, 1 fmol/mL	1.10 (1.03–1.18)	0.004

CI: confidence interval. In the multivariate analysis using stepwise regression model, all factors that had a significant association in the univariate analysis, i.e., previous coronary artery disease, creatinine clearance, AM, and hs-CRP, were included as possible independent variables.

predictor of future CV events, in contrast to the powerful prognostic value of AM. Several large epidemiological studies have suggested that CRP measurement predicts the risk of future CV events [1,31–34,36], whereas others have failed to identify CRP as a significant independent risk factor, especially after using multivariate analysis [28,42,44]. Hs-CRP was one of the significant predictors of CV events in univariate Cox regression analysis of the present study. However, since there was a close correlation between hs-CRP and AM levels (data not shown) and the predictive power of hs-CRP was weaker than that of AM in univariate analysis, hs-CPR might not become an independent predictor in multivariate analysis. As for adiponectin, it has been shown that low levels of plasma adiponectin are a predictor of CV events and mortality [4,5,9,16,37,47], but some studies reported that adiponectin did not predict future risk of coronary artery disease after adjusted for classical risk factors [18,35]. In addition, recent studies revealed that high, rather than low, adiponectin levels were associated with increased mortality and incidence of myocardial infarction in patients with chronic heart failure, chronic kidney disease, and stable angina [2,13,21]. Thus, the value of adiponectin as an independent risk marker for CV events and mortality remains controversial at present.

Although the exact reason behind the superiority of plasma AM over hs-CRP and adiponectin as a predictor of CV events in the present study remains to be elucidated, a number of mechanisms may be involved. AM is produced in various organs and tissues, but the main source of circulating AM is the blood vessels (especially vascular endothelial cells) [38], in contrast to the major sites of the production of CRP and adiponectin. Therefore, AM may directly reflect vascular inflammation and endothelial injury during the initiation and development of atherosclerosis. In fact, increased plasma levels of AM were reported to be associated with the progression of atherosclerotic lesions [7,40]. Furthermore, since several studies have shown that ischemic and hypoxic conditions stimulate the production and secretion of AM [26,43,46], it is possible that the increase in baseline AM might be induced by silent cerebral or cardiac ischemia before attack. Plasma AM has also been shown to increase in response to left ventricular systolic and diastolic dysfunction [23,25,45], suggesting the possibility that baseline AM in our subjects could detect latent cardiac disorders. Therefore, as AM comprehensively reflects vascular inflammation and injury, atherosclerotic change, systemic and myocardial ischemia, and cardiac dysfunction, plasma AM might become a sensitive marker of future CV disease.

There were some limitations in the present study. The sample size of our subjects was small to evaluate the predictive power of AM discretely for cerebrovascular, coronary, and heart failure events. In addition, the prognostic value of AM for all-cause and CV death could not be investigated. As another limitation of this study, we did not consider the influence of medication during follow-up on the occurrence of CV disease. Therefore, the use of statin, aspirin, renin angiotensin system inhibitors, and β-blockers and the alteration of dosage of these drugs after the initial assessment might bias the outcome of the present study. Furthermore, we did not examine the change of plasma AM levels during follow-up periods. It is possible that the prognostic potential of AM may be raised by serially evaluating its plasma level in high-risk patients.

In conclusion, the present findings indicate that plasma AM is a powerful independent predictor of future CV events in patients with multiple CV risk factors, and suggest that its prognostic value is superior to that of hs-CRP or adiponectin. However, further investigations using larger population of high-risk patients will be required to establish the usefulness of AM as a novel predictive marker for CV diseases.

