twice that seen in vessels from DR rats ($P < 0.01$) (Fig. 5A); moreover, Western blotting showed the pattern of protein expression to parallel that of the mRNA (Fig. 5B). Infusion of AM reversed this effect, however, suppressing eNOS expression to levels comparable to those seen in DR rats. In addition, the mRNA expression of GTP cyclohydrolase I, the rate limiting enzyme catalyz-

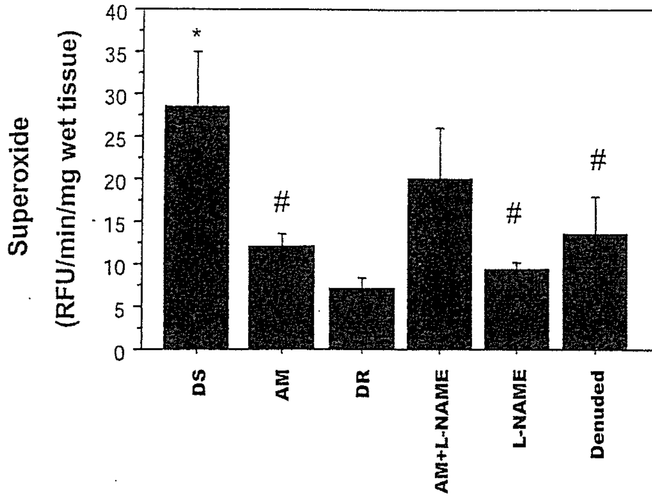

Fig. 3. Superoxide production measured as a function of lucigenin-enhanced chemiluminescence. Aortic rings were incubated with lucigenin (5 μmol/L), after which chemiluminescence was measured using a luminometer. Data are presented as relative light units (RLU) per milligram of wet tissue; $n = 6$–8 in each group; $^*P < 0.05$ vs. DR, $^{\#}P < 0.05$ vs. DS.

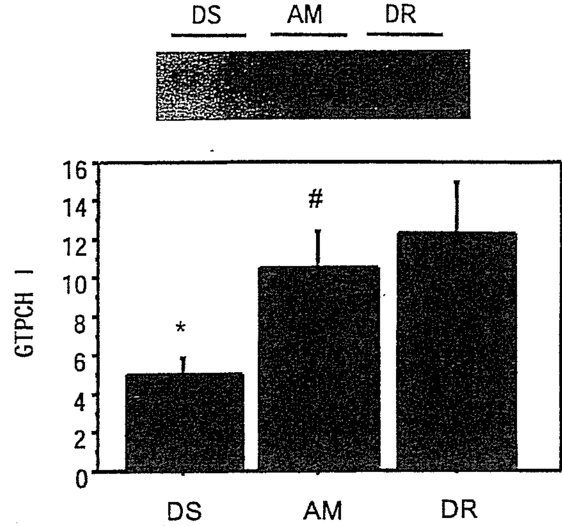

Fig. 6. Expression of mRNAs encoding GTP cyclohydrolase I (GTPCH I). Target genes were amplified by direct RT-PCR, after which the densities of the amplified products on agarose gels were scanned and normalized to GAPDH mRNA, which had been quantified using real-time RT-PCR. Data are expressed as means ± SEM; $n = 6$ in each group; $^*P < 0.05$ vs. DR, $^{\#}P < 0.05$ vs. DS.

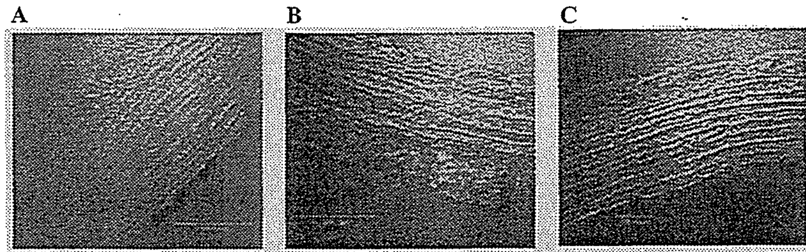

Fig. 4. Confocal images depicting $O_2^{\cdot -}$ production in representative vessels from the rats in DS (A), AM (B), and DR (C) groups. Frozen sections cut from aortic rings were labeled with dihydroethidine and examined under a confocal laser scanning microscope. The red signal reflects production of $O_2^{\cdot -}$. Scale bars: (A) and (B), 100 μm; (C) 50 μm.

