Molecular forms of circulating adrenomedullin in patients with congestive heart failure

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Abstract

In the biosynthesis of adrenomedullin (AM), an intermediate form, AM(1-52)-glycine-COOH (iAM), is cleaved from proAM and subsequently processed to a biologically active mature form, AM(1-52)-NH₂ (mAM), by enzymatic amidation. We recently reported that immunoreactive AM in human plasma consists of mAM and iAM. To clarify the pathophysiological roles of mAM and iAM in heart failure, we established an assay method to specifically detect mAM, and we determined the plasma concentrations of mAM and iAM in 68 patients with congestive heart failure (CHF). The plasma mAM concentrations of the CHF patients classified as being class I or II of New York Heart Association (NYHA) functional classification were significantly greater than those of the 28 healthy controls, and a further increase was noted in the class III or IV patients. Similar increases in plasma iAM

were also observed in these patients compared with controls. The increased plasma mAM and iAM in 12 patients with exacerbated CHF were significantly reduced by treatment of their CHF for 7 days. In addition, the plasma concentrations of both mAM and iAM were significantly correlated with pulmonary capillary wedge pressure, pulmonary artery pressure, right atrial pressure, cardiothoracic ratio, heart rate, and the plasma concentrations of atrial and brain natriuretic peptides in the CHF patients. Thus the plasma concentrations of both mAM and iAM were increased progressively in proportion to the severity of CHF. These results suggest that, though the role of iAM remains to be clarified, mAM acts against the further deterioration of heart failure in patients with CHF.

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Introduction

Human adrenomedullin (AM) is a potent vasodilator peptide consisting of 52 amino acid residues with an amidated C-terminal tyrosine and a ring structure formed by an intramolecular disulfide bond (Kitamura *et al.* 1993). Besides its vasodilator effect, AM has been shown to increase the glomerular filtration rate (Hirata *et al.* 1995) and to inhibit aldosterone secretion (Yamaguchi *et al.* 1996). The development of a radioimmunoassay (RIA) for AM revealed that immunoreactive AM is present in human blood in the low picomolar range (Ichiki *et al.* 1994, Kitamura *et al.* 1994a, Lewis *et al.* 1998). These findings imply a possible role of AM in modulating cardiovascular homeostasis. The structures of porcine (Kitamura *et al.* 1994b), rat (Sakata *et al.* 1993) and mouse (Okazaki *et al.* 1996) AM showed a well-conserved amino acid sequence among species, particularly in the ring structure and the C-terminal amidated tyrosine residue, suggesting the importance of these two portions of the AM

molecule for its biological activity. In fact, both the ring and C-terminal amide structures were reported to be essential for the receptor binding and the production of intracellular cAMP, a major second messenger of AM, in cultured vascular smooth muscle cells (Eguchi *et al.* 1994). In the processing of AM, an intermediate form, AM(1-52)-glycine-COOH (iAM), is believed to be cleaved from proAM and subsequently processed to a biologically active mature form, AM(1-52)-NH₂ (mAM), by an amidation enzyme. We recently reported that immunoreactive AM in the human plasma consists, not only of mAM, but also of iAM, which is converted to mAM in the presence of the amidation enzyme *in vitro* (Kitamura *et al.* 1998). A number of reports have shown that the plasma concentrations of AM are increased in patients with congestive heart failure (CHF) (Jougasaki *et al.* 1995, Nishikimi *et al.* 1995, Kato *et al.* 1996, Kobayashi *et al.* 1996); however, the molecular forms of plasma AM have not been examined. In those previous studies, the plasma AM concentration was determined by

Table 1 Clinical characteristics of controls and heart failure patients. Values are means ± S.E.M.

