in vivo and in vitro. We describe here the biological actions of AM in cardiovascular systems.

The basic characteristic effect of AM is a potent, long-lasting hypotension that is dose-dependent in several species, including humans. AM dilates resistance vessels in the kidney, brain, lung, hind limbs, and mesentery in animals. In conscious sheep, Parkes has reported details on the cardiovascular and hemodynamic changes induced by human AM [17]. AM produced a dose-dependent decrease in blood pressure accompanied by an increase in heart rate and cardiac output. Decreased peripheral resistance and blood pressure (BP) induce reflex tachycardia; however, the heart rate increases less than after other vasodilators inducing comparable hypotension.

Several papers show that the vasodilating effect by AM is abrogated by blockade of nitric oxide (NO) synthase activity with L-NAME, suggesting that the decrease in total peripheral resistance subsequent to AM infusion is in part due to NO generation [19]. AM activates endothelial nitric oxide synthase (eNOS) by at least two mechanisms. First, AM elevates the intracellular calcium level, which increases eNOS activity. Second, ADM activates phosphatidylinositol 3-kinase (PI3K) and protein kinase B/Akt, which phosphorylate eNOS and increase its activity even at low calcium concentration. However, the diminution of vasodilation by L-NAME seems to vary greatly from study to study. Pulmonary vasodilator responses are significantly reduced by L-NAME in rats, but L-NAME had no significant effect in the pulmonary vascular bed of the cat [15]. Hence, it appears that nitric oxide may at least be an important mediator for AM despite its regional and interspecies variation.

In cultured cardiac myocytes and fibroblasts, AM may inhibit the protein synthesis and hypertrophy of myocytes, proliferation of fibroblasts, and production of extracellular matrix. Because AM is synthesized and secreted by cultured cardiac myocytes and fibroblasts, this peptide may regulate myocardial hypertrophy and remodeling in arterial hypertension or heart failure in a paracrine/autocrine manner.

AM may bidirectionally regulate VSMC proliferation. It stimulates the proliferation of quiescent VSMC in the absence of other stimulating factors, but inhibits proliferation induced by PDGF or fetal bovine serum. AM inhibits endothelial cell apoptosis induced by serum deprivation. This effect is mediated by nitric oxide, but is cGMP-independent. In addition, AM stimulates the proliferation of endothelial cells that may be involved in angiogenesis and reendothelialization of injured vessels. Considering the results on vascular tissue expression of AM mRNA, AM seems to regulate vascular proliferation and remodeling as well as vascular tone.

TABLE 1. Cardiovascular Actions of Adrenomedullin.

Vasculature	Hypotension, antiproliferation, survival factor inhibition of Ca increase by endothelin, decreased endothelin production
Heart	Positive chronotropism and inotropism, increased coronary blood flow, increased ANP gene transcription, antimitogenesis, increased hypertrophy
Lung	Vasodilation, bronchodilation, antiinflammatory
Adrenal gland	Inhibition of aldosterone secretion
Kidney	Increased renal flow, diuresis, natriuresis, inhibition of mesangial cell proliferation
Pituitary	Inhibition of ACTH secretion, inhibition of AVP secretion
Brain	Increased collateralization and cerebral blood flow, inhibition of thirst and salt appetite, stimulation of sympathetic outflow (hypertension)

PROADRENOMEDULLIN N-TERMINAL 20 PEPTIDE

PAMP consists of 1–20 amino acids of proadrenomedullin (pro-AM) whose C-terminus is Arg-CONH2. The distribution of PAMP in mammalian tissues is similar to AM, consistent with their origin from a common precursor. The PAMP/AM ratio in tissue extracts and cell culture homogenates varies depending on the cell studied, from 1–2% in the lung to ~50% in the heart atria. PAMP elicits a potent hypotensive effect in anesthetized rats [6]. An intravenous bolus injection of human PAMP causes a rapid and strong hypotensive effect in a dose-dependent manner, although this peptide is less potent than AM.

PAMP is found to inhibit carbachol-induced catecholamine secretion in cultured bovine adrenal medullary cells, but AM showed no effect on catecholamine secretion. Fujita et al. demonstrated that AM infused into pithed rat showed hypotensive action dose-dependently but PAMP did not [20]. After BP was increased to a level of 80–100 mmHg by electrical stimulation of the pithed rat, PAMP exhibited hypotensive effects. Furthermore, during augmentation of peripheral sympathetic nerve activity with periarterial electrical stimulation, norepinephrine released in the perfusate was measured as an indication of neural transmission. PAMP decreased norepinephrine overflow dose-dependently, whereas AM did not. These results suggest that the hypotensive effect of PAMP may be due to the inhibition of neural transmission at nerve endings

rather than via a direct vasodilating effect. Recently, it has been reported that PAMP may antagonize the stimulatory effect of AM on endothelial NO production, suggesting a more complex role of this peptide in the regulation of vascular tone [12]. Very recently, it has been reported PAMP exhibits an extremely potent angiogenic potential. Exposure of endothelial cells to PAMP increases gene expression of other angiogenic factors such as adrenomedullin, vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived growth factor C.

Consequently, PAMP as well as AM is biosynthesized from the AM precursor and may participate in circulation control in different mechanisms (Fig. 2).

INFORMATION ON MOLECULAR FORM OF AM

The mature form of AM (mAM, AM[1–52] NH2) is produced by C-terminal amidation of glycine-extended AM, AM-glycine (AM-Gly). AM-Gly, an intermediate form of AM (iAM), is processed from proAM. Biological activity is exerted only by mAM. We have found that most of the circulating AM in human plasma is not mAM but iAM [8]. The plasma concentration of mAM was much lower than that of AM-Gly or of total AM (tAM), which is mAM and iAM. All forms of AM increased significantly as the severity of congestive heart failure increased [2]. The ratio of mAM/tAM did not differ significantly in any of the study groups, suggesting that the amidation process of AM is unaffected in the patients with congestive heart failure. On the other hand, the major immunoreactive AM in tissue or cell is determined to be mAM.

CLINICAL IMPLICATION OF AM IN CARDIOVASCULAR DISEASES

AM circulates in human blood at a considerable concentration, and the plasma AM levels were increased in patients with a variety of diseases, including congestive heart failure, renal diseases, hypertensive diseases, diabetes mellitus, and septic shock [1].

The plasma AM concentration in patients with essential hypertension or primary aldosteronism was significantly higher than that in normotensive controls. The plasma AM levels increase with the severity of hypertension [4]. Furthermore, in the patients with malignant hypertension and renovascular hypertension, the increase in plasma AM is marked in comparison with the increases in primary aldosteronism and essential hypertension. AM, therefore, seems to act as a humoral regulator in blood pressure control and to counteract pressor factors that may cause and promote hypertension. In patients with hypertension complicated with chronic renal failure, the increment of AM is obvious and the plasma AM concentration was elevated in relation to the degree of renal failure.

In patients with congestive heart failure, the plasma AM was significantly correlated with pulmonary artery pressure, pulmonary capillary wedge pressure, left atrial dimension, plasma renin activity, and plasma concentrations of atrial and brain natriuretic peptides. Intravenous infusion of human AM into patients with congestive heart failure predominantly improved cardiac function [14]. The AM elicited dilatation of the resistance arteries and increases in cardiac stroke index and urinary sodium excretion. An improvement of the cardiac pre- and after-loads and cardiac contractility elicited by AM is the mechanism for recovery of function of the failing heart.

Intravenous injection of lipopolysaccharide (endotoxin) produces a marked increase in plasma immunoreactive AM in a dose-dependent fashion in the anesthetized rat, suggesting AM may be involved in sepsis. Actually, plasma AM concentration was markedly increased in patients with septic shock. As suggested by an experimental model of septic shock in transgenic mice [21], the large amount of plasma AM observed in patients with septic shock may play a role in protecting against peripheral circulatory failure, organ damage, and mortality characteristic of endotoxic shock.

In summary, regarding the clinical features of a variety of diseases, the plasma and tissue concentrations of AM are increased in response to pathological conditions such as hypervolemia, hypertension, tissue ischemia or hypoxia, and inflammatory damage by cytokines. The increased plasma and tissue concentrations of AM seems to function as a counterregulator for pathologically altered circulation, tissue ischemia, or tissue injury by toxic factors through its humoral or paracrine-autocrine actions.

CONCLUSION

AM is a potent vasodilatory peptide discovered in 1993 from the extract of human pheochromocytoma. AM consists of 52 amino acids and belongs to the calcitonin, CGRP, and amylin family. In addition to AM, the AM precursor generates another bioactive peptide, proadrenomedullin N-terminal 20 peptide (PAMP). Though AM and PAMP show hypotensive effect, their mechanisms are different. During the 12 years since its discovery, over 1600 papers concerning AM and PAMP have been published. Considering the multifunctional characteristics of AM and significant increase in plasma

immunoreactive AM levels in patients with cardiovascular diseases, AM should be recognized as an important factor regulating circulation and involved in cardiovascular diseases.

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Isolation and identification of proangiotensin-12, a possible component of the renin–angiotensin system

Sayaka Nagata [a], Johji Kato [a,*], Kazuki Sasaki [b], Naoto Minamino [b], Tanenao Eto [a], Kazuo Kitamura [a]

[a] *Circulatory and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan*
[b] *National Cardiovascular Center Research Institute, Fujishirodai, Suita, Osaka 565, Japan*

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Abstract

The renin–angiotensin (RA) system plays an important role in regulating blood pressure and fluid balance. In the search for bioactive peptides with an antibody binding to the N-terminal portion of angiotensin II (Ang II), we isolated a new angiotensinogen-derived peptide from the rat small intestine. Consisting of 12 amino acids, this peptide was termed proangiotensin-12 based on its possible role of an Ang II precursor. Proangiotensin-12 constricted aortic strips and, when infused intravenously, raised blood pressure in rats, while both the vasoconstrictor and pressor response to proangiotensin-12 were abolished by captopril and by CV-11974, an Ang II type I receptor blocker. Proangiotensin-12 is abundant in a wide range of organs and tissues including the small intestine, spleen, kidneys, and liver of rats. The identification of proangiotensin-12 suggests a processing cascade of the RA system, different from the cleavage of angiotensinogen to Ang I by renin.