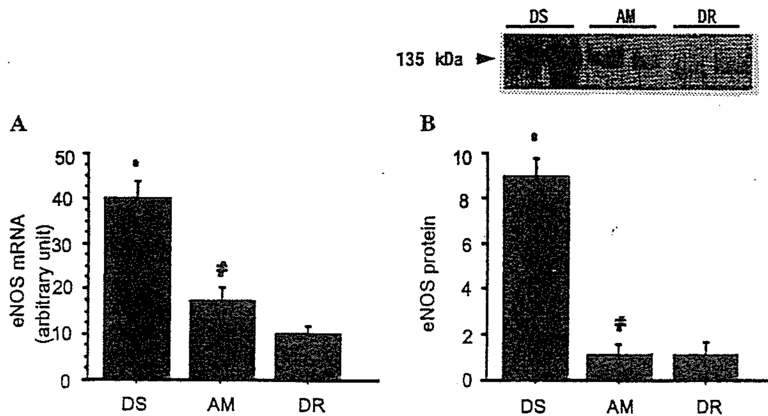

Fig. 5. Endothelial NO synthase (eNOS) mRNA and protein expression. eNOS mRNA (A) and protein (B) expression were evaluated using real-time RT-PCR and Western blot analysis, respectively. Target mRNA expression was normalized by the level of GAPDH mRNA. Data are expressed as means ± SEM; $n = 6$ in each group; $^*P < 0.05$ vs. DR, $^{\#}P < 0.05$ vs. DS.

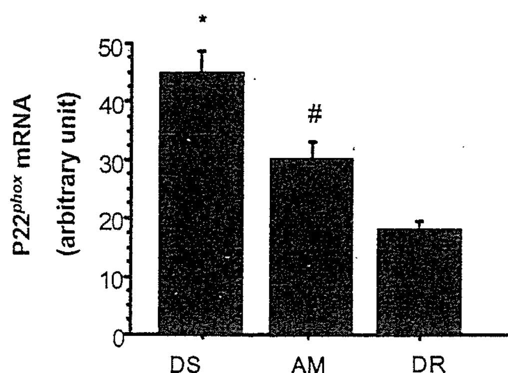

Fig. 7. Expression of mRNA encoding NADPH oxidase subunit p22^{phox} using real-time RT-PCR. Expression of the target mRNA was normalized to the level of GAPDH mRNA. Data are expressed as means ± SEM; $n = 6$ in each group; $^{*}P < 0.05$ vs. DR, $^{\#}P < 0.05$ vs. DS.

ing the synthesis of the NOS cofactor BH4, was significantly ($P < 0.01$) downregulated in DS rats, and that effect, too, was reversed by infusion of AM (Fig. 6).

Finally, expression of one of the subunits of NADPH oxidase, p22^{phox}, was upregulated in DS rat vessels, and infusion of AM attenuated that effect (Fig. 7).

Discussion

With this study, we have shown for the first time that GTP cyclohydrolase I, the rate limiting enzyme in the de novo synthesis of the NOS cofactor BH4, is downregulated in DS rats. This likely leads to insufficient synthesis of BH4, which would in turn lead to the uncoupling of eNOS activity and production of excess $O_2^{\cdot -}$. We also found that eNOS expression was upregulated in DS rats, which, in the absence of adequate BH4, would be expected to exacerbate the uncoupled NOS activity under some pathophysiological conditions. Excess $O_2^{\cdot -}$ production in DS rat vessels could be reduced by removing the endothelium, by administering the NOS inhibitor L-NAME, or by infusing AM, which alleviated the uncoupled eNOS activity, thereby diminishing $O_2^{\cdot -}$ production and increasing NO bioavailability.

BH4 is an essential NOS cofactor that mediates the homodimerization of the enzyme necessary for NO production. Although eNOS synthesizes mainly NO under normal conditions, when concentrations of BH4 are suboptimal, it can produce $O_2^{\cdot -}$ instead through "uncoupled" electron transfer from its reductase domain to O_2 to form $O_2^{\cdot -}$ [27–29], which in turn leads to endothelial dysfunction. Conversely, exogenous administration of BH4, or the BH4 precursor sepiapterin, inhