### Statistical analysis

All the data were analyzed using IBM SPSS Software, version 22.0 (IBM, Armonk, NY, USA). Two groups were compared by the unpaired *t*-test or $\chi^2$ test, while multiple comparisons were made by ANOVA followed by Scheffe's test. Simple regression analysis was used to examine the relationships between plasma levels of the peptides and the other parameters, and these relationships were further tested by Spearman's rank correlation coefficient. A multiple linear regression analysis with a stepwise method was used to extract factors significantly associated with the plasma AM levels. All data are expressed as the means±S.D. and $P<0.05$ was considered to be statistically significant.

## Results

The basal profiles and peptide measurements of the residents examined in this study are given in Table 1. The plasma level of AM in the female residents was significantly ($P<0.01$) lower than that in the males, while in contrast, the BNP and NT-proBNP levels were slightly higher in women than in men. When men and women were analyzed together by simple regression analysis, the AM levels were significantly correlated with BMI ($r=0.153$, $P<0.01$) and waist circumference (WC; $r=0.132$, $P<0.05$). As there were substantial differences in the basal profiles between the two genders (Table 1), we further analyzed the data to examine whether gender is independently associated with the plasma AM levels by multiple regression analysis with a stepwise method. The parameters included as explanatory covariates in this analysis were the BMI, mean blood pressure, and fasting blood glucose level, because these parameters, in addition to prevalence of hypertension, significantly differed between men and women (Table 1 and Supplementary Table 1, see section on supplementary data given at the end of this article). Also those included were age and eGFR, which have been reported to be the factors influencing plasma AM levels (15, 22). As given in Table 2, although marginally significant ($P=0.043$), gender was extracted as an independent determinant of the plasma AM levels, in addition to BMI and eGFR, in the study subjects.

**Table 1** Basal profiles and plasma levels of the peptides of the male and female residents examined in this study. Means±S.D.

| | Men | Women |
|---|---|---|
| *n* | 172 | 174 |
| Age (years) | 62.1±9.1 | 61.9±8.6 |
| BMI (kg/m$^2$) | 23.5±2.5 | 22.0±3.0* |
| Waist circumference (cm) | 85.1±7.1 | 82.0±9.1* |
| Mean blood pressure (mmHg) | 96±12 | 90±12* |
| Fasting blood glucose (mg/dl) | 96±13 | 92±9* |
| HbA1c (%) | 5.5±0.3 | 5.5±0.3 |
| eGFR (ml/min per 1.73 m$^2$) | 74±14 | 76±14 |
| AM (pmol/l) | 7.14±1.29 | 6.77±1.18* |
| BNP (pg/ml) | 19.1±23.4 | 21.7±15.5 |
| NT-proBNP (pg/ml) | 60.5±101.8 | 68.1±48.2 |

eGFR, estimated glomerular filtration rate; AM, adrenomedullin; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP. *$P<0.01$ vs male residents.

**Table 2** Identification of significant factors for plasma AM levels by multiple regression analysis with a stepwise method.

| Independent variables | β | P |
|---|---|---|
| BMI | 0.129 | 0.022 |
| eGFR | −0.119 | 0.028 |
| Gender (male=1 and female=2) | −0.114 | 0.043 |

eGFR, estimated glomerular filtration rate.

We then analyzed the data of the male and female residents separately. The relationships between plasma levels of the peptides and the BMI or WC are given in Table 3 as Pearson's correlation coefficients (*r*). The plasma levels of AM were found to be correlated with the BMI and WC in women, but such a relationship was not detected in men, as also shown in Fig. 1A and B. These results were confirmed by Spearman's rank correlation coefficient, which showed significant relationships between the plasma AM and BMI or WC in women but not in men (data not shown). In contrast to AM, as given in Table 3, inverse correlations were found between the plasma levels of BNP or NT-proBNP and the BMI or WC in women.