Keywords: Proangiotensin-12; Renin–angiotensin system; Pressor peptide; Angiotensinogen; Processing

A number of mechanical or humoral mechanisms are involved in blood pressure and body fluid homeostasis in mammals. Among them, the renin–angiotensin (RA) system plays a pivotal role, regulating the vascular tone of resistant vessels and glomerular filtration or re-absorption of electrolytes in the kidneys. The protease enzyme renin secreted from kidneys cleaves specifically angiotensinogen circulating in the blood to produce angiotensin I (Ang I), a peptide consisting of 10 amino acid residues, which in the presence of angiotensin-converting enzyme (ACE), is in turn converted to Ang II, a potent pressor peptide mediating the major actions of the RA system as a circulating hormone [1,2]. On the other hand, recent research has revealed new aspects of the RA system. For example, the tissue RA system has been vigorously studied, and its activation is assumed to be regulated independently of the systemic RA system [3–5]. Another example is the identification of Ang II-derived peptides that are shorter than Ang II, such as Ang(1–7), Ang III, or Ang IV, having pharmacological properties different from those of Ang II [6–11]. Meanwhile, consisting of 10 amino acid residues, Ang I has been thought to be produced by renin directly from angiotensinogen, a protein of 452 amino acids for humans or 453 for rats [12,13], but there has been no report on the occurrence of angiotensinogen-derived peptides of amino acid sequences longer than Ang I. In the present study, on searching for peptides structurally related to Ang II, we purified an angiotensinogen-derived pressor peptide of 12 amino acids, which is thought to be one of the major components of the RA system.

Materials and methods

Reagents and animals. Ang I and Ang II were purchased from Peptide Institute Inc. (Osaka, Japan) and proangiotensin-12 was synthesized by Bex. Co., Ltd. (Tokyo, Japan). CV-11974 was kindly provided by Takeda

* Corresponding author. Fax: +81 985 85 6596.
E-mail address: jkjpn@med.miyazaki-u.ac.jp (J. Kato).

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Pharmaceutical Company Limited (Osaka, Japan). Wistar rats of 6–7 weeks of age were purchased from Charles River Laboratories (Kanagawa, Japan) and New Zealand white rabbits from Kyudo Co., Ltd. (Saga, Japan). The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2004-100-2, 2004-101-2).

Detection of immunoreactive N-terminal Ang II. To prepare antiserum against the N-terminal of Ang II, synthesized Ang II-Cys was conjugated with *keyhole limpet* hemocyanin at room temperature over 2 h. After dialyses repeated four times in 50 mmol/L PBS, the conjugate solution was emulsified with an equal volume of Titer Max Gold (Sigma–Aldrich, Tokyo, Japan) and injected subcutaneously into New Zealand white rabbits every two weeks over three months. A specific radioimmunoassay (RIA) was prepared with the antiserum obtained from an immunized rabbit, as previously described [14,15]. This RIA cross-reacted with Ang I and Ang III at levels of 50% and 12.5%, respectively, without cross-reactions with Ang IV and Ang(1–7). To characterize the immunoreactive N-terminal Ang II in tissues, 1.0 g of sample from various rat tissues was immediately boiled for 10 min and acidified with acetic acid to a final concentration of 1.0 mol/L to inactivate proteases. Then the samples were homogenized and centrifuged for 20 min at 12,000 rpm, and the supernatant was applied to Sep-Pak C18 cartridges. After elution with 60% CH_3CN in 0.1% trifluoroacetic acid, the peptide extracts were subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) with an ODS-120A column, and the immunoreactive N-terminal Ang II of each fraction was measured by the RIA.

Purification procedure. The peptide extract was obtained with the above-described method from 380 g of small intestine, a rat tissue showing the largest peak of immunoreactive N-terminal Ang II at a position clearly different from that of Ang I or Ang II in the RP-HPLC analysis. The sample was applied to a SP Sephadex C-25 column eluted with 2.0 mol/L pyridine, and subjected to gel filtration with Sephadex G-50 and an affinity column (Affi-Gel 10 Active Ester Agarose, Bio-Rad) which had been prepared with the anti-N-terminal Ang II antiserum. These purification steps were done while monitoring immunoreactive N-terminal Ang II with the RIA. The extract was further purified by three steps of HPLC with columns of ODS-120A, diphenyl, and Chemco sorb3-ODS-H. The amino acid sequence of the finally purified peptide was analyzed by a time-of-flight mass spectrometric method.

Mass spectrometry. To determine the amino acid sequence and molecular weight of the purified peptide, a tandem mass spectrometric analysis was performed with positive electrospray ionization using a QTof-2 quadrupole time of flight mass spectrometer (Micromass, UK). The sample was dissolved in a solution of water/methanol/acetic acid mixed at 49:49:2 by volume and nanosprayed from off-line MS emitters (Proxeon, Denmark) with a capillary voltage of 1.5 kV and a cone voltage of 30 V. The mass spectrum was acquired from m/z 200 to 2000. For tandem mass spectrometry, the triply charged ion with m/z 524.97 was subjected to collision-induced dissociation with argon gas in the 30 eV collision energy range. Data acquisition and processing were performed using MassLynx v4.0 (Micromass). The resultant tandem mass spectrum was subjected to a Mascot MS/MS ion search (Matrixscience, UK) and also interpreted by PepSeq (Micromass).

Radioimmunoassay for the C-terminal portion of proangiotensin-12. To specifically detect proangiotensin-12 in tissues and plasma, we developed a RIA, as previously described, with antiserum raised against the C-terminal portion of the peptide [14,15]. Synthetic proangiotensin-12 was conjugated with bovine thyroglobulin at room temperature for 15 min by the glutaraldehyde method. New Zealand white rabbits were immunized with the dialyzed conjugate, according to the procedure described above. After the immunization, specific antibody was purified from the antiserum using an affinity column (NHS-activated Sepharose 4 Fast Flow) with the C-terminal peptide of proangiotensin-12, Ile-His-Pro-Phe-His-Leu-Leu-Tyr. The proangiotensin-12 levels in tissues and plasma of male Wistar rats were determined by RIA with the purified antibody, following extraction with a Sep-Pak C18 cartridge, as described previously [14,15]. This RIA detected 1.6 and 3.1% of angiotensinogen(1–14) and angiotensinogen(1–17), respectively, but showed no cross-reactivity with Ang I, Ang II, Ang III, Ang IV, or Ang(1–7). The Ang I or Ang II levels in tissues and plasma were similarly determined by RIAs with anti-C-terminal of Ang I and Ang II antisera purchased from Miles and Cortex Biochem, Inc. (San Leandro, USA), respectively [16,17]. To characterize the immunoreactive C-terminal proangiotensin-12 in the extract of rat small intestines, a RP-HPLC analysis was done with an ODS-120A column as described above.

Pharmacological studies ex vivo and in vivo. We examined the effects of proangiotensin-12 on vascular tone with perfused aorta isolated from rats as reported previously [18]. The aortic rings were mounted under a passive tension of 1.0 g in organ baths containing Krebs–Henseleit solution oxidized with 95% O_2 and 5% CO_2 at 37 °C, and left for 60 min for equilibration. The viability of the aortic rings was confirmed by exposing the samples to 60 mmol/L KCl, 10^{-7} mol/L phenylephrine, and 10^{-6} mol/L acetylcholine. Contractions of the aortic samples were recorded before and after Ang I, Ang II, or proangiotensin-12 was added to the perfusion solution at the indicated concentration. The responses to these peptides were also tested in the presence of 10^{-7} mol/L captopril, an ACE inhibitor, or 10^{-8} mol/L CV-11974, an Ang II type 1 (AT1) receptor antagonist. Next, the effects of proangiotensin-12 on blood pressure were examined *in vivo* with rats fed a normal rat chow, as previously described [19]. Male Wistar rats weighing 220–260 g were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Synthetic proangiotensin-12 dissolved in 100 μl saline was injected into the jugular vein at the bolus-dose indicated, and blood pressure and heart rate were monitored before and after the injection by a carotid artery catheter connected to a pressure transducer with the Power Lab system (MLT0699 Disposable BP Transducer, AD Instruments, Australia). The responses to proangiotensin-12 were similarly tested 2 min after the intravenous injection of 0.03 mg/kg captopril or 0.3 mg/kg CV-11974.

Statistical analysis. Comparisons of all data were made with an analysis of variance (ANOVA) followed by Scheffe's test. Values are presented as means ± SE and statistical significance was set at $P < 0.05$.

Results

Purification of proangiotensin-12

As a first step in the isolation of Ang II-related peptides, we analyzed immunoreactive N-terminal Ang II in peptide samples extracted from various tissues of rats with RP-HPLC and a RIA. In those analyses, we found three clear peaks of immunoreactive N-terminal Ang II in rat tissues including the small intestine (Fig. 1): the earliest peak and the next peak corresponded to Ang II and Ang I, respectively. Interestingly, the largest peak emerged later

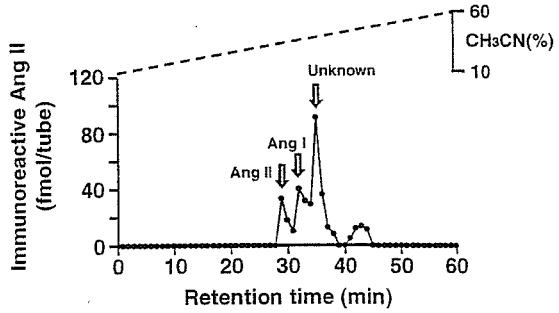

Fig. 1. Immunoreactive N-terminal Ang II in rat small intestine. An extract of the small intestine was subjected to RP-HPLC, where the immunoreactive N-terminal Ang II of each fraction was determined by specific radioimmunoassay, as described. An elution gradient of 10% to 60% CH_3CN was made in 0.1% trifluoroacetic acid over 60 min with a flow rate at 1 ml/min.

than Ang I in the small intestine, suggesting the presence of an unknown peptide similar in sequence to the N-terminal portion of Ang II. Therefore, we tried to purify this unknown peptide from 380 g of rat small intestine by several steps of ion-exchange, gel-filtration, and affinity chromatography, and RP-HPLC, as described in the Materials and methods section. Fig. 2A shows the final step of purification with RP-HPLC, where the unknown peptide was obtained as a single peak. The purified peptide sample was then subjected to the tandem mass spectrometry and found to consist of 12 amino acid residues (Fig. 2B). In comparison of the sequences of Ang I and the N-terminal portion of angiotensinogen, the unknown peptide was deduced to be a C-terminal extended form of Ang I (Fig. 3). We have termed this novel peptide proangiotensin-12, based upon the results of the tissue distribution and pharmacological experiments of the present study, suggesting a role for this peptide as a precursor of Ang II.

Measurement of tissue and plasma levels of proangiotensin-12

Next, we developed a radioimmunoassay specifically detecting the C-terminal portion of proangiotensin-12 to clarify the presence of this novel peptide in rat tissues including the small intestine. As shown in Table 1, proangiotensin-12 was abundantly detected in a variety of tissues, where its levels were higher than those of Ang I or Ang II, except for the lungs, adrenal glands, pancreas, and aorta. These tissue levels determined by the RIA specific to the C-terminal were found to be mostly identical to those estimated by an immunoreactive peak corresponding to proangiotensin-12 (Fig. 1) by RP-HPLC analyses with RIA detecting the N-terminal Ang II. In contrast to the tissue levels, the plasma concentration of proangiotensin-12 was lower than that of Ang I or

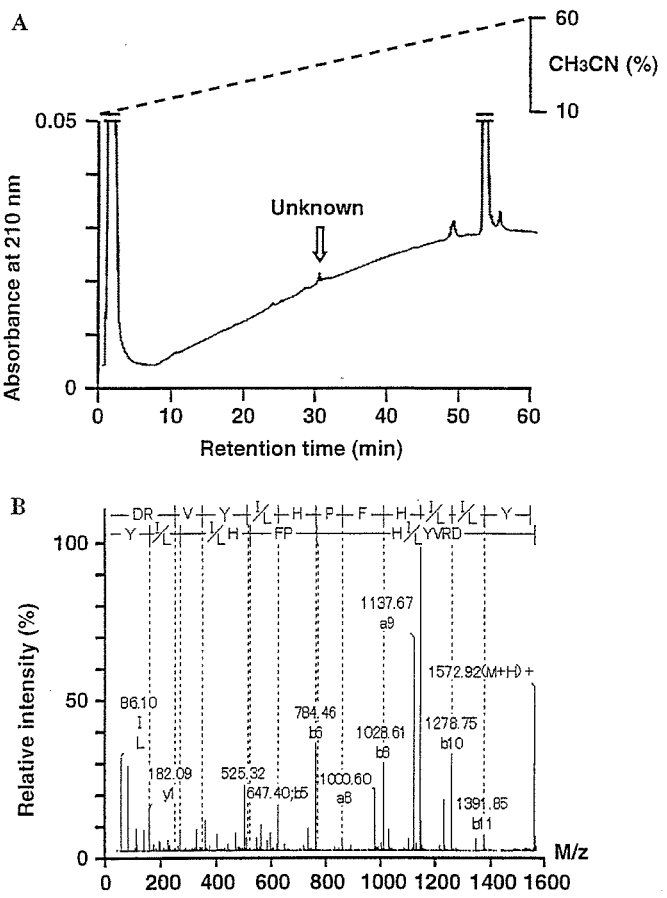

Fig. 2. Final purification by RP-HPLC (A) and tandem mass spectrometric analysis of the purified peptide (B).

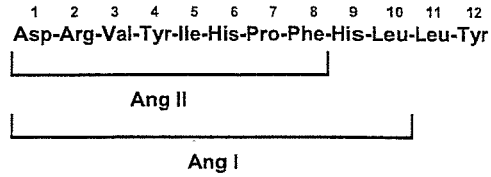

Fig. 3. Amino acid sequence of proangiotensin-12.

Table 1
Specific measurement of immunoreactive proangiotensin-12, Ang I, and Ang II levels in rat tissues and plasma

	Proangiotensin-12	Ang I	Ang II
Small intestine	663.1 ± 123.3	283.6 ± 43.0	254.9 ± 48.3
Spleen	419.1 ± 18.4	71.1 ± 4.0	302.0 ± 8.7
Kidneys	380.3 ± 27.0	365.2 ± 29.7	116.4 ± 6.9
Liver	252.0 ± 13.8	79.6 ± 2.6	87.9 ± 4.6
Stomach	241.7 ± 15.5	148.2 ± 23.0	81.5 ± 6.8
Lungs	227.8 ± 20.1	56.7 ± 5.7	236.2 ± 31.7
Adrenal glands	223.8 ± 8.2	121.4 ± 27.7	3049.2 ± 779.5
Heart	150.6 ± 11.3	84.9 ± 7.8	42.3 ± 7.3
Brain	147.4 ± 8.1	52.0 ± 2.7	29.6 ± 3.6
Pancreas	87.2 ± 13.8	37.8 ± 11.6	93.0 ± 17.6
Aorta	24.1 ± 7.5	39.6 ± 2.4	118.5 ± 6.6
Plasma	10.1 ± 2.7	382.5 ± 79.9	28.9 ± 8.8

The tissue or plasma levels of proangiotensin-12 were determined by a radioimmunoassay specifically detecting the C-terminal portion of the peptide as described in the Materials and methods section. The tissues are listed in order of immunoreactive proangiotensin-12 levels. The results are shown as means ± SE for eight rats examined (fmol/g tissue or fmol/ml plasma).

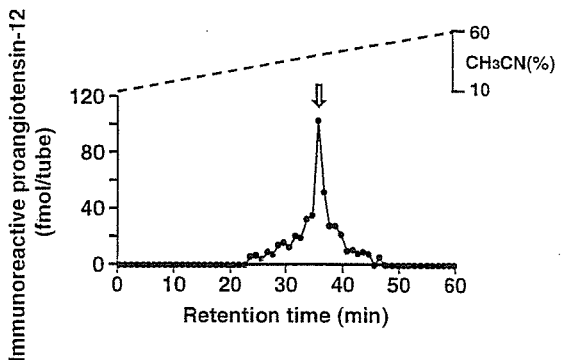

Fig. 4. Characterization of immunoreactive C-terminal proangiotensin-12 in rat small intestine by RP-HPLC and radioimmunoassay. RP-HPLC was conducted as described in the legend for Fig. 1. The arrow indicates the position of the full-length synthetic proangiotensin-12 peptide.

Ang II. The HPLC analysis revealed that immunoreactive proangiotensin-12 in the small intestine was eluted at the same position as the complete synthetic peptide (Fig. 4), further confirming the amino acid sequence and presence of proangiotensin-12.

Pharmacological studies ex vivo and in vivo

To study the biological actions of proangiotensin-12, we first looked at the effects on the vascular tonus of perfused rat aortic rings *ex vivo*. As shown in Fig. 5, proangiotensin-12 dose-dependently constricted the rat aorta as did Ang I or Ang II. Ang I and proangiotensin-12 had weaker vasoconstrictor effects than Ang II at concentrations of 3–30 nmol/L, but similar maximum contractions were obtained at 100 nmol/L of all three peptides. The constrictor action of proangiotensin-12 was mostly abolished in the presence of captopril, an ACE inhibitor, or CV-11974, an AT1 receptor blocker. Next, we examined the effects of proangiotensin-12 on blood pressure levels *in vivo* in anesthetized rats. A rise in arterial blood pressure was observed immediately after the intravenous injection of a bolus of proangiotensin-12 in rats, with a return to the basal level in 3–4 min (Fig. 6A). The pressor effects were dose-dependent and attenuated by pre-administration of captopril or CV-11974 (Figs. 6B and C), a result consistent with the *ex vivo* study.

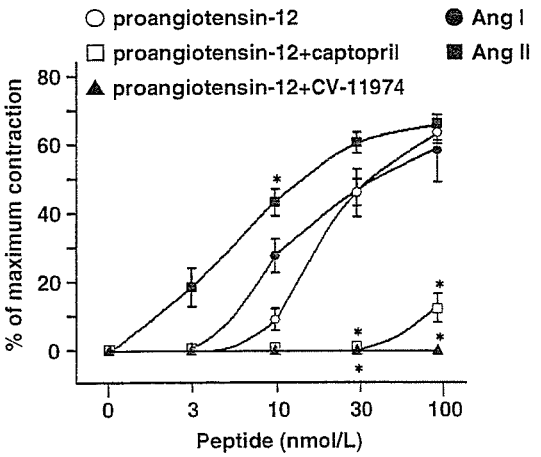

Fig. 5. Vasoconstrictor effects of Ang I, Ang II, and proangiotensin-12 on perfused rat aorta *ex vivo*. Captopril and CV-11974, an AT1 receptor antagonist, were used at 10^{-7} and 10^{-8} mol/L, respectively. The results are shown as means ± SE of % of maximum contraction induced by 60 mmol/L KCl for six to seven samples. $^{*}P < 0.05$ vs proangiotensin-12 alone.

Discussion

In this report, we describe the purification, sequence determination, tissue distribution, and vasoconstrictor properties of the novel angiotensinogen-derived peptide, Ang I-Leu-Tyr, which consists of 12 amino acid residues. We have termed this novel peptide proangiotensin-12, based upon the present results that suggest its role as a precursor of Ang II.

A concern may be raised over non-specific cleavage between Tyr-12 and Ser-13 of rat angiotensinogen, resulting in the occurrence of proangiotensin-12 as a non-specifically fragmented product during the extraction procedure; however, this possibility is unlikely based on the following reasons or findings. First, samples of rat small intestine were immediately boiled and acidified after resection in

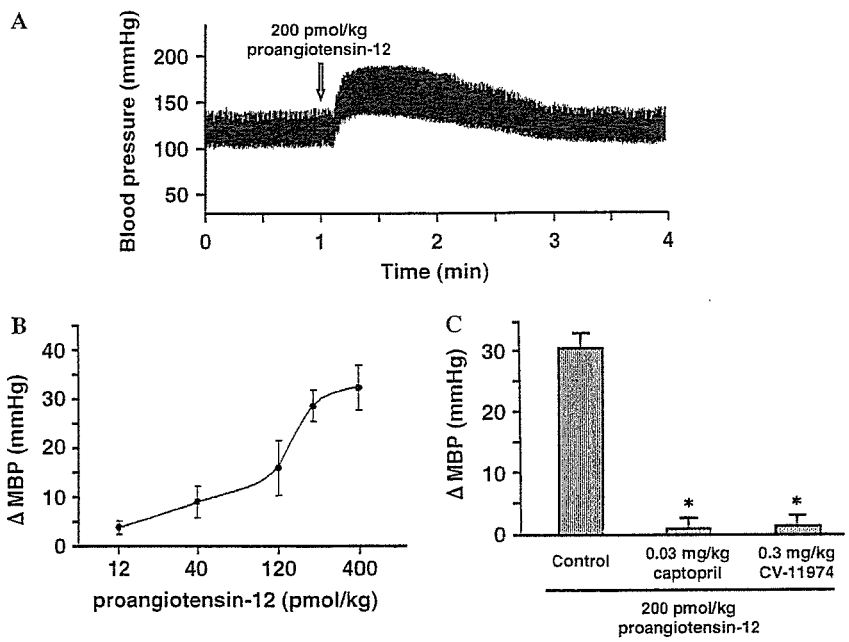

Fig. 6. (A) Representative blood pressure recording following the intravenous injection of a 200 pmol/kg bolus of proangiotensin-12 in anesthetized rats, (B) dose-dependent pressor effects, and (C) attenuation of the effects of proangiotensin-12 by captopril or CV-11974, an AT1 receptor blocker. ΔMBP indicates the maximum rise of mean blood pressure from the baseline. Either captopril or CV-11974 was intravenously injected at the indicated dose 2 min before the injection of proangiotensin-12. The results are shown as means ± SE for five rats examined. $^{*}P < 0.05$ vs proangiotensin-12 alone.

the extraction procedure. Non-specific cleavage of peptides can be avoided with this step which inactivates enzymes with proteolytic activity [20,21]. Second, the HPLC analysis of immunoreactive N-terminal Ang II (Fig. 1) showed three clear peaks, which correspond to Ang II, Ang I, and proangiotensin-12, without fragmentation of peptides. The specific radioimmunoassay (Table 1) also revealed that proangiotensin-12 is ubiquitously present in various organs and tissues, as are Ang I and Ang II. Last, the immunoreactive proangiotensin-12 in the small intestine was eluted at the position of the full-length peptide in the RP-HPLC analysis and radioimmunoassay detecting the C-terminal portion of proangiotensin-12 (Fig. 4). These findings are clearly indicative of the endogenous occurrence of proangiotensin-12.