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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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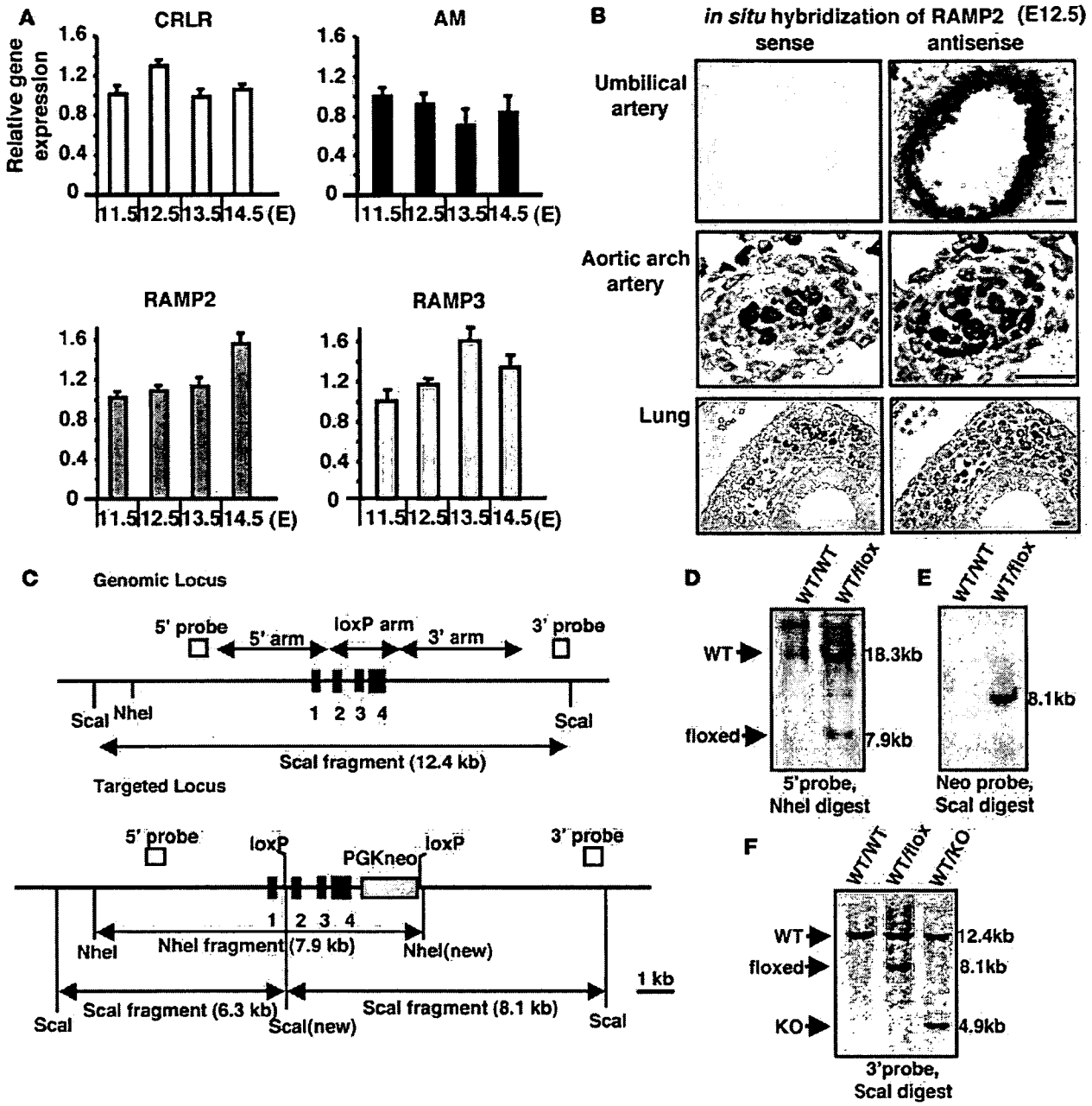


Figure 1

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.

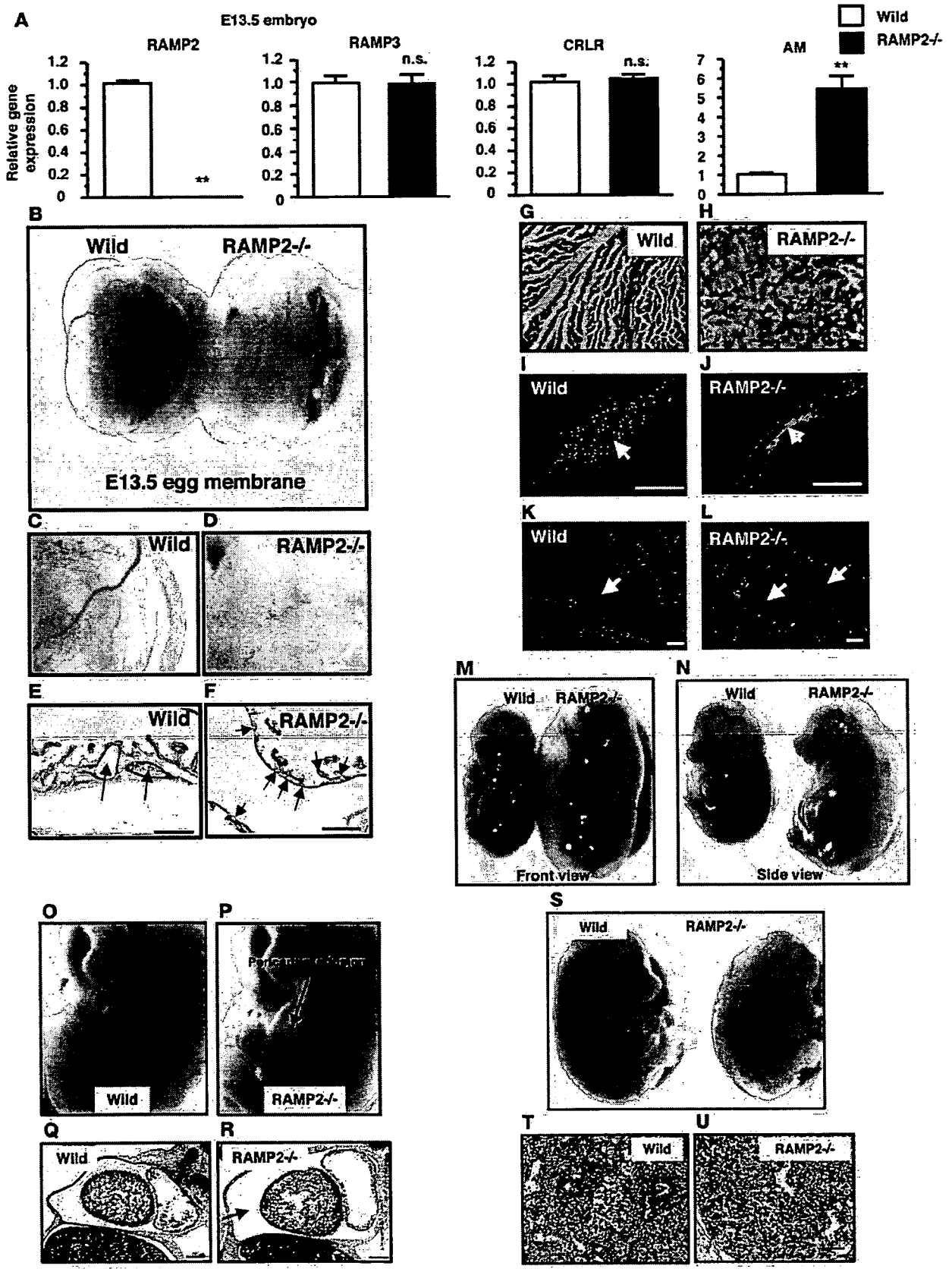
Vascular abnormalities and gene expression in RAMP2$^{-/-}$ *mice.* To determine whether the developmental abnormalities described above were the cause of the vascular fragility seen in *RAMP2*$^{-/-}$ embryos, we analyzed in detail the vascular structure at E12.5. Electron microscopic observation of 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
C
D
E
F
G
H
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J
K
L
M
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Front view
Side view
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Figure 2
Quantitative real-time PCR analysis, macroscopic analysis, and histology of *RAMP2*$^{-/-}$ embryos. (**A**) Gene expression of AM, CRLR, and RAMPs in E13.5 WT and *RAMP2*$^{-/-}$ embryos, assessed by real-time PCR of total RNA. No RAMP2 expression was detected in *RAMP2*$^{-/-}$ mice, confirming *RAMP2* was successfully destroyed. Conversely, RAMP3 expression did not differ between *RAMP2*$^{-/-}$ and WT mice, showing that the absence of RAMP2 did not induce compensatory upregulation of RAMP3 during development. AM expression was upregulated more than 5-fold in *RAMP2*$^{-/-}$ mice. $n = 6$ per group. $^{**}P < 0.01$ vs. WT. (**B–L**) Development of blood vessels in E13.5 WT and *RAMP2*$^{-/-}$ mice. Appearance of the yolk sac (**B**) and vitelline arteries (**C** and **D**). (**E** and **F**) CD31 immunostaining of sections of yolk sacs. Arrows indicate sections of vitelline arteries. (**G** and **H**) Whole-mount immunofluorescence staining of CD31 in yolk sacs. In **C–H**, vitelline arteries were well developed on the yolk sacs of WT mice but poorly developed on those of *RAMP2*$^{-/-}$ mice. (**I–L**) TUNEL staining of sections of vitelline artery (**I** and **J**) and umbilical vessel (**K** and **L**) in E13.5 WT and *RAMP2*$^{-/-}$ embryos. Apoptosis was visualized in green fluorescence. Arrows indicate vessel lumens. Some ECs in *RAMP2*$^{-/-}$ mice were TUNEL positive. (**M** and **N**) Severe systemic edema observed in *RAMP2*$^{-/-}$. Front (**M**) and side (**N**) views of WT and *RAMP2*$^{-/-}$ embryos at midgestation. Some *RAMP2*$^{-/-}$ embryos showed severe systemic edema. (**O–R**) Pericardial effusion in *RAMP2*$^{-/-}$ mice. (**O** and **P**) Magnified side view of embryos at midgestation revealing the appearance of the pericardial space in *RAMP2*$^{-/-}$ embryos. (**Q** and **R**) Sagittal sections showing the pericardial space in embryos at midgestation. The pericardial space was larger in *RAMP2*$^{-/-}$ than WT embryos and showed the accumulation of pericardial effusion. (**S–U**) Severe hemorrhagic changes in *RAMP2*$^{-/-}$ mice. (**S**) Side view of WT and *RAMP2*$^{-/-}$ embryos at midgestation. (**T** and **U**) Sections of the liver at the same stage. Some *RAMP2*$^{-/-}$ embryos showed severe hemorrhagic changes that were apparent on their surface and within the liver. Scale bars: 20 μm (**E** and **F**); 50 μm (**I–L**, **T**, and **U**); 200 μm (**Q** and **R**).