Basal profiles of the study subjects with or without BW gain of 10 kg or more since 20 years old are given in Table 4. Compared with the residents without BW gain, significantly higher ($P<0.01$) values were noted in the BW, BMI, and WC in those with BW gain, although there were no significant differences in the other clinical parameters, including age, blood pressure, blood glucose, HbA1c, and renal function, between the two groups. Prevalence of hypertension in the male residents was higher than that in the females (Supplementary Table 1), but when compared within the same gender, no differences were noted for hypertension, dyslipidemia, or diabetes mellitus (Supplementary Table 2, see section on supplementary data given at the end of this article). As given in Table 5, when comparing the residents with and without BW gain, we failed to detect a difference in the plasma AM levels of the males, but found a significantly



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**Table 3** Correlation coefficients (*r*) of simple regression analysis for relationships between BMI or WC and AM, BNP, or NT-proBNP.

| | Men | | Women | |
|---|---|---|---|---|
| *r* | BMI | WC | BMI | WC |
| AM | 0.002 | 0.008 | 0.231† | 0.195* |
| BNP | −0.009 | 0.027 | −0.151* | −0.156* |
| NT-proBNP | 0.012 | 0.057 | −0.155* | −0.108 |

AM, adrenomedullin; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP; WC, waist circumference. *$P<0.05$ and †$P<0.01$, Pearson's correlation.

higher level of plasma AM in the female residents with BW gain ($P<0.01$). In comparison between the two genders without BW gain, the plasma AM level in women was significantly lower than that in men ($P<0.01$). The plasma levels of BNP and NT-proBNP in the male and female residents with BW gain were slightly lower than that in those without, but the differences did not reach statistically significant levels. As expected, the plasma levels of two BNP peptides were slightly, but not significantly, higher in the female residents than that in the males, irrespective of BW gain.

## Discussion

Plasma levels of AM, a bioactive peptide with pleiotropic actions, are increased in various human diseases including hypertension, heart failure, and obesity (3, 8), but little is known about gender-related differences in plasma AM. According to an animal study, BW gain via a high-fat diet resulted in augmented AM expression in adipose tissue with concomitant elevation of plasma AM levels in rats (10), but BW gain-induced elevation of plasma AM level has not been proven in humans yet. Examining the general population in this study, we revealed that i) plasma AM levels in women might be lower than that in men; ii) AM levels in women are associated with BW gain and a possible gender-related alteration is noted in the plasma AM–BW gain relationship; and iii) the lower AM levels in women are likely due to those without BW gain.

An important issue that arises in this study is the mechanism for the closer relationship between BW gain and the plasma AM levels in the female residents compared with that in the males. Currently, there is no clear explanation for this, but we can discuss some possibilities based on previous reports. It has been shown that the factors affecting plasma AM levels in humans without overt cardiovascular or renal diseases are age, BMI, blood pressure, and renal function (6, 22). In this study, no differences were noted in those parameters between the subjects with or without BW gain in both genders, except for BW, BMI, and WC.

As fat tissue appears to be an organ contributing to AM circulation in human blood (10, 23), it is possible that a gender difference in BW gain-induced production of AM in the adipose tissue accounts for the present phenomenon. According to a report by Paulmyer-Lacroix *et al.* (23), expression of AM is augmented in the omental adipose tissue of obese women compared with that in the non-obese. In this study, the BW gain-induced elevation of plasma AM may have resulted from increased expression of AM in the visceral fat of the female residents. This is unlikely to be the case in male residents because there was no difference in the plasma AM levels in those with or without BW gain, despite the substantial differences in BW, BMI, and WC; however, there have been no reported studies comparing AM expression in adipose tissue between non-obese and obese men.