	Controls	Patients with CHF (NYHA functional class)	
		Class I or II	Class III or IV
<i>n</i>	28	48	20
Age (years)	61.9 ± 1.8	62.1 ± 1.7	65.5 ± 3.0
Sex (M/F)	18/10	34/14	8/12
Primary cause of CHF			
IHD	—	27	9
Valvular	—	9	7
DCM	—	3	2
HHD	—	3	1
Congenital	—	3	0
Other	—	3	0
Serum creatinine (mg/dl)	0.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Body mass index (kg/m ²)	23 ± 0.5	22.9 ± 0.6	23.0 ± 1.4
Systolic BP (mmHg)	125 ± 5	133 ± 5	130 ± 10
Diastolic BP (mmHg)	75 ± 2	72 ± 3	74 ± 6
Heart rate (beats/min)	66.5 ± 2.8	69.1 ± 2.0*	102.0 ± 6.0*†
CTR (%)	47.1 ± 1.5	51.9 ± 1.4*	65.6 ± 0.9*†
LVEF (%)‡	71.5 ± 1.0	65.3 ± 2.5*	41.5 ± 5.3*†
Plasma ANP (fmol/ml)	3.3 ± 0.4	12.0 ± 2.8*	50.0 ± 9.8*†
Plasma BNP (fmol/ml)	2.5 ± 0.4	24.7 ± 5.7*	255.2 ± 54.5*†

P < 0.001: *compared with controls; †compared with class I or class II. IHD, ischemic heart disease; DCM, dilated cardiomyopathy; HHD, hypertensive heart disease; BP, blood pressure; CTR, cardiothoracic ratio. ‡Determined by echocardiography.

RIAs unable to distinguish mAM from iAM, and was thereby measured as total AM (tAM) concentration.

In the present study, we established a specific, sensitive assay method to measure the human plasma mAM concentration. Using this assay, we examined the plasma concentrations of mAM and iAM in patients with CHF and compared them with those in control subjects, in order to clarify the pathophysiological roles of mAM and iAM.

Materials and Methods

Study patients and blood sampling

Sixty-eight patients with chronic CHF caused by various heart disorders were enrolled in the study (42 men and 26 women; 26–85 years of age) and classified into two groups according to their New York Heart Association (NYHA) functional class (Table 1). Patients who had impaired renal function with a serum creatinine concentration >177 µmol/l (2.0 mg/dl), severe liver dysfunction, or lung or inflammatory disease were excluded. The diagnosis of the primary cause of CHF was made on the basis of clinical histories, physical examinations, chest X-rays, electrocardiograms, and echocardiograms, in addition to the cardiac catheterization data. All the patients with symptomatic heart failure were receiving medications such as diuretics, digitalis, β-blockers, angiotensin-converting enzyme inhibitors, Ca channel blockers or nitrates as

needed. The mAM and iAM concentrations in 12 of the 20 patients in class III or IV, who were admitted to our hospital because of an exacerbation of CHF, were evaluated repeatedly before and during the treatment for CHF. Their clinical symptoms were relieved after the treatment, and echocardiography and chest X-ray were performed at day 7 after admission in these patients. Twenty-eight healthy subjects aged 37–79 years served as controls. Informed consent for all the procedures was obtained from each study participant.

Blood was drawn from the antecubital vein or the femoral vein after the subject had undergone an overnight fast and 30 min of supine rest, on the day when the heart catheterization study or echocardiography was performed. Blood samples were collected in chilled tubes with 1 mg/ml disodium ethylenediaminetetra-acetic acid (EDTA-2Na) and 500 KIU/ml aprotinin, and centrifuged at 4 °C. Plasma samples were kept at –40 °C until required for use.

Cardiac catheterization

Right-sided heart catheterization was performed in 47 of the CHF patients, with a 7F Swan–Ganz catheter (model 131H, Baxter Healthcare Corporation, Irvine, CA, USA), and left ventricular contrast studies were performed in 39 patients with 6F pigtail catheters (model 7779-B3, Mallinckrodt Medical Inc., St Louis, MO, USA). Cardiac output was measured by the thermodilution method in 43

patients and by Fick's method in four patients. The left ventricular ejection fraction (LVEF) and left ventricular end-diastolic/systolic volume were calculated by the area-length method.

Preparation of antisera for AM

Three types of antisera were raised against human AM and its fragment peptides. Entire-AM-antibody (E-AM-Ab) shows 100% cross-reactivity with mAM (AM(1–52)-NH₂) and iAM (AM(1–52)-glycine-COOH) (Kitamura *et al.* 1998). C-terminal-AM-antibody (C-AM-Ab) specifically recognizes the C-terminal amide structure of the AM molecule and has no cross-reactivity with iAM, calcitonin gene-related peptide (CGRP) or neuropeptide Y (Ichiki *et al.* 1994). E-AM-Ab and C-AM-Ab were prepared as reported elsewhere (Ichiki *et al.* 1994, Kitamura *et al.* 1994a). A monoclonal antibody for the ring structure (R-AM-Ab) was raised by a standard method. Briefly, human AM(12–25) was conjugated to bovine thyroglobulin (Sigma) by the carbodiimide coupling procedure. The conjugate containing 18.1 µg peptide dissolved in saline was emulsified with Freund's complete adjuvant and then injected subcutaneously into 5-week-old female BALB/c mice eight times at 3-week intervals. Spleen cells of the immunized mice were fused with cells of the mouse myeloma line, X63-Ag8.653 (Galfré & Milstein 1981). The hybridoma culture media were screened periodically for the ability to bind [¹²⁵I]human AM. Cells from the well with the highest titer were cloned by limiting dilution and then injected intraperitoneally to BALB/c mice. R-AM-Ab specifically recognizes the ring structure of the AM molecule and has no cross-reactivity with CGRP, amylin or rat AM.

Assays for mAM and tAM

Ten milliliters plasma were acidified by adding 120 µl 1.0 mol/l HCl and diluted with 10 ml saline. The plasma was then loaded to a Sep-Pak C18 cartridge that had been activated by washing with 5 ml 60% acetonitrile containing 0.1% trifluoroacetic acid followed by 5 ml saline. After washing with 5 ml saline, absorbed material was eluted with 4 ml 60% acetonitrile containing 0.1% trifluoroacetic acid. The eluate was lyophilized and dissolved in 450 µl RIA buffer, 50 mmol/l sodium phosphate buffer (pH 7.4) containing 0.5% BSA treated with 0.5% N-ethylmaleimide, 0.5% Triton X-100, 80 mmol/l NaCl, 25 mmol/l EDTA-2Na, 0.05% NaN₃, and 500 KIU/ml aprotinin. AM peptides having the ring structure were purified by immunoprecipitation. Fifty microliters 1% γ-globulin containing R-AM-Ab diluted at 1:1000 were added to the plasma extract. After an incubation for 4 h at 4 °C, 500 µl 23% polyethyleneglycol was added and the mixture stirred vigorously, allowed to stand for 20 min, and centrifuged for 30 min at 3000 r.p.m. After the

supernatant was aspirated, the precipitate was dissolved in 500 µl 1 mol/l acetic acid and boiled for 10 min to inactivate R-AM-Ab. The samples were then lyophilized and stored at –40 °C until the RIAs. About 75% of the [¹²⁵I]AM or synthetic AM added to the plasma sample was reproducibly recovered by this immunoprecipitation procedure. The extract was dissolved in 300 µl RIA buffer and subjected to either an RIA for tAM (E-AM-RIA) with E-AM-Ab or an RIA for mAM (C-AM-RIA) with C-AM-Ab. These RIA procedures have been described previously (Ichiki *et al.* 1994, Kitamura *et al.* 1994a). The intra- and interassay coefficients of variance in the RIA for tAM were 5% and 8%, and those in the RIA for mAM were 6% and 9%, respectively.

Characterization of tAM and mAM in human plasma

The plasma extract was analyzed by reverse-phase high-performance liquid chromatography (HPLC). The extract of 37 ml human plasma was immunoprecipitated by the R-AM-Ab. After the precipitate had been boiled in 1 mol/l acetic acid for 10 min, the solution was subjected to an HPLC column of TSK-ODS 120A (4.6 × 150 mm, Tosoh, Japan). A 60-min linear gradient of CH₃CN from 10 to 60% was made in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. One milliliter eluate was collected every 1 min, and each fraction was subjected to the C-AM-RIA or E-AM-RIA.

Assay procedures for ANP and BNP

As markers of severity of the heart failure, the plasma concentrations of atrial and brain natriuretic peptides (ANP and BNP) were measured by RIAs (Shiono RIA ANP kit and Shiono RIA BNP kit, Shionogi Co., Osaka, Japan). The accuracies and procedural details of these assays have been described previously (Yasue *et al.* 1994).