A number of mechanisms, either mechanical or humoral, have been known to be involved in regulating blood pressure and body fluid balance in mammals. Among the humoral factors, the RA system plays a pivotal role: renin secreted mainly from kidneys cleaves angiotensinogen circulating in the blood to produce Ang I, which is then converted to Ang II exerting various effects particularly important for blood pressure and body fluid homeostasis. In the meantime, recent progress in research has revealed new aspects of the RA system. One example is identification of the Ang II-derived bioactive peptides with amino acid sequences shorter than Ang II such as Ang(1–7), Ang III or Ang IV [6–11]. Given this fact, it should be noted that proangiotensin-12 is a peptide longer than Ang I and such an angiotensinogen-derived peptide had not been identified. The second example is an active role of the tissue RA system probably independent of the systemic RA system [3–5]. In the present study, radioimmunoassay specifically detecting the C-terminal portion of proangiotensin-12 revealed ubiquitous presence of this novel peptide in various tissues and organs at the concentrations comparable with those of Ang I and Ang II, while the plasma concentration was lower than that of Ang I or Ang II. The relatively higher tissue levels suggest a significant role of proangiotensin-12 as a molecule of the tissue RA system.

In the present study, proangiotensin-12 exerted vasoconstrictor activity *ex vivo* on rat aortic rings, potency of which was similar to Ang I, while somehow weaker than Ang II at concentration of 3–30 nmol/L, showing the maximum contraction similar to Ang II. When injected intravenously in rats, proangiotensin-12 immediately raised blood pressure levels, and both the vasoconstrictor and pressor effects mostly disappeared following administration of captopril or CV-11974, an AT1 receptor blocker. These findings suggest prompt conversions of proangiotensin-12 to Ang I, and then Ang I to Ang II by ACE. Although the enzymes involved in the conversion to Ang I remain to be identified, the prompt cleavages of proangiotensin-12 to produce Ang II support the hypothesis of a significant role of proangiotensin-12 as an important molecule of the RA system.

Angiotensinogen is recognized to be produced and supplied to the blood mainly by the liver, though this precursor protein has been shown to be expressed widely in other tissues [22–25]. Consistent with this, according to our quantitative PCR, angiotensinogen mRNA was detected in various rat organs and tissues including the small intestine with the highest level in liver (data not shown). Because renin is an exclusively specific enzyme which cleaves angiotensinogen directly to produce Ang I, it is unlikely that renin is involved in the production of proangiotensin-12 [1,2]. An important question related to this is where the cleavage of angiotensinogen to proangiotensin-12 occurs: in tissues or in plasma. Clarifying the processing cascade of angiotensinogen would therefore provide us with information not only on the production of proangiotensin-12 but also on the mechanism activating the RA system and the role of proangiotensin-12 in blood pressure and body fluid homeostasis. Indeed, we are currently working on characterization and purification of enzymes that produce or cleave proangiotensin-12. Additionally, it should be clarified whether or not Ang I precursor peptides similar to proangiotensin-12 are present in other species including humans. Thus, the identification of proangiotensin-12 warrants future research on the RA system, which should be aimed at addressing a number of these unanswered questions.

Acknowledgments

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Roles of Adrenomedullin in Hypertension and Hypertensive Organ Damage

Johji Kato*, Kazuo Kitamura and Tanenao Eto

Circulatory and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan

Abstract: Adrenomedullin (AM) is a bioactive peptide having a wide range of biological actions such as vasodilatation, natriuresis, diuresis and inhibition of aldosterone secretion. Various organs and tissues, including the myocardium, vascular wall and kidneys, produce AM, and AM is also present in the bloodstream. Plasma levels of AM were elevated in patients with essential or secondary hypertension as compared with normotensive controls. When infused in a relatively short period of time, AM reduced blood pressure in humans and experimental animals largely through vasodilatation. The blood pressure of transgenic mice overexpressing AM was lower than that of their wild-type littermates; while heterozygotes of AM knockout mice showed higher blood pressure, suggesting a role for endogenous AM in the regulation of blood pressure. Studies with cultured cardiac cells suggest a role for AM in inhibiting hypertrophy or fibrosis of the heart as an autocrine or paracrine factor. Animal experiment studies showed that either prolonged infusion or virally mediated overexpression of AM ameliorated the cardiovascular and renal damage associated with hypertension. Thus, a body of evidence accumulated in this field suggests that AM functions to counteract the elevation in blood pressure and progression of hypertensive organ damage.

Key Words: Adrenomedullin, hypotensive peptide, hypertension, end organ damage.

INTRODUCTION

In mammalians, blood pressure and the water-electrolyte balance are regulated by a number of neurohumoral factors, among which, peptide hormones including angiotensin II, endothelins and natriuretic peptides have a particular importance. These peptides have been shown to not only participate in the mechanism regulating blood pressure but also modulate progression of the cardiovascular and renal diseases associated with elevations of blood pressure. In 1993, a new peptide hormone, adrenomedullin (AM), was isolated from tissue extract of human pheochromocytoma by monitoring cAMP levels in rat platelets [1]. Substantial levels of the AM peptide and gene expression were detected in the heart, blood vessels and kidneys, and AM was found to exert a wide range of biological actions related to cardiovascular and renal functions such as vasodilatation, natriuresis and inhibition of aldosterone secretion [1-3]. Ever since the discovery of AM, efforts have been made to clarify the role of this bioactive peptide and a substantial amount of basic and clinical data has been accumulated. In this review, after summarizing the biochemical and pharmacological features of AM, we comprehensively discuss the pathophysiological role of AM in hypertension and hypertensive organ damage.

BIOCHEMISTRY OF AM

Human AM is a 52-amino-acid peptide with a ring structure formed by a disulfide bond and amidated tyrosine at the C-terminal (Fig. (**1**)), portions essential for the binding to receptors and biological actions [1-3]. Based on sequence homology, AM is thought to belong to the calcitonin gene-related peptide (CGRP) superfamily [1-3]. Cloning of the cDNA encoding AM revealed the sequence of the AM precursor peptide preproAM of 185 amino acids, in which the C-terminal of AM is found to be followed by a pair of basic amino acids, Arg-Arg, a typical processing signal (Fig. (**2**)) [4]. In addition to AM, preproAM was found to contain another bioactive peptide, proadrenomedullin N-terminal 20 peptide (PAMP), in the N-terminal portion [4]. PAMP lowered blood pressure when injected intravenously, but the hypotensive action is weaker than that of AM and there has currently been little information as to the role of PAMP in regulating blood pressure [3, 4]. Ishimitsu *et al.* reported the sequence of genomic DNA for human preproAM [5], where AM is encoded in the fourth exon and PAMP in the second and third exons (Fig. (**2**)). When processed from preproAM, AM-Gly, an intermediate form (iAM), is produced, and then, iAM is converted by amidation enzyme to the mature form of AM (mAM) having an amide structure at the C-terminal [6]. The mature form of PAMP is thought to be produced by a similar process (Fig. (**2**)).

BIOLOGICAL ACTIONS OF AM

Soon after the discovery of AM peptide, a number of pharmacological studies were performed to see the biological actions of AM in experimental animals or human subjects [2, 3]. As listed in Table **1**, AM has been shown to exert a wide range of biological effects in various organs and tissues. Some of them are thought to occur at physiological levels of AM in plasma or tissue, but it is unclear whether or not the others do. Nonetheless, one can realize that many of the effects of AM are closely related to the regulation of blood pressure and the body fluid-electrolyte balance. We summarize the biological effects of AM below, based mainly on the results of pharmacological studies. The cardiovascular pro-

*Address correspondence to this author at the Circulatory and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan; Tel: 81-985-85-0872; Fax: 81-985-85-6596; E-mail: jkjpn@med.miyazaki-u.ac.jp

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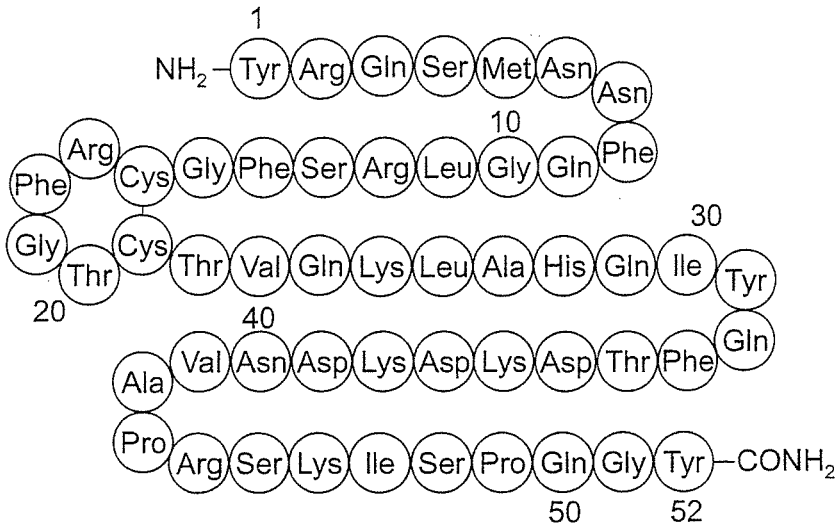

Fig. (1). Amino-acid sequence of human AM.

tective effect and long-term blood pressure-modulating effect of AM are discussed later in this review.

Blood Vessels

AM was initially characterized as having a potent, long-lasting blood pressure-lowering effect with reduced peripheral resistance following intravenous bolus injection or infusion in a relatively short period of time [1, 7, 8]. The hypotensive effect of AM observed in the short-term was shown to be largely secondary to direct vasodilatation [7, 8], which was further demonstrated by studies *ex vivo* with isolated rat aorta and with perfused rat mesenteric artery [9, 10]. Endothelium denudation abolished the vasodilator action of AM in a rat aortic ring preparation, indicating an endothelium-dependent vasodilatation [10]. Consistent with this, AM was found to dilate blood vessels *via* the nitric oxide and cyclic GMP (NO-cGMP) pathway; but there has been some inconsistency regarding the role of this intracellular pathway [10-14]. For example, Nossaman *et al.* reported that pulmonary vasodilator responses were reduced by N-nitro-L-arginine methyl ester (L-NAME) in rats, but not in cats [14]. The mechanism by which AM dilates blood vessels may differ depending on species or possibly on the regions in which the vessels are isolated from [10-14].

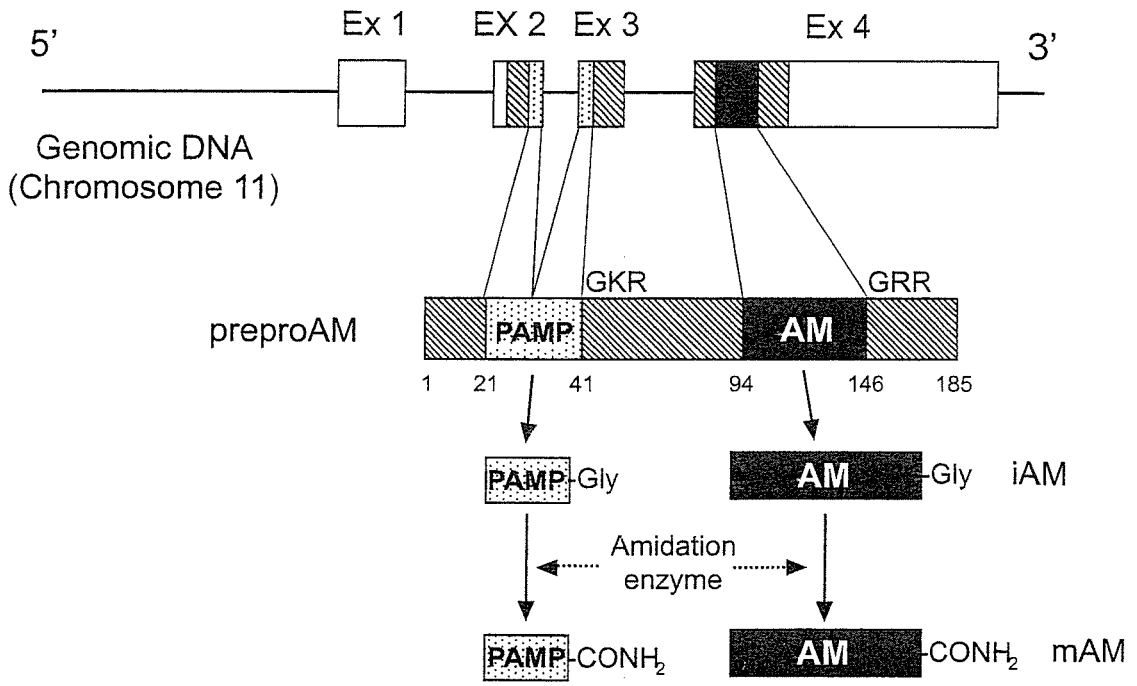

Fig. (2). Schematic representations of the AM gene and of processing of AM and PAMP from preproAM. Ex, exon: PAMP, proadrenomedullin N-terminal 20 peptide; iAM and iPAMP, intermediate forms of AM and PAMP; mAM and mPAMP, mature forms of AM and PAMP.

Table 1. Biological Actions of AM

Blood vessels	Vasodilatation Stimulation of nitric oxide (NO) production Inhibition of proliferation and migration of smooth muscle cells Inhibition of apoptosis of vascular endothelial cells Inhibition of endothelin production
Heart	Inhibition of myocardial hypertrophy and fibrosis
Kidney	Augmentation of urinary output and sodium excretion
Endocrine system	Inhibition of aldosterone production Inhibition of ACTH and vasopressin secretion Inhibition of insulin secretion
Central nervous system	Elevation of blood pressure Inhibition of dringking and salt intake

Heart

When injected intravenously, AM increased heart rate and cardiac output through the sympathetic nerve system activated by a reduction of blood pressure [15, 16]. In addition, AM has been reported to have direct actions on cardiac muscle [17, 18]. Parkes *et al.* showed a possible, positive inotropic action of AM in sheep [17], and this was supported by experiments *ex vivo* using rat myocardium [18]. In contrast, Ikenouchi reported that AM exerted a negative inotropic action toward cultured cardiac myocytes isolated from rabbit hearts [19]. These inconsistencies may have resulted from differences in the experimental conditions or species used.

Kidneys

When infused into experimental animals, AM has been shown to increase urinary volume and sodium excretion, increasing renal blood flow, with little or no change in the glomerular filtration rate [20, 21]. Ebara *et al.* reported a natriuretic action of AM resulting from inhibition of tubular sodium reabsorption in rats presumably at a level of AM in plasma within the physiological range [22]. According to an experiment *ex vivo* by Hirata *et al.*, AM reduces renal vascular resistance, dilating both afferent and efferent arterioles, and this renal action appears to be mediated by the cAMP and NO-cGMP pathways [23]. Consistent with these findings are the diuresis and natriuresis observed in a clinical setting following intravenous infusion of AM [24].

Endocrine System

Samson *et al.* showed that AM inhibited the basal and corticotropin releasing hormone-stimulated secretion of adrenocorticotropic hormone (ACTH) from cultured rat anterior pituitary cells [25]. *In vivo*, AM inhibited the release of arginine vasopressin release induced by hyperosmolality and by hypovolemia in conscious rats [26]. Yamaguchi *et al.* reported an inhibitory effect of AM *in vitro* on the secretion of aldosterone induced by angiotensin II or potassium from rat adrenal zona glomerulosa cells [27]. The inhibition of aldosterone secretion by AM was confirmed with experiments *in vivo* by the same group [28]. In isolated pancreatic islets of rats, AM had an inhibitory effect on insulin secretion, which was reversed by blocking endogenous AM with a monoclonal antibody, suggesting an autocrine or paracrine role in islet cells [29].

Central Nervous System

In contrast to peripheral infusion, the intracerebroventricular administration of AM produced a pressor response with an increased heart rate in rats [30-32]. The responses were attenuated by pretreatment with an α-adrenergic blocker, suggesting an action mediated by activation of the sympathetic nerve system [31]. AM has also been shown to centrally modulate water and salt intake [33-35]. Samson *et al.* found an inhibitory effect of centrally administered AM on the drinking of water induced by angiotensin II or dehydration in rats [33]. According to this group, the appetite for salt of volume-depleted rats was inhibited by AM, and conversely, augmented following the central injection of an anti-AM antibody or an antisense oligonucleotide of AM mRNA [34, 35], a finding indicative of the role of endogenous AM. The mechanisms of the central actions of AM remain to be clarified, though these findings imply a role for AM in central modulation of blood pressure or the water-electrolyte balance.

AM Actions in Human Studies

Blood pressure-lowering effects accompanied by an increased heart rate and cardiac output have been observed following intravenous infusion of AM in a relatively short period of time in humans [16, 36]. In those studies, plasma aldosterone levels remained unchanged despite increased plasma renin activity [16], a finding consistent with the *in vitro* and animal experiments showing an inhibitory effect of AM on aldosterone secretion [27, 28]. Troughton *et al.* found that the blood pressure-lowering effect of AM was greater in patients with essential hypertension than in normotensive controls [37]. Conversely in patients with congestive heart failure, both the hypotensive and renal responses to AM were found to be small, compared with those in control subjects [24]. Meanwhile, McGregor *et al.* reported that the hypotensive and renal responses to AM were not necessarily attenuated in patients with chronic renal failure secondary to IgA nephropathy, despite the increased plasma levels of AM and reduced renal function, as compared with those of healthy

volunteers or uncomplicated essential hypertensive patients [38].

Receptors Mediating AM Actions

Many of the AM actions were shown to be mediated by intracellular cAMP, though the subtype of AM receptors has been controversial [2, 3]. McLatchie *et al.* identified three subtypes of receptor-activity-modifying protein (RAMP1 to 3), an accessory protein required for the transport of calcitonin-receptor-like receptor (CRLR) to the cell membrane [39]. CRLR can function as either an AM receptor or a calcitonin gene-related peptide (CGRP) receptor, depending on co-expression with the subtype of RAMP: CRLR serves as a CGRP receptor when coexpressed with RAMP1, while it functions as an AM receptor when coexpressed with either RAMP2 or 3 [39]. Meanwhile, not all the actions of AM can be fully explained by this receptor system linked to adenylate cyclase: some have been shown to be independent of cAMP [40, 41]. This suggests the presence of unknown receptor systems and further studies are required to clarify the intracellular signaling systems for AM.

PRODUCTION OF AM IN VARIOUS ORGANS OR TISSUES AND CIRCULATING AM IN THE BLOODSTREAM

AM was initially isolated from pheochromocytoma tissue, but subsequently, the AM gene was found to be expressed in various organs and tissues in humans as well as in rats: those are adrenal glands, cardiac atria and ventricles, kidneys, blood vessels and lungs [4, 42, 43]. Consistent with the gene expression, AM was found to be produced and secreted from cultured cells isolated or derived from these organs and tissues [43-50]. For example, cultured vascular endothelial and smooth muscle cells have been shown to express the AM gene, secreting AM peptide into culture media [43, 44]. We previously reported the production of AM in cultured cardiomyocytes and cardiac fibroblasts isolated from ventricles of neonatal rats [45, 46]. In addition, a number of types of cultured renal cells, such as mesangial cells and tubular cell lines derived from different species, were found to produce and secrete AM [47-49]. The secretion from cultured cells isolated from the organs and tissues where AM has been shown to exert biological actions, suggests a role as an autocrine or paracrine factor [45-47, 50]. Another important finding we should mention is that both AM gene expression and AM peptide secretion in these cultured cells increased in response to mechanical stress and humoral factors, such as angiotensin II and endothelin-1, which not only participate in regulating blood pressure but also are involved in the development of end organ damage associated with hypertension [45, 46, 50]. The factors affecting AM production will be discussed under the section "Production of AM in animal models of hypertension".

Radioimmunoassays for AM revealed that immunoreactive AM was circulating in the blood at mean plasma levels ranging from 2.4 to 10 fmol/ml in healthy human subjects [51-54]. Immunoreactive AM in plasma or tissues consists of two molecular forms: a mature form with an amidated C-terminal (mAM) and an intermediate form with an unamidated glycine-extended C-terminal (iAM) (Fig. (**2**)) [6]. In the process producing mAM, iAM is cleaved from the AM precursor peptide, and then converted to mAM by amidation enzyme [6]. The major molecular form of AM in plasma was found to be iAM, while that in tissues is mostly mAM [6, 51-55]. As detailed in the next section, plasma levels of immunoreactive AM have been shown to be elevated in patients with hypertension, while both mAM and iAM levels are elevated without a notable difference in the ratio of mAM to total AM (tAM), which is the sum of mAM and iAM, when compared with control subjects [53]. Similarly, there was found to be no significant difference in the ratio of mAM to tAM between Dahl salt-sensitive hypertensive and control rats [56]. iAM is thought to have no biological effects by itself, while our *ex vivo* study showed that iAM dilated rat aorta following its conversion to mAM probably in the aortic wall [57]. In any case, very little information is currently available as to the role of iAM, which should be clarified further with experiments *in vivo*.

PLASMA AM IN ESSENTIAL HYPERTENSION

The biological action originally ascribed to AM was a blood pressure-lowering effect largely due to potent vasodilatation [1, 7], therefore initial interest focused on the role of this bioactive peptide in the regulation of blood pressure. Measurements with specific radioimmunoassays revealed plasma levels of AM to be higher in patients with essential hypertension than in normotensive controls [58, 59]. Cross-sectional, observational studies showed that the increase was related to blood pressure levels and to the severity of damage to target organs [58-60]. Kohno *et al.* demonstrated that plasma AM levels in untreated hypertensive patients remained unchanged after treatment with calcium channel blockers, despite a reduction in blood pressure [61]. Instead, they found an intimate relationship between the plasma AM and serum creatinine levels, which suggests that an increase in the plasma AM concentration is associated with impaired renal function [61]. In addition, a single administration of angiotensin converting enzyme (ACE) inhibitor lowered blood pressure in a short period of time, but the plasma AM level remained unchanged [62]. Sumimoto *et al.* reported that the plasma AM level of hypertensive patients complicated with left ventricular hypertrophy (LVH) was higher than that in those without LVH, despite the similar blood pressure levels in the two groups of patients [63]. Thus, the increased plasma AM levels in patients with essential hypertension seem to be correlated not directly with blood pressure levels but with organ damage associated with the elevation of blood pressure. This, however, is not the case in patients with a more severe form of hypertension: plasma AM levels in patients with malignant hypertension rose to a much higher level, and dropped, along with blood pressure levels, following antihypertensive treatment within one to three weeks [60].

Because AM peptide was abundantly detected in the adrenal medulla, we measured plasma AM concentrations of the adrenal vein, but found no step-up compared with the inferior vena cava [64]. Moreover, Nishikimi *et al.* were unable to find any site in the blood vessels showing a higher plasma AM concentration in patients with ischemic heart disease, though a step-down was observed in the aorta compared with pulmonary artery [65]. According to our previous

study with patients with ischemic heart disease, significant step-ups were observed in plasma mature AM (mAM) levels between the femoral artery and vein and between the aortic root and coronary sinus [66]. These data were not necessarily from patients with essential hypertension, though various organs or tissues including the heart and vasculature may contribute to plasma AM in hypertensive patients.

PLASMA AM IN PATIENTS WITH PHEOCHROMOCYTOMA AND OTHER FORMS OF SECONDARY HYPERTENSION

In addition to essential hypertension, there has been a number of reports on plasma AM levels in patients with secondary hypertension. Interest was aroused in plasma AM levels of patients with pheochromocytoma, in which AM was originally isolated and abundant AM expression is detected [1, 4]. Unexpectedly, plasma AM levels were not necessarily higher in patients with pheochromocytoma than those with essential hypertension [65]. Moreover, no elevation of plasma AM was observed during the paroxysms of this disease despite the elevation of blood pressure and plasma catecholamine levels [65]. This appears consistent with the notion that the adrenal medulla is not the major site secreting AM, but rather multiple organs or tissues contribute to the plasma AM level.

The plasma AM levels are higher in patients with primary aldosteronism, renovascular hypertension and Cushing's syndrome than controls, as they are in those with essential hypertension [62, 64, 67]. A significant positive correlation was found between the plasma AM level and mean blood pressure in patients with primary aldosteronism [64]. Meanwhile, no significant reduction in the plasma AM level was observed following surgical resection of an aldosterone-producing adrenal adenoma despite reduced blood pressure [62], a finding comparable with the effect of anti-hypertensive treatment on plasma AM levels in essential hypertensive patients [61].

PLASMA LEVELS OF AM IN CARDIOVASCULAR AND RENAL DISEASES ASSOCIATED WITH HYPERTENSION

Heart Failure

It has been demonstrated that plasma AM levels are increased in patients with congestive heart failure as compared with controls [68-70]. Interestingly, in comparison with other humoral factors or hemodynamic parameters, the plasma AM level was significantly correlated with pulmonary capillary wedge pressure, pulmonary artery pressure, plasma renin activity (PRA) and plasma levels of atrial and brain natriuretic peptides (ANP and BNP) [69, 70]. The elevated plasma levels gradually decreased following successful treatment, together with the levels of ANP and BNP, in patients with heart failure [71]. Kobayashi *et al.* subgrouped patients with chronic heart failure based on the primary cause of heart disease, but failed to find any particular heart disease in which the plasma AM level rises higher than in the others [70]. Thus, the increased levels appear to be closely related to the degree of depressed cardiac function, not to the primary cause of heart failure.

Arteriosclerosis

There has also been a number of reports showing increased plasma AM levels in patients with arteriosclerotic vascular disorders [72-75]. Kuwasako *et al.* found a possible association between plasma AM levels and endothelial damage by comparing the plasma levels of AM with those of endothelin and thrombomodulin, markers of endothelial damage, in patients with cerebrovascular disease [72]. Similarly, in patients with chronic ischemic stroke, the increased plasma AM level was shown to be associated with the degree of carotid atherosclerosis [73]. Recently, Suzuki *et al.* reported that the plasma AM concentration was elevated in patients with peripheral arterial occlusive disease in proportion to its severity [74]. It has been recognized that arterial stiffness is an important cardiovascular risk factor. Kita *et al.* measured plasma AM levels in patients with various degrees of atherosclerosis and compared the plasma levels with indirectly measured pulse wave velocity, a parameter often used to assess arterial stiffness and sclerosis [75]. Their multiple regression analysis revealed a significant correlation between the plasma AM levels and pulse wave velocity, a relationship independent of blood pressure [75]. These findings are indirect, but indicative of a possible pathophysiological role of AM in arteriosclerotic vascular diseases.

Chronic Renal Failure and End-Stage Renal Disease

The plasma AM concentration progressively increased in proportion to the impairment of renal function in patients with chronic renal failure, irrespective of the basal renal disease [59, 76]. Since peptide hormones and small fragments of peptides are metabolized in the kidneys, the possibility of a decreased clearance of AM should be taken into account as an explanation for the increased AM concentration. Meanwhile, according to Nishikimi *et al.*, no significant step-down of the plasma AM levels was noted between the renal artery and vein of hypertensive patients [65]. This finding does not support the notion that the kidneys are the major clearance sites of AM circulating in the blood. The elevated plasma AM levels in patients with chronic renal failure may not be simply attributable to the impaired renal function.

In line with the findings in patients with chronic renal failure, plasma AM levels are markedly increased in those with end-stage renal disease. It should be of interest to compare the plasma AM levels before and after hemodialysis, where reductions of blood pressure or fluid volume occur in a short period of time, but there have been inconsistencies regarding changes in plasma levels following hemodialysis. Some investigators found reduced plasma AM concentrations in a hemodialysis session [77-79], while others reported that AM levels remained unchanged [80, 81]. The plasma AM levels were shown to be reduced by hemodialysis with a high-flux dialyzer, but not with a low-flux dialyzer [82]. Kanozawa *et al.* reported that AM levels were lowered in patients undergoing ultrafiltration, but remained unchanged in those without ultrafiltration [83]. The discrepancy in changes of plasma AM levels in the hemodialysis patients may be partly explained by differences in subtracted fluid volume or in the types of dialyzer used, though according to Tokura *et al.*, other factors also appear to be involved [79].

PLASMA AM LEVEL AND PRODUCTION OF AM IN ANIMAL MODELS OF HYPERTENSION

Substantial efforts have so far been made to clarify the pathophysiological role of AM in hypertension by measuring AM concentrations of plasma or tissues and AM mRNA expression in various rat models of hypertension [56, 84-87]. Shimokubo *et al.* reported that both AM peptide and gene expression levels in the cardiac atrium and ventricle were higher in spontaneously hypertensive rats (SHR) than the control Wistar-Kyoto rats (WKY) [84]. The Dahl salt-sensitive rat, a model of volume-dependent hypertension, was examined for AM by at least two groups including ours. Similar to SHR, left ventricular AM content and mRNA expression of Dahl salt-sensitive rats fed on a high salt diet were found to be progressively elevated in association with left ventricular hypertrophy (LVH) and with transition from LVH to congestive heart failure [56, 85]. The plasma AM levels also increased in Dahl-salt sensitive rats on a high salt diet as compared with those on a low salt diet or Dahl salt-resistant rats [56, 85]. In addition to the increased AM expression, mRNA levels for the AM receptor components of calcitonin-receptor-like receptor (CRLR) and receptor-activity-modifying protein (RAMP) type 2 and 3 were found to increase in the left ventricle of Dahl salt-sensitive rats, suggesting up-regulation of the AM effector systems [56]. To examine AM expression in hypertension dependent on the renin-angiotensin system, we measured plasma and tissue AM levels in 2-kidney, 1-clip (2K-1C) renovascular hypertensive rats [86]. Although the plasma level was found to be similar between the 2K-1C and control rats, the AM concentration in the left ventricle of the hypertensive rats increased, showing a significant correlation with the degree of left ventricular hypertrophy [86] (Fig. (**3**)).

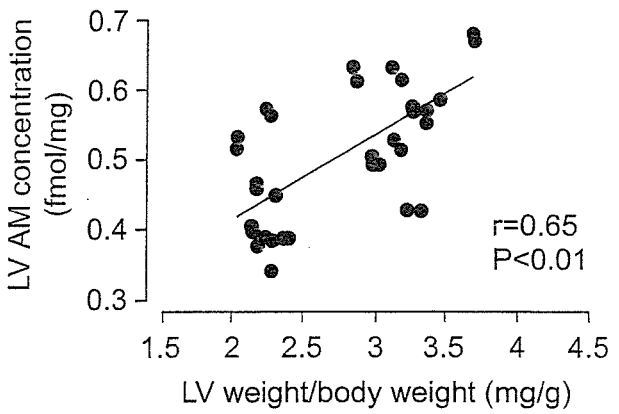

Fig. (3). Relationship between degree of left ventricular (LV) hypertrophy and LV AM concentration in 2K-1C renovascular hypertensive rats. Reprinted from reference 86 with permission from the Japanese Society of Hypertension.

In kidneys of SHR and Dahl salt-sensitive rats, neither the peptide nor mRNA expression level of AM was increased in comparison with the respective controls [84, 85]. In the meantime, Nishikimi *et al.* reported that both AM content and mRNA expression were augmented in renal medulla of SHR given deoxycorticosterone acetate and salt (DOCA salt SHR), a model of malignant hypertension, concomitantly with elevated plasma AM levels and with increased urinary excretion of AM [87]. Although the mRNA level for CRLR in the renal medulla remained unchanged, levels for RAMP1 and 3 were augmented in this model of hypertension [87]. Taking the blood pressure-lowering properties of AM into account, we hypothesize that AM functions as a factor acting systemically or locally against elevations of blood pressure in the animal models of hypertension.

The mechanisms for augmented AM production in the heart or kidneys of hypertensive rats are not totally understood at present, but we may be able to discuss this important issue based on the findings from cell culture studies, as well as from animal experiments. According to our previous studies, AM production in cultured cardiomyocytes and cardiac fibroblasts was increased by the pressor peptides angiotensin II and endhothelin-1 or simply by mechanical stretching of the cells [45, 46, 88]. Yoshihara *et al.* showed that production of AM in cultured cardiomyocytes was increased by hydrogen peroxide (H_2O_2), suggesting the involvement of oxidative stress [89]. The production of AM by cultured mesangial cells and vascular smooth muscle cells was found to increase in response to such humoral factors or cytokines as angiotensin II, aldosterone, interleukin-1β and tumor necrosis factor-α [44, 47, 90]. In vascular endothelial cells, AM production has been shown to be modulated by glucocorticoid or the alteration of shear stress [91, 92]. Hence, various factors seem to be involved in augmented AM production in the animal models of hypertension and possibly in human hypertension.

Another approach to clarifying the mechanisms behind the augmented AM expression is to examine the changes in levels of AM peptide or mRNA expression following antihypertensive treatment. Pressure overload to the heart by aortic banding elevated left ventricular AM levels in rats, but this increase was inhibited by reducing blood pressure with quinapril, an angiotensin-converting enzyme inhibitor [93]. Using Dahl-salt sensitive rats, Kobayashi *et al.* demonstrated that the increased AM level in the left ventricle was reduced by a sub-depressor dose of the angiotensin II type 1 receptor blocker TCV-116 [94]. Consistent with the cell culture experiments, either mechanical stress applied to the myocardium or the renin-angiotensin system appears to be involved in the augmented AM expression in the left ventricle in these rat models of hypertension.

LONG-TERM MODULATION OF BLOOD PRESSURE BY AM

Soon after the discovery of AM, the hypotensive effects of bolus AM injection or infusion in a relatively short period of time were well characterized in animal experiments and in human studies [7, 8, 16]. However, the plasma AM concentration was shown to rise to a level 100-fold higher than the physiological concentration following such a bolus injection [8], therefore we are unable to discuss the roles of endogenous AM in the long-term regulation of blood pressure using these reports. To examine the effects of chronically administered AM, we continuously infused synthetic human AM for two weeks, into spontaneously hypertensive rats (SHR) and 2-kidney, 1-clip (2K-1C) renovascular hypertensive rats [95, 96]. As shown in Fig. (**4**), the systolic blood pressure of the

2K-1C rats was reduced by the chronic AM infusion, which raised the plasma AM level to within the physiological range [96]. Blood pressure reduction by prolonged AM infusion at a physiological plasma AM level was also observed by Rademaker *et al.*, who infused AM over four days into an ovine model of heart failure [97]. Meanwhile, in other rat models of hypertension, such as the Dahl salt-sensitive rat or SHR given deoxycoricosterone acetate and salt (DOCA salt SHR), chronically infused AM failed to reduce blood pressure despite the infusion of AM at doses similar to those of the above-mentioned studies [98, 99].

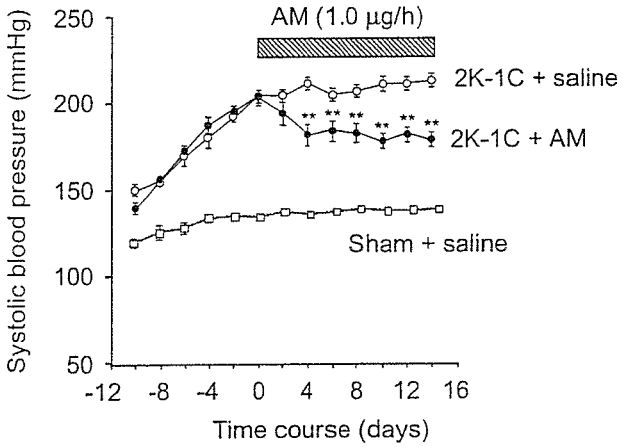

Fig. (4). Blood pressure-lowering effect of long-term infusion of AM in 2K-1C renovascular hypertensive rats. The 2K-1C rats were infused with 1.0 μg/h of synthetic human AM for 14 days. **P<0.01, *vs.* 2K-1C rats infused with saline. Reprinted from reference 96 with permission from Elsevier.

Also, we may be able to discuss the role of endogenous AM in long-term BP modulation based on the findings from AM gene-manipulated mice. The homozygote of AM gene-knockout mice was found to be embryo-lethal, but mean blood pressure of the heterozygote was significantly higher by about 10 mmHg than that of the wild-type littermates [100]. Comparable with this, transgenic mice over-expressing the AM gene showed a lower blood pressure with higher plasma AM levels [101]. Although the blood pressure-lowering effects of continuous, long-term infusion of AM appear dependent on animal models of hypertension, the results obtained by the AM gene manipulation suggest the participation of AM in the mechanisms acting against the elevation of blood pressure.

When injected in a relatively short period of time, AM exerts a potent blood pressure-lowering effect largely secondary to vasodilatation [7, 9, 16]. In such experiments, plasma renin activity was elevated probably by the activation of the sympathetic nerve system resulting from an acute reduction of blood pressure [16]. However, chronically infused AM reduced the plasma renin activity and aldosterone concentration of 2K-1C renovascular hypertensive rats [96]. Consistent with the direct inhibitory action of AM on the secretion of aldosterone [27], AM suppression of plasma aldosterone levels was observed not only in chronic experiments but also in relatively short-term infusion studies [102, 103].

As mentioned in the section "Biological actions of AM", vasodilatation is partly mediated *via* the NO-cGMP pathway [10, 12], which may also be involved in the chronic modulation of blood pressure by AM. Nishikimi *et al.* found an increased plasma level and urinary excretion of cGMP following prolonged AM infusion in Dahl salt-sensitive rats [104]. An increased plasma level of cGMP was also observed in the AM gene-overexpressing mice, whose blood pressure was lower compared with the wild type, but rose to a greater degree following the inhibition of NO production with N-monomethyl-L-arginine (L-NMMA), offsetting the blood pressure difference [101]. Conversely in mice deficient in a single copy of the AM gene, showing reduced production of NO in the kidneys, the blood pressure elevation induced by L-NMMA was smaller compared with their wild-type littermates [101]. The notion of reduced production of NO in AM knockout mice was further supported by the higher renal perfusion pressure *ex vivo*, which rose to less in response to N-nitro-L-arginine methyl ester (L-NAME), compared with the controls [105]. Collectively, though bolus injection of AM reduces blood pressure largely *via* vasodilatation, the blood pressure-lowering effects of chronically infused or overly expressed AM appear to be mediated by multiple mechanisms: inhibition of the renin-angiotensin system, direct inhibition of aldosterone secretion, stimulation of the NO-cGMP system, etc.

CARDIOVASCULAR AND RENAL PROTECTIVE EFFECTS OF AM *IN VITRO*

A number of cell culture experiments have been done to see whether or not AM has the potential to protect end organs from the damage associated with an elevation of blood pressure. AM production was found to progressively increase in cardiac ventricles of Dahl salt-sensitive or 2K-1C renovascular hypertensive rats in relation to the degree of ventricular hypertrophy [85, 86] (Fig. (3)). To examine the effect of AM on cardiac hypertrophy, we carried out a series of experiments *in vitro* with cultured cardiomyocytes isolated from cardiac ventricles of neonatal rats [45, 50, 88, 106]. Incubation with synthetic AM reduced *de novo* protein synthesis, cell size and atrial natriuretic peptide (ANP) production induced by angiotensin II or by mechanical stretching in the cultured cardiac myocytes, a finding indicative of an inhibitory effect of AM on cardiac ventricular hypertrophy [45, 50, 88, 106].

Making up two-thirds of the total number of cells in the heart, cardiac fibroblasts also play an important role in the myocardium: they contribute to the morphological integrity of the myocardium, while excessive proliferation and collagen production by the fibroblasts result in impairments of systolic and diastolic function. In addition to its effect on cardiomyocytes, AM inhibited the proliferation and protein synthesis of cultured cardiac fibroblasts [46]. Similar results were obtained by Horio *et al.*, who raised the possibility that AM inhibits the synthesis of collagen in fibroblasts [107]. Interestingly, a blockade of the actions of endogenous AM with a monoclonal antibody increased the protein synthesis or cell proliferation of cultured cardiac myocytes and fibroblasts, suggesting an autocrine or paracrine inhibition of cardiomyocyte hypertrophy and the proliferation of cardiac fibroblasts by AM [45, 46, 106]. These results led us to the

hypothesis that AM functions to inhibit cardiac hypertrophy or fibrosis induced by inappropriate mechanical or humoral stimuli associated with elevations of blood pressure.

The proliferation of vascular smooth muscle cells in the media and intima of arteries is involved in the progression of vascular remodeling or atherosclerotic lesions. Because AM is produced by the vascular wall [43, 108], its effects on the proliferation of vascular smooth muscle cells were tested *in vitro*; however, there has been an inconsistency regarding the actions of AM. Kano *et al.* reported that AM inhibited the proliferation of cultured vascular smooth muscle cells [109]. The same group also showed an inhibitory effect of AM on the migration of these cultured cells, and according to their experiments, the effects were mediated by intracellular cAMP [110]. Conversely, Iwasaki *et al.* found that AM stimulated the proliferation of cultured vascular smooth muscle cells in a mitogen-activated protein kinase-dependent manner [111]. These discrepancies may result from differences in the experimental conditions or types of cultured cells used. Meanwhile, as discussed in the next section, recent studies with AM gene knockout or transgenic mice have shown that AM inhibits fatty streak formation and periarterial cuff-induced intimal thickening [112, 113].

Secreting various vasoactive substances, vascular endothelial cells have an important role not only in regulating vascular tone but also in modulating the progression of vascular remodeling and atherosclerotic lesions. In line with this, cultured vascular endothelial cells were found to actively produce and secrete AM [43]. Kato *et al.* reported that AM inhibited serum deprivation-induced apoptosis of cultured rat vascular endothelial cells *via* a cAMP-independent manner [114]. Sata *et al.* also observed an anti-apoptotic effect of vascular endothelial cells, which was presumably mediated by a non-cAMP and non-cGMP dependent mechanism [115]. In addition to inhibition of apoptosis, an *in vitro* wound healing assay by Miyashita *et al.* showed that AM promoted endothelial regeneration in a protein kinase A- or phosphatidylinositol-3-kinase-dependent pathway [116]. Though the intracellular mechanisms of the action remain to be clarified, these results suggest that AM has a protective effect against the progression of vascular remodeling or atherosclerotic lesions by inhibiting apoptosis and promoting the regeneration of vascular endothelial cells.

A substantial level of AM mRNA was detected in kidneys [4, 42], and in accord with this, AM has been reported to be produced by cultured renal cells including mesangial cells [47-49]. Although mesangial proliferation is not a main feature of benign nephrosclerosis, hypertension is a factor exacerbating glomerular diseases, in which the inhibition of mesangial proliferation should be beneficial. There have been a significant number of reports showing an inhibitory effect of AM on the proliferation of cultured mesangial cells *in vitro* [47, 117-119]. This effect seems to be partly mediated by intracellular cAMP, because forskolin, a direct adenylate cyclase activator, mimicked the effect and H-89, a protein kinase A inhibitor, attenuated it [119]. In addition to the inhibition of mesangial cell proliferation, Parameswaran *et al.* reported AM-induced apoptosis of mesangial cells, showing the possibility that p38 mitogen-activated protein kinase was involved in the mechanism [119, 120]. These findings suggest that AM exerts a protective effect on the glomerulus by inhibiting the proliferation of mesangial cells in renal diseases.