Low-grade inflammation in adipose tissues associated with obesity seems involved in the mechanism of the increased plasma AM level in obese subjects, because the AM production is up-regulated by inflammatory cytokines such as TNFα or IL1β (6, 8, 9, 11). It was reported that body fat distribution differs from between two genders: ratios of the visceral fat to the subcutaneous or lower body fat mass were higher in men than in women (24). According to an epidemiological study by Pou *et al.* (25), increased volumes of the visceral and subcutaneous fat were associated with elevation of inflammatory markers, while the former was more closely related to these markers than the latter. In this context, the intimate relationship between the plasma AM and BW gain in women of this study is somehow contradictory. Although there are no data available about menopausal state in this study, it seems

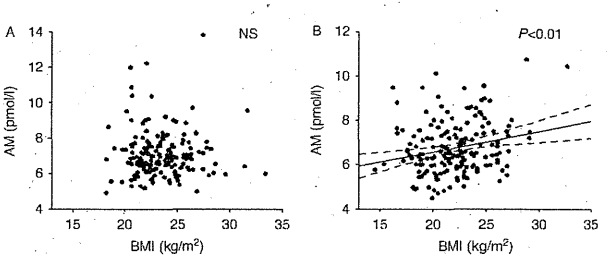


**Figure 1**
Relationships between BMI and plasma AM levels in the male (A) and female (B) residents. The regression line and the 95% confidence limits are shown by solid and broken lines respectively. NS, not significant.

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**Table 4** Basal profiles of the male and female residents with or without BW gain of 10 kg or more. Means±S.D.

| | Men | | Women | |
|---|---|---|---|---|
| **BW gain ≧10 kg** | (−) | (+) | (−) | (+) |
| *n* | 112 | 60 | 122 | 52 |
| Age (years) | 62.7±9.4 | 61.1±8.4 | 61.3±8.8 | 63.2±8.1 |
| BW (kg) | 60.7±7.3 | 69.7±8.6* | 49.1±5.9 | 56.8±7.0* |
| BMI (kg/m$^2$) | 22.4±1.9 | 25.5±2.5* | 21.0±2.5 | 24.4±2.6* |
| Waist circumference (cm) | 82.3±5.6 | 90.4±6.6* | 79.3±8.0 | 88.4±8.0* |
| Mean blood pressure (mmHg) | 95±11 | 98±13 | 89±12 | 91±11 |
| Fasting blood glucose (mg/dl) | 96±15 | 97±11 | 91±8 | 94±10 |
| HbA1c (%) | 5.4±0.3 | 5.5±0.3 | 5.4±0.2 | 5.5±0.3 |
| eGFR (ml/min per 1.73 m$^2$) | 73±14 | 75±14 | 76±13 | 76±15 |

eGFR, estimated glomerular filtration rate. *$P$<0.01 vs without body weight (BW) gain ≧10 kg.

unlikely that sex steroids are involved in the gender difference in BW gain-induced AM production: neither testosterone nor estradiol has much effect on AM production (26). Clearly, further studies are necessary to clarify the mechanism behind the gender difference in BW gain-induced alteration in plasma AM levels.

BNP has natriuretic and vasodilatory effects, exerting cardiovascular protective actions, and plasma levels of BNP are elevated in patients with hypertension and heart failure, as are those of AM (14). The increased BNP levels are thought to be a mechanism counteracting blood pressure elevation and excess body fluid retention in patients with hypertension or heart failure (12, 14). In contrast to these phenomena, plasma BNP level has been shown to be decreased in obesity, where reduced BNP action is assumed to be involved in BW gain-induced elevation of blood pressure (27). Indeed, higher BNP or NT-proBNP levels were found to be associated with favorable adipose tissue distribution by a population-based study (28). Chainani-Wu *et al.* (29) reported increased plasma BNP levels in obese patients with coronary heart disease (CHD) or high risk of CHD following comprehensive life style modification, suggesting that the BNP elevation associated with BW reduction does not necessarily indicate deterioration of heart disease.

In this study, consistent with the notions discussed above, plasma levels of BNP and NT-proBNP in the subjects with BW gain were slightly lower than in those without BW gain in both genders. In addition, plasma levels of these peptides were inversely correlated with BMI or WC in the female subjects; however, the differences between the residents with or without BW gain were not statistically significant in both genders. This study also showed the higher plasma BNP and NT-proBNP levels in women than in men, a finding accordant with the previous notions (12), while those gender difference were less clear as compared with plasma AM. Thus, the present results suggest that both gender- and BW gain-related alterations in plasma levels of the peptides are clearer in AM than in BNP.

Next, we need to discuss the biological or clinical significance of the present findings. AM has been shown to exert a wide range of biological actions including blood pressure lowering, cardiovascular protection, and alleviation of insulin resistance (3, 5). As mentioned above, in the case of BNP, BW gain-related reduction in plasma BNP levels is assumed to be involved in obesity-induced elevation of blood pressure (27). In contrast to this, we speculate, based on the AM actions, that BW gain-induced increase in AM level in the female subjects is a counter-regulatory mechanism against obesity-related disorders such as insulin resistance and hypertension.

Lastly, there are limitations we need to mention in this study. First, a lack of statistical power may need to be taken into account, because we examined a relatively small number of subjects with BW gain data based on the simple questionnaire. For example, differences in the plasma BNP or NT-proBNP levels between two genders or between those with and without BW gain were statistical insignificant. Meanwhile, a significant finding of this study is that the gender-related alterations were clearly seen in the plasma levels of AM despite insignificant differences in those of the BNP peptides. Second, we have been unable to completely exclude residents with inflammatory, respiratory, or liver diseases, which had possibly affected the AM measurement from the study subjects (3, 6, 8). In our health check-up, when physicians notice

**Table 5** Plasma levels of AM, BNP, and NT-proBNP of the male and female residents with or without BW gain of 10 kg or more. Means±S.D.

| | Men | | Women | |
|---|---|---|---|---|
| **BW gain ≧10 kg** | (−) | (+) | (−) | (+) |
| AM (pmol/l) | 7.11±1.22 | 7.20±1.42 | 6.53±1.02$^\dagger$ | 7.34±1.33* |
| BNP (pg/ml) | 19.6±26.8 | 18.2±15.5 | 22.8±15.9 | 19.2±14.4 |
| NT-proBNP (pg/ml) | 64.7±120 | 52.6±51.8 | 70.4±47.8 | 62.6±49.4 |

AM, adrenomedullin; BNP, brain natriuretic peptide; NT-proBNP, N-terminal proBNP. *$P$<0.01 vs without body weight (BW) gain in the identical gender and $^\dagger P$<0.01 vs men without BW gain.

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possibilities of these diseases in history taking or physical examination, they are supposed to describe it on the medical files; but there were no reports about such a disease. Thirdly, this study lacks parameters or clinical tests, with which we could seek further the relationships between AM levels and low-grade inflammation associated with obesity or alterations of body fat distribution (6, 11), such as C-reactive protein and magnetic resonance imaging, and these points need to be clarified in future studies.

In summary, there appear to be gender-related differences in the plasma AM levels and in the BW gain–plasma AM relationship in the general population. The AM levels in the female residents without BW gain during the adolescent period were partly attributed to the lower plasma AM of women.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EC-14-0131.

**Declaration of interest**
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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# Biological properties of adrenomedullin conjugated with polyethylene glycol

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ABSTRACT

Adrenomedullin (AM) is a vasodilator peptide with pleiotropic effects, including cardiovascular protection and anti-inflammation. Because of these beneficial effects, AM appears to be a promising therapeutic tool for human diseases, while intravenous injection of AM stimulates sympathetic nerve activity due to short-acting potent vasodilation, resulting in increased heart rate and renin secretion. To lessen these acute reactions, we conjugated the N-terminal of human AM peptide with polyethylene glycol (PEG), and examined the biological properties of PEGylated AM in the present study. PEGylated AM stimulated cAMP production, an intracellular second messenger of AM, in cultured human embryonic kidney cells expressing a specific AM receptor in a dose-dependent manner, as did native human AM. The pEC50 value of PEGylated AM was lower than human AM, but no difference was noted in maximum response (Emax) between the PEGylated and native peptides. Intravenous bolus injection of 10 nmol/kg PEGylated AM lowered blood pressure in anesthetized rats, but the acute reduction became significantly smaller by PEGylation as compared with native AM. Plasma half-life of PEGylated AM was significantly longer than native AM both in the first and second phases in rats. In summary, N-terminal PEGylated AM stimulated cAMP production in vitro, showing lessened acute hypotensive action and a prolonged plasma half-life in comparison with native AM peptide in vivo.



## 1. Introduction

Adrenomedullin (AM) is a pluripotent bioactive peptide initially isolated from human pheochromocytoma, while AM circulates in the blood and is expressed in a number of tissues and organs, including the heart and blood vessels in humans [9–11,13,14]. The characteristic feature initially found was blood pressure-lowering action due to potent vasodilation [14], but AM has been shown to possess various biological properties: inhibition of cardiac hypertrophy and fibrosis, inhibition of vascular remodeling, neovascularization, inhibition of apoptosis, and anti-inflammation [9–11,13]. A number of experimental studies have been carried out to test where this endogenous peptide exerts beneficial effects by using animal models of various human diseases [1,2,9,10,19]. For example, AM was shown to inhibit cardiac remodeling following acute myocardial infarction and to alleviate hind limb ischemia in rats or mice, and also suppressed inflammatory cytokines, facilitating the healing of colon ulcer, in rat models of inflammatory bowel diseases [1,2,9,10,19]. These findings suggest the potential of AM as a therapeutic tool in the treatment of ischemic heart disease, peripheral vascular disease, and inflammatory bowel diseases, and indeed, AM was shown to be effective in treating patients with those diseases [3,8]. Meanwhile, AM should be infused continuously with careful dose settings in human patients because the short-acting potent vasodilator property of AM could result in acute hypotension, activated sympathetic nerve activity, and increased renin secretion [12]. In an attempt to reduce these acute unfavorable actions, we synthesized molecularly modified AM by conjugating the N-terminal of the peptide with polyethylene glycol (PEG), and characterized the biological effects of the PEGylated AM peptide using cultured cells in vitro and anesthetized rats in vivo.

*Abbreviations:* AM, adrenomedullin; PEG, polyethylene glycol; Boc, t-butyloxycarbonyl; HEK, human embryonic kidney; CLR, calcitonin receptor-like receptor; RAMP, receptor activity-modifying protein.

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## 2. Materials and methods

### 2.1. Preparation of peptide

$N^{\varepsilon}$-t-butyloxycarbonyl-lysyl[25,36,38,46] human AM ($H_2N$-[Lys(Boc)]$_4$-human AM) was chemically synthesized by the solution method with the Boc strategy according to a previous report [6]. $H_2N$-[Lys(Boc)]$_4$-human AM solved in dimethyl sulfoxide (DMSO) was reacted overnight with PEG5000-NHS (SUNBRIGHT ME-050-HS; NOF Corporation, Tokyo, Japan) at room temperature in the presence of diisopropylethylamine. PEG5000-[Lys(Boc)]$_4$-human AM was purified by high-performance liquid chromatography, and then N-terminal PEGylated human AM peptide was obtained by treatment with 95% trifluoroacetic acid for 40 min and further purification with high-performance liquid chromatography.

### 2.2. Cell culture experiments

To test the pharmacological effects of PEGylated AM in vitro, we used human embryonic kidney (HEK)-293 cells stably expressing the AM type I receptor (AM1 receptor), which had been prepared as previously described [16]. This receptor subtype is highly specific to AM and formed by co-expression of calcitonin receptor-like receptor (CLR) and activity-modifying protein-2 (RAMP2) [15,18]. The HEK-293 cell were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G, 100 units/ml streptomycin, 500 μg/ml amphotericin B, 0.4 mg/ml hygromicin B, and 0.25 μg/ml geneticin in a 24-well plate coated with human fibronectin (Invitrogen) in 5% $CO_2$ at 37 °C. After culturing for 3 days, 90% confluent cells were subjected to experiments stimulating intracellular cAMP accumulation by PEG-conjugated or native AM peptides. The culture media were replaced with Hanks' buffer containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 0.1% bovine serum albumin. The cells were then incubated with the indicated concentrations of the peptides in the presence of 0.5 mM isobutylmethylxanthine for 15 min at 37 °C. The reactions were terminated by the addition of cell-lysis buffer, and cAMP levels of the supernatants were measured with an enzyme immunoassay kit (GE Healthcare UK Limited, UK). The pEC50 value and maximum response (Emax) were calculated based on intracellular cAMP concentrations stimulated by the AM peptides at $10^{-11}$ to $10^{-6}$ mol/L.

### 2.3. Animal experiments

Animal experiments were performed in accordance with the Animal Welfare Act and with approval from the University of Miyazaki Institutional Animal Care and Use Committee (No. 2012-501-3). Male Wistar rats of 7–8 weeks of age were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and maintained under a 12-h light/dark cycle in specific pathogen-free conditions with standard chow. After anesthetizing rats by inhalation of 1.5–2.5% isoflurane at a flow volume of 0.6–0.8 L/min, tracheotomy was performed and the trachea was intubated with a PE-240 catheter. A PE-10 catheter was inserted into the left jugular vein for intravenous injection of AM peptides. Similarly, the carotid artery was cannulated with a PE-50 catheter for either blood pressure monitoring or blood sampling before and after peptide injections.

In the blood pressure-monitoring experiment, the catheter inserted into the carotid artery was connected to a pressure transducer (MLT0699; ADInstruments, Australia), the outputs of which were analyzed with a blood pressure-monitoring system (PowerLab and LabChart; ADInstruments) before and after intravenous bolus injections of 3 or 10 nmol/kg PEGylated AM or native human AM peptides. Mean blood pressure prior to the injections was 88.9 ± 2.1 mmHg (mean ± S.D.) and, throughout the experiment, 0.9% saline was infused at a constant rate of 4.8 ml/kg/h. To determine plasma half-lives, either PEGylated or native AM was injected through the jugular vein catheter. Three hundred microliters of blood were drawn via the carotid artery with 21 μg aprotinin and 0.3 mg EDTA-2Na at the indicated time points, and plasma samples were obtained by centrifugation at 3000 rpm. Human AM levels in plasma were measured by a fluorescence enzyme immunoassay using two anti-human AM antibodies: one binds to the ringed structure and the other to the amidated C-terminal of the AM peptide [20]. Cross-reactivity of this method for PEGylated AM was found to be 78% on a molar basis by assaying PEGylated peptide added to rat plasma.

### 2.4. Statistical analysis

All data were analyzed with IBM SPSS software version 21.0 (IBM, Armonk, NY, USA). Plasma half-lives of the AM peptides were calculated by the two-compartment model. Unpaired *t*-test was used to compare two parameters, and multiple comparisons were made by analysis of variance (ANOVA) followed by Tukey's HSD test. All data are expressed as the means ± S.E.M., unless otherwise indicated, and $P < 0.05$ was considered to be significant.

## 3. Results

First, we tested the biological activity of PEGylated AM to stimulate intracellular accumulation of cAMP in HEK-293 cells stably expressing AM1 receptor. As shown in Fig. 1, native human AM peptide elevated intracellular cAMP levels in a dose-dependent manner with pEC50 and Emax values of 8.59 ± 0.90 and 9.30 ± 0.26 nmol/well, respectively. PEGylated AM exerted similar effects, augmenting cAMP production with pEC50 of 8.19 ± 0.10 and Emax of 9.44 ± 0.30 nmol/well. pEC50 of PEGylated AM was significantly ($P < 0.05$) smaller than that of native AM, while Emax values of the two forms of peptide were similar.

Fig. 2 shows the time course of mean blood pressure following bolus intravenous injection of PEGylated or native human AM into anesthetized rats. At the dose of 3 nmol/kg, slight blood pressure reductions were observed, while no significant differences were noted in the hypotensive effects between PEGylated and native peptides. Meanwhile, injection of 10 nmol/kg native AM resulted in a substantial reduction of mean blood pressure of −24.5 ± 3.2 mmHg at 2 min, followed by a gradual rise. An identical dose of PEGylated AM exerted hypotensive effects, which were significantly less than native AM 2 and 4 min after injection. This difference in the hypotensive effects became insignificant at 6 min

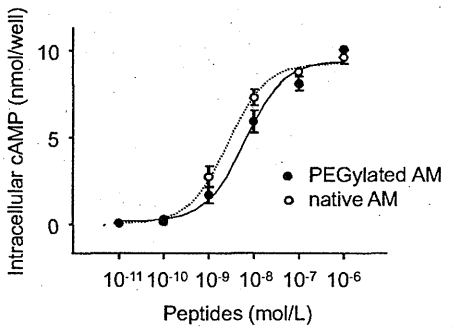


**Fig. 1.** Intracellular cAMP accumulation by PEGylated or native AM in cultured cells. HEK-293 cells stably expressing AM1 receptor were incubated with the indicated concentrations of PEGylated or native human AM peptides for 15 min, and intracellular cAMP levels were measured with enzyme immunoassay. Data are presented as the means ± S.E.M. of 6 samples examined.

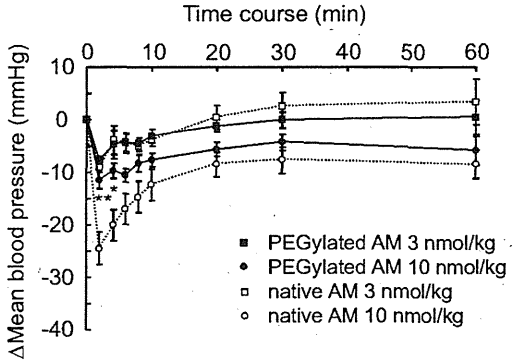


**Fig. 2.** Reduction in blood pressure following intravenous bolus injection of PEGylated or native AM. Indicated doses of either PEGylated or native human AM peptides were injected intravenously at time 0 into anesthetized rats. Data are presented as the means ± S.E.M. of 5 rats examined. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with an identical dose of native human AM.

or later; meanwhile, in contrast to the groups of 3 nmol/kg, reductions of mean blood pressure by the native AM injection remained larger throughout the experiment in comparison with the PEGylated AM injection. No differences in heart rate were noted among the four groups throughout the experiment.

Fig. 3A and B is plasma disappearance curves of PEGylated and native human AM following bolus intravenous injection of 3 nmol/kg into anesthetized rats. The first and second plasma half-lives of PEGylated AM were 4.87 ± 0.68 and 108 ± 12 min (Fig. 3A), while those of native AM were 0.62 ± 0.02 and 15.2 ± 1.9 min (Fig. 3B), respectively. The half-lives of the PEGylated peptide were significantly prolonged when compared with the native peptides ($P < 0.05$).

## 4. Discussion

Consisting of 52 aminoacids, AM is an endogenous bioactive peptide with a wide range of biological actions [13,14]. Both amidated C-terminal $Tyr^{52}$ and a ringed structure formed by $Cys^{16}$-$Cys^{21}$ were shown to be essential for the actions of AM [13,14]. We therefore conjugated $Tyr^{1}$ of the N-terminus of human AM peptide with polyethylene glycol (PEG) and looked at the biological properties of the PEGylated human AM peptide in the present study. PEGylated AM stimulated intracellular cAMP production in cultured cells stably expressing AM type I receptor (AM1 receptor) in vitro, exerting lower acute hypotensive action at a high dose of 10 nmol/kg and a longer plasma half-life in comparison with the native human AM peptide. This is the first report on the pharmacological features of AM molecularly modified by PEG.