Statistical analysis

All values are means ± s.e.m. Multiple groups were compared by either one-way analysis of variance followed by Scheffé's test or χ^2 test. Correlations between two variables were evaluated by simple regression analysis. The time course data before and during the treatment were examined by the Wilcoxon signed-rank test. A *P* value of less than 0.05 was considered statistically significant.

Results

Characterization of immunoprecipitated materials

To characterize the immunoreactive AM obtained by the R-AM-Ab immunoprecipitation procedure, the extract was analyzed by reverse-phase HPLC coupled with either

Figure 1 Reverse phase HPLC of immunoreactive AM in human plasma extract immunoprecipitated by the anti-AM antibody against the ring structure (R-AM-Ab). The arrows show the elution position of synthetic human AM(1–52)-NH₂ and human AM(1–52)-glycine-COOH.

the C-AM-RIA specifically recognizing the C-terminal amide structure or the E-AM-RIA, which detects the entire AM structure whether or not the C-terminus is amidated. One major immunoreactive peak emerged in the E-AM-RIA at a position where authentic AM(1–52)-NH₂ and AM(1–52)-glycine-COOH were eluted (Fig. 1A). Similarly, more than 90% of the immunoreactive AM detected by the C-AM-RIA was eluted at exactly the same position as that of the E-AM-RIA (Fig. 1B). However, the immunoreactive AM measured by the C-AM-RIA was much less than that measured by the E-AM-RIA. On the basis of the findings of these HPLC analyses and the cross-reactivities of E-AM-Ab and C-AM-Ab, in addition to the results of our previous HPLC analyses (Kitamura *et al.* 1998), in the present study we calculated the iAM values by subtracting the mAM values from the tAM values.

Clinical characteristics of study subjects

Table 1 gives the clinical profiles of the study subjects. There were no significant differences in age, sex, serum concentration of creatinine, body mass index, or systolic and diastolic blood pressures among the three subject groups. There were no significant differences between the two CHF groups in the primary causes of CHF. The heart rate, cardiothoracic ratio and plasma ANP and BNP concentrations in the CHF groups were significantly ($P < 0.001$) increased compared with those in the control group, and values of these parameters in the functional

classes III or IV were significantly ($P < 0.001$) greater than those in classes I or II. As expected, the LVEF was progressively ($P < 0.001$) reduced in relation to the severity of CHF.

Plasma mAM, iAM and tAM concentrations

Table 2 shows the plasma concentrations of mAM, iAM and tAM and the ratio of mAM to tAM in the CHF patients, subgrouped according to their NYHA functional classification, and in the control subjects. The plasma mAM in the class I or II patients was significantly greater than that in the control group ($P < 0.01$), and a further increase was noted in the class III or IV patients compared with the controls ($P < 0.001$) and the class I or II patients ($P < 0.001$). Similar increases in the plasma iAM and tAM were also observed in the class III or IV patients compared with the controls ($P < 0.001$) and class I or II patients ($P < 0.001$). However, the mAM/tAM ratio, ranging from 0.24 to 0.29, showed no significant differences among the three groups. Table 3 shows the correlations between the plasma mAM or iAM concentrations and clinical parameters. Plasma mAM and iAM were significantly correlated with the mean pulmonary capillary wedge pressure, systolic pulmonary artery pressure and mean right atrial pressure. The cardiothoracic ratio, heart rate and ANP and BNP concentrations were also significantly correlated with both the plasma mAM and iAM. The systolic blood pressure of the aorta, cardiac index, left ventricular end-diastolic/systolic volumes, LVEF and left ventricular dimension were not correlated with the plasma mAM and iAM concentrations.

Plasma mAM and iAM concentrations before and after treatment

As shown in Table 4, in 12 class III or IV patients with exacerbated CHF, the cardiothoracic ratio, left atrial dimension, left ventricular dimension and plasma ANP and BNP concentrations were significantly decreased and the LVEF was significantly increased after CHF treatment for 7 days. The increased plasma mAM and iAM concentrations were significantly reduced, from 1.54 ± 0.12 to 1.03 ± 0.08 ($P < 0.01$) and from 6.24 ± 0.89 to 3.64 ± 0.27 fmol/ml ($P < 0.05$), respectively, after the treatment (Fig. 2).