CARDIOVASCULAR AND RENAL PROTECTIVE EFFECTS OF AM *IN VIVO*

As discussed earlier, the increased plasma AM concentration in patients with hypertension appears to be largely related to target organ damage such as left ventricular hypertrophy, reduced cardiac function, impaired renal function and arteriosclerosis [58-63]. To investigate whether or not AM protects the target organs against hypertensive damage *in vivo*, three experimental approaches have so far been taken: long-term administration of AM, virally-mediated overexpression of AM and genetic manipulation of the AM gene. Using the long-term infusion method, Nishikimi and his colleagues showed that in Dahl salt-sensitive hypertensive rats fed a high-salt diet, AM had a minimal effect on blood pressure, but significantly inhibited left ventricular hypertrophy (LVH) and the transition from LVH to heart failure [98]. The infusion also lessened pulmonary congestion and the deterioration of left ventricular function, improving survival in this animal model of hypertension [98]. The inhibitory effect of AM on LVH without a significant reduction of blood pressure suggests a possibly direct action on cardiac myocytes, which was shown previously by our cell culture experiments [45, 46].

Either preload or afterload to the heart results not only in LVH but also in cardiac fibrosis, where excessive fibroblast proliferation and collagen deposition occur, leading to impairment of diastolic dysfunction. To test *in vivo* the antifibrotic action of AM observed in the cell culture experiments [46], we infused recombinant human AM into a rat model of acute myocardial infarction (MI) [121, 122]. Consistent with the study *in vitro*, the infusion significantly reduced the myocardial collagen deposition following MI, while the exact mechanism of action remains to be specified [121, 122]. As shown in Fig (**5**), we recently found that long-term administration of AM reduced perivascular fibrosis of the coronary arteries in rats with hypertension induced by chronic infusion of angiotensin II [123]. This effect was accompanied by the suppression of fibroblast activation and transforming growth factor-β1 (TGF-β1) expression, but not by a significant reduction of blood pressure [123], suggesting an AM action independent of hemodynamic factors.

Renoprotective effects of AM have been implied by *in vitro* experiments as mentioned above. Indeed, the long-term infusion of AM has been reported to reduce glomerular and arterio-arteriolar scleroses in Dahl salt-sensitive rats given a high-salt diet and spontaneously hypertensive rats administered deoxycorticosterone acetate and salt (DOCA-salt SHR) [99, 104]. As a result, urinary protein excretion was reduced and creatinine clearance preserved by the AM infusion. The renoprotective effects were observed without a significant reduction of blood pressure, suggesting again direct actions of AM on kidneys [99, 104]. Increased urinary excretion of cAMP and cGMP suggests roles for these cyclic nucleotides in mediating the actions of AM [104], but the mechanisms involved remain to be specified as do those for the effects of AM on the heart. Irrespective of the mechanisms, the plasma levels of exogenous AM were found to be within the physio-

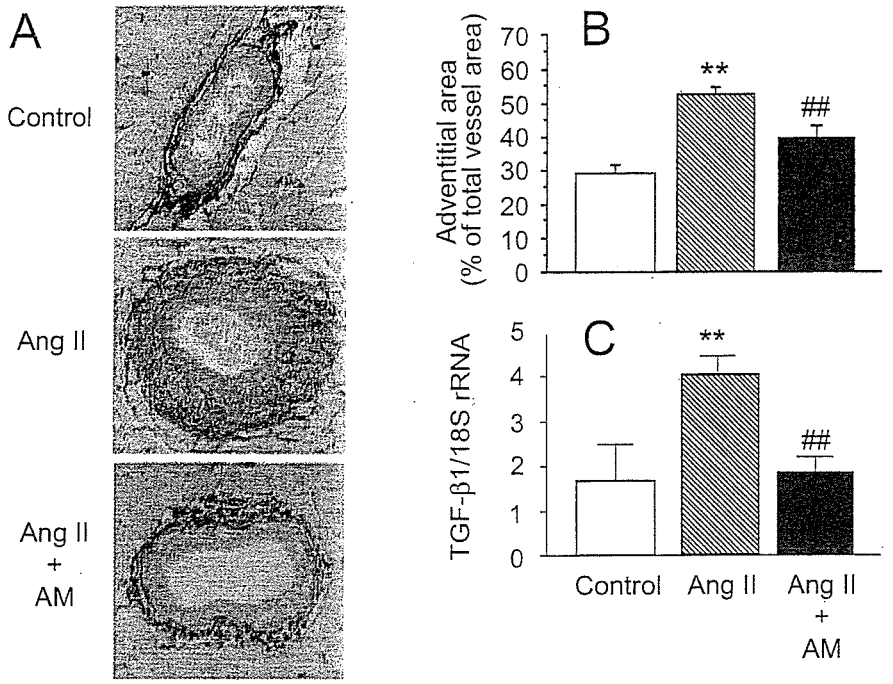

Fig. (5). Effects of angiotensin II (Ang II) and AM on perivascular fibrosis of coronary arteries. The two-week infusion of 200 ng/kg/min Ang II thickened the adventitial area of coronary arteries of rats as determined by Sirius red staining (**A** and **B**). The infusion of AM at 60 ng/kg/min alleviated the adventitial thickening concomitantly with a reduction in TGF-β1 expression (**C**), but did not have a significant effect on blood pressure (Ang II 180+/-7, Ang II+AM 191+/-4 mmHg). Values are the means+/-SEM; **P<0.01, *vs.* control; ##P<0.01, *vs.* Ang II. Reprinted from reference 123 with permission from the European Society of Cardiology.

logical range in the above-mentioned long-term infusion studies, where 0.2 to 1.0 μg/h AM was used [95, 96, 98, 99, 104]. It is therefore possible that endogenous AM functions to inhibit cardiac and renal damage in the rat models of hypertension.

Chao and coworkers demonstrated that adenovirus-mediated systemic delivery of the human AM gene ameliorated cardiac and renal damage in rats with deoxycorticosterone acetate (DOCA) salt-induced and 2K-1C renovascular hypertension and in Dahl salt-sensitive hypertensive rats fed a high-salt diet [124-126]. Similar to the results of the long-term infusion experiments, the overexpression of AM lessened cardiac hypertrophy and fibrosis and glomerular sclerosis, improving urinary protein excretion and creatinine clearance [124-126]. Urinary excretion of cAMP was increased by the overexpression [124, 125]; while systolic blood pressure was reduced by about 20 to 30 mmHg [124-126], therefore blood pressure-dependent mechanisms may have also contributed to the beneficial effects of AM. In addition to the systemic overexpression of the AM gene, the same group reported that the local delivery of AM to balloon-injured carotid artery inhibited neointimal formation in rats [127]. The plasma AM concentration rose beyond the physiological range following the systemic delivery of the AM gene; nonetheless, these overexpression studies suggest the potential of AM as a therapeutic tool inhibiting organ damage resulting from elevations of blood pressure.

Lastly, the cardiovascular and renal protective effects have been suggested by genetic manipulation of the AM gene in mice. Shimosawa *et al.* showed that, when given angiotensin II and excessive salt, heterozygotes of AM knockout mice showed more severe perivascular fibrosis and intimal thickening of coronary arteries, compared with their wild-type littermates, despite a similar elevation of blood pressure between the knockout and wild-type mice [128]. Based on increases in the production of reactive oxygen species (ROS) and in NAD(P)H oxidase expression in the AM knockout mice, the possibility of augmented oxidative stress was raised as the mechanism responsible for the severe vascular lesions [128]. Periarterial cuff-induced intimal thickening of the femoral artery was also found to be more severe in the knockout mice, compared with the control mice [112]. The enhanced neointimal formation was reversed by delivery of the AM gene and by an NAD(P)H oxidase inhibitor or tempol, a superoxide dismutase mimetic, further suggesting augmentation of oxidative stress in the AM knockout mice [112]. More recently, Niu *et al.* subjected heterozygotes of another strain of AM gene-knockout mice to the cardiovascular stress of aortic constriction or angiotensin II infusion, resulting in left ventricular hypertrophy and perivascular fibrosis, changes that were more pronounced in the knockout mice than in the controls [129]. In addition, the renal damage characterized by reduced creatinine clearance and glomerular sclerosis was worse in the knockout mice than in the controls [129]. The precise mechanisms remain to be clarified; though, it appears convincing that AM has protective effects on the progression of the cardiovascular or renal damage associated with elevations of blood pressure.

INSULIN RESISTANCE AND AM

Insulin resistance with resultant hyperinsulinemia is often seen in patients with hypertension, particularly in those with

obesity-related hypertension. Chan *et al.* reported that the blood pressure-lowering effect of AM was attenuated in the Zucker obese rat, an animal model of insulin resistance, compared with the control [130]. A comparable result was recently obtained in a human study by Kita *et al.*, where the effects of intravenous AM infusion were examined in subjects without or with insulin resistance [131]. The infusion reduced not only blood pressure but also pulse wave velocity (PWV), a parameter for arterial stiffness. Interestingly, the reduction in PWV was smaller in the subjects with insulin resistance than those without [131]. It therefore seems likely that insulin resistance blunts the actions of AM in humans as well as in rats. On the other hand, AM deficiency has been shown to be related with insulin resistance by using heterozygotes of AM gene-knockout mice [132, 133]. Shimosawa *et al.* found that AM knockout mice developed insulin resistance on aging with increased urinary 8-iso-prostaglandin F2-α excretion and reactive oxygen species (ROS) production in skeletal muscle. Both the reduced response to insulin and augmented oxidative stress were partially reversed by supplements of AM or tempol, a superoxide dismutase-mimetic compound [132]. The same group also showed that angiotensin II-induced insulin resistance with increased oxidative stress was more prominent in the AM knockout mice than in the controls, and this was reversed by the AM or tempol supplements [133]. These findings from the AM gene-knockout mice suggest that reduced AM actions would result in insulin resistance, probably by augmentation of oxidative stress.

CONCLUSIONS AND PERSPECTIVES

Thirteen years has passed since AM was discovered as a novel vasodilator peptide. During this period, much research, basic and clinical, has been done to clarify its pathophysiological role in hypertension and hypertensive organ damage. As discussed in this review, a substantial amount of data accumulated in this field suggests that AM functions to counteract the elevation in blood pressure and progression of the cardiovascular and renal diseases associated with hypertension. Research on AM now seems to be entering a new phase, with clinical benefits to be examined and specified. AM itself is orally inactive, but the development of either AM analogues or drugs inhibiting AM degradation would provide a new therapeutic tool for treating patients with hypertension or those with the cardiovascular and renal diseases associated with hypertension.

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