Biological actions of AM are mediated by a unique receptor system: calcitonin receptor-like receptor (CLR) can function as AM type 1 and 2 (AM1 or AM2) receptors when co-expressed with receptor activity-modifying protein-2 and 3 (RAMP2 and RAMP3), respectively [15,18]. AM1 receptor (CLR/RAMP2) has been shown to be highly specific to AM in humans and rats [15]. In the present study, we found that PEGylated AM stimulated the production of cAMP, an intracellular second messenger of AM, as did native AM in cultured cells stably expressing the AM1 receptor. Consistent with the previous findings [7], we observed slight reductions of blood pressure following bolus injection of 3 nmol/kg PEGylated or native AM into anesthetized rats. There was no significant difference in blood pressure reduction at a low dose of 3 nmol/kg, but the acute hypotensive action of PEGylated AM was lower than that of native AM at a high dose of 10 nmol/kg. This reduced action of PEGylated AM in vivo is comparable with the result in vitro showing that intracellular cAMP accumulation by PEGylated AM is lower than native AM at $10^{-9}$ mol/L. According to the results of the plasma half-life study, this concentration of peptide is expected to be reached in the blood of rats at an acute phase following bolus injection in vivo.

A notable finding of this study is that the first and second phases of the plasma half-life of PEGylated AM were prolonged 7.9- and 7.1-fold, respectively. PEGylation has been used as a method to enhance or prolong the pharmacological efficacy of peptides or proteins used to treat patients with various diseases. Examples are interferon-α for viral hepatitis, growth hormone (GH) receptor antagonist for acromegaly, and erythropoietin for renal anemia, while there has been no report on AM peptides [4,17,21,22]. In the present study, the mechanisms of the prolonged plasma half-life of PEGylated AM remain to be specified, while those proposed so far are reduced renal clearance due to a larger molecular size and protection from enzymatic degradation [5]. As shown in Fig. 2, the acute hypotension by native AM was lessened by PEGylation, while it remains unproven in the present study whether the prolonged plasma half-life results in the prolonged duration of the action: no significant differences were noted in the hypotensive effects between native and PEGylated AM at 6 min or later. AM has been shown to exert pharmacological actions, including cardiovascular protection, angiogenesis or anti-inflammation, that seem beneficial in the treatment of particular diseases [1,10,11]. Indeed, there are a number of reports implying the potential of AM as a therapeutic tool for patients with acute myocardial infarction, peripheral vascular disease and inflammatory bowel disease [3,8–10]. Meanwhile, when used as an intravenous agent, AM doses should be carefully monitored, because of the acute hypotensive action which activates sympathetic nerve activity, resulting in increased heart rate and renin secretion [12]. The present findings suggest that PEGylation can lessen these acute unfavorable effects and possibly prolong the duration of the action when compared with native

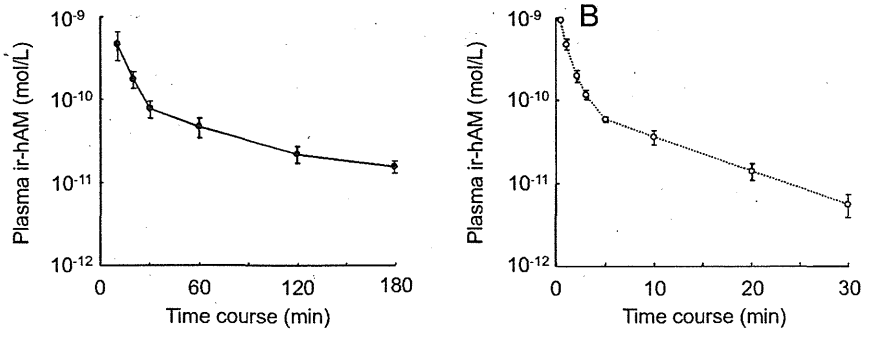


**Fig. 3.** Plasma disappearance curves of PEGylated (A) and native (B) AM peptides. Either PEGylated or native human AM peptide at a dose of 3 nmol/kg was injected intravenously into anesthetized rats, and blood samples were collected at the indicated time points. Data are presented as the means ± S.E.M. of 3 rats examined.

AM peptide. Further studies will be aimed to test whether the longer plasma half-life can be translated into better outcomes in the above-mentioned, diseased settings.

In summary, PEGylated AM peptide stimulated cAMP production in cultured cells expressing AM1 receptor, as did native human AM peptide, while it showed lower acute hypotensive action and longer plasma half-lives than native peptide. These results suggest the potential of PEGylated AM as a therapeutic tool devoid of the unfavorable effect of acute hypotension of native AM.

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