Discussion

Human AM consists of 52 amino acid residues with a C-terminal amidated Tyr⁵² and a ring structure formed by an intramolecular disulfide bond of Cys¹⁶ and Cys²¹ (Kitamura *et al.* 1993). Immunoreactive AM has been shown to be present in human plasma and tissues such as adrenal medulla, lung, kidney, cardiac atrium and

Table 2 Plasma concentrations of AMs and ratio of mAM/tAM in controls and patients with heart failure. Values are means $\pm$ S.E.M.

	Controls	Patients with CHF (NYHA functional class)	
		Class I or II	Class III or IV
mAM (fmol/ml)	0.65 $\pm$ 0.06	1.01 $\pm$ 0.07*	1.66 $\pm$ 0.12**††
iAM (fmol/ml)	2.15 $\pm$ 0.12	2.51 $\pm$ 0.19	5.39 $\pm$ 0.66**††
tAM (fmol/ml)	2.80 $\pm$ 0.14	3.52 $\pm$ 0.24	7.04 $\pm$ 0.71**††
mAM/tAM (%)	23.9 $\pm$ 1.6	29.3 $\pm$ 1.5	26.6 $\pm$ 2.4

* $P < 0.01$, ** $P < 0.001$, compared with controls; †† $P < 0.001$, compared with class I or class II.

Table 3 Coefficients for correlation between the plasma mAM or iAM and clinical parameters. Statistically significant values of P are in *italics*

Parameter	mAM		iAM	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Pressure				
Pulmonary capillary wedge (mean)	0.63	<0.01	0.64	<0.01
Pulmonary artery (systolic)	0.54	<0.01	0.45	<0.01
Right atrium (mean)	0.64	<0.01	0.58	<0.01
Left ventricle (end-diastolic)	-0.07	0.66	0.33	<0.05
Aorta (systolic)	-0.11	0.27	-0.03	0.74
Cardiac index	0.12	0.45	0.15	0.36
End-diastolic volume (left ventricle)	-0.12	0.48	-0.03	0.85
End-systolic volume (left ventricle)	-0.07	0.69	-0.12	0.46
LVEF	-0.07	0.65	-0.21	0.20
Left atrial dimension	0.25	<0.05	0.18	0.12
Left ventricular dimension (diastolic)	0.03	0.80	0.16	0.17
CTR	0.51	<0.01	0.55	<0.01
Heart rate	0.59	<0.01	0.56	<0.01
Plasma ANP	0.49	<0.01	0.49	<0.01
Plasma BNP	0.40	<0.01	0.50	<0.01

Table 4 Effect of treatment for CHF in 12 patients of NYHA class III or IV. Values are means $\pm$ S.E.M.

Parameter	Before treatment	After treatment
CTR (%)	65.2 $\pm$ 1.6	60.2 $\pm$ 1.6*
Left atrial dimension (mm)	44.4 $\pm$ 1.3	37.6 $\pm$ 1.8*
Left ventricular dimension (diastolic) (mm)	60.3 $\pm$ 2.5	54.1 $\pm$ 2.1*
LVEF (%)	29.8 $\pm$ 4.6	40.6 $\pm$ 5.1*
Plasma ANP (fmol/ml)	40.9 $\pm$ 7.3	30.0 $\pm$ 7.1**
Plasma BNP (fmol/ml)	247.4 $\pm$ 53.5	116.5 $\pm$ 48.8*

* $P < 0.01$, ** $P < 0.001$, compared with values before the treatment.

ventricle (Ichiki *et al.* 1994, Kitamura *et al.* 1994a), and the plasma AM was reported to be increased in patients with hypertension or heart failure (Ishimitsu *et al.* 1994, Kitamura *et al.* 1994a, Jougasaki *et al.* 1995, Nishikimi *et al.* 1995, Kato *et al.* 1996, Kobayashi *et al.* 1996). We recently found, by chromatographic analyses, that the immunoreactive AM in human plasma consists of two molecular forms of AM: mAM, AM(1–52)-NH₂, and

iAM, a glycine-extended intermediate form of AM that is converted to mAM by an amidation enzyme (Kitamura *et al.* 1998). There have been a number of reports on the measurement of plasma AM; however, the plasma AM concentrations were determined by RIAs unable to distinguish mAM from iAM in these studies. It has been shown that both the ring and C-terminal amide structures of AM are essential for the receptor binding and the

Figure 2 Plasma mAM and iAM concentrations before and after treatment in 12 patients with congestive heart failure. NS, not significant.

production of intracellular cAMP in cultured vascular smooth muscle cells (Eguchi *et al.* 1994). In accordance with this, we found that synthetic iAM exerts a much weaker hypotensive effect compared with that of mAM when injected intravenously to rats (unpublished observation). In the present study, we established a specific assay method to measure the mAM in human plasma. As described in Materials and Methods, AM peptide having the ringed structure in plasma was purified by immunoprecipitation with a monoclonal antibody against human AM(12–25), and subsequently subjected to either an RIA that specifically detects the C-terminal amide structure of AM, for the measurement of mAM, or an RIA recognizing mAM and iAM equally, for the measurement of tAM. As shown in Table 2, the mean plasma concentrations of mAM and tAM in the control subjects were found to be 0.65 and 2.80 fmol/ml, respectively, and thus the calculated iAM value was 2.15 fmol/ml.

In the present study, we determined the plasma concentrations of the two molecular forms of AM separately in patients with CHF, and found that both the mAM and iAM concentrations were similarly increased in these patients compared with the controls. The increased mAM concentrations were significantly correlated with the pulmonary capillary wedge pressure, pulmonary artery pressure, right atrial pressure, and the plasma concentrations of ANP and BNP. Nishikimi *et al.* (1995) reported a significant correlation between plasma tAM and LVEF, an indicator for left ventricular dysfunction, in patients with heart failure, whereas a significant relationship was not found in this study, despite the constant ratio of mAM/tAM. This discrepancy may be partially explained by the primary causes of heart failure or by severity of the left ventricular dysfunction in the patients studied. In any case, the present results appear to support our hypothesis that the increased plasma AM is related to the retention of fluid volume in patients with CHF, probably acting as a

defensive mechanism in heart failure, through its vasodilator and natriuretic effects. It should be noted that the plasma concentrations of mAM were lower than those of iAM, and the major molecular form of immunoreactive AM in human plasma is iAM. However, a chronically infused low dose of AM(1–52)-NH₂ has been shown to decrease blood pressure, reducing plasma renin activity and the aldosterone concentration, at a plasma concentration of 1 fmol/ml (Khan *et al.* 1997) – a value observed for the plasma mAM in the present study. Taken together with the reduction in the plasma mAM after the treatment for CHF, this suggests that mAM may have a role acting against a further deterioration of heart failure in the CHF patients.

Another finding noted in the present study was that the plasma iAM concentration was similarly increased in patients with CHF, and reduced by the treatment for heart failure, as was plasma mAM. In addition, similar correlations were found between the plasma iAM and clinical parameters. As mentioned above, it is true that synthetic iAM is much less bioactive than mAM. We previously found a conversion of iAM in human plasma to mAM in the presence of recombinant amidation enzymes *in vitro* (Kitamura *et al.* 1998). Recently, it was reported that the glycine-extended substance P (SP) is converted to SP-NH₂, a biologically active form, in the wall of rat aorta (Oldham *et al.* 1997). It may therefore be possible that iAM in the plasma is enzymatically processed to mAM, presumably acting as a hormone reservoir for mAM, in patients with CHF. In any case, further studies are necessary to determine the pathophysiological role of plasma iAM in patients with heart failure.

In summary, we developed a sensitive assay method to specifically detect mAM in human plasma, and we determined the plasma concentrations of mAM and iAM in patients with CHF. The plasma concentrations of mAM and iAM were similarly increased in relation to the severity of heart failure in these patients compared with controls. Although the role of the plasma iAM remains to be elucidated, mAM may act as defense mechanism against a further deterioration of heart failure in patients with CHF.

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