WT, 18.4 ± 1.4 mmHg; *RAMP2*$^{+/-}$, 13.3 ± 1.3 mmHg; $P < 0.05$, $n = 6$ per group). In contrast, CGRP-induced depressor effects did not differ in *RAMP2*$^{+/-}$ and WT mice (maximum percent change in systolic BP, WT, 15.9 ± 1.2 mmHg; *RAMP2*$^{+/-}$, 14.4 ± 1.3 mmHg; NS, $n = 6$ per group). Interestingly, AM expression was significantly upregulated in the aortas of *RAMP2*$^{+/-}$ mice, which suggests that reducing the number of functional AM receptors caused a compensatory upregulation of AM expression and implies that the AM-RAMP2 system is also important in the vascular function of adults. This prompted us to analyze the angiogenic properties of the AM-RAMP2 system in adult mice. We found that aortic ring explants cultured in collagen gel sprouted microvessels when stimulated with VEGF and that this

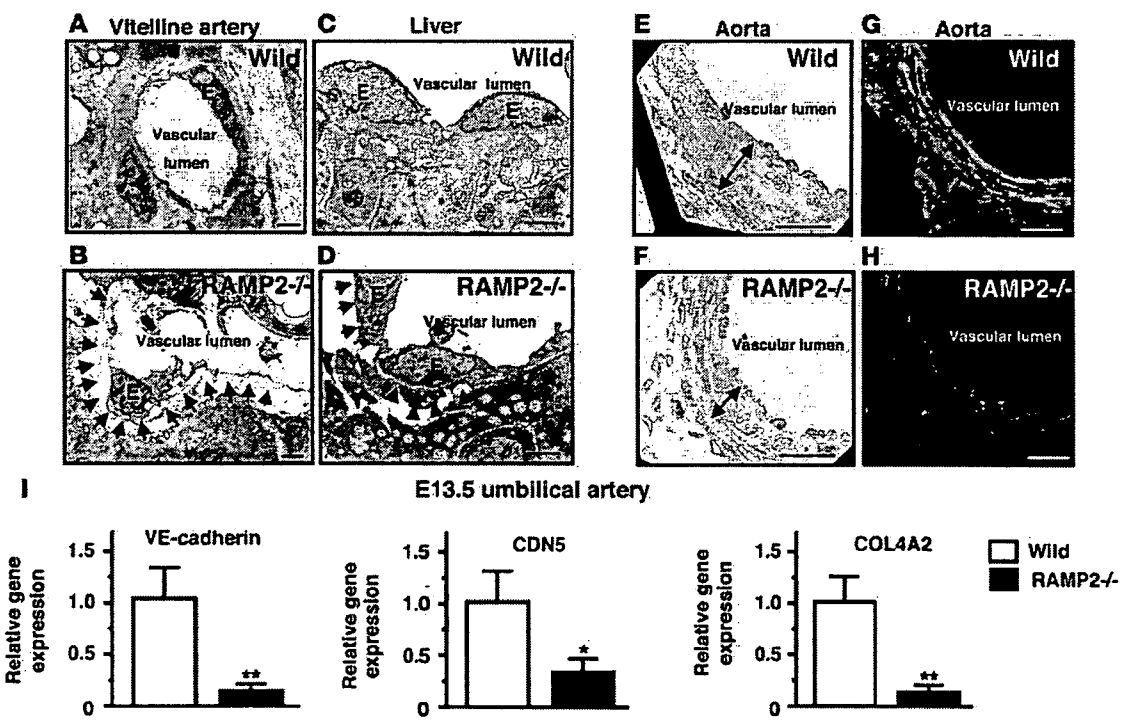

Figure 3
Abnormalities of vascular structure and gene expression in *RAMP2*$^{-/-}$ embryos. (**A–H**) Vascular structure of WT and *RAMP2*$^{-/-}$ embryos. Transmission electron micrographs of vitelline arteries (**A** and **B**), hepatic vessels (**C** and **D**), and aortas (**E** and **F**) from E12.5 *RAMP2*$^{-/-}$ and WT embryos. The vitelline arteries and hepatic vessels from *RAMP2*$^{-/-}$ mice showed the detachment of ECs (E) from basement membrane (arrows, **B** and **D**). In aortas from *RAMP2*$^{-/-}$ mice, the smooth muscle cell layer was thinner and rougher than in aortas from WT mice (double-headed arrows, **E** and **F**). (**G** and **H**) Immunohistochemical staining for type IV collagen and actin in aortas from WT and *RAMP2*$^{-/-}$ mice. Green, immunohistochemical staining using anti–mouse type IV collagen antibody; red, phalloidin (actin); blue, DAPI (nuclei). The structure of the smooth muscle cell layer and the basement membrane showed severe deformity in *RAMP2*$^{-/-}$ mice. (**I**) Quantitative real-time PCR analysis of gene expression in the umbilical artery from E13.5 embryos. Expression levels are shown relative to the level in WT embryos. VE-cadherin, CDN5, and α2 type IV collagen (COL4A2) expression was reduced in arteries from *RAMP2*$^{-/-}$ mice. $n = 6$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. WT. Scale bars: 2 μm (**A–D**); 25 μm (**E–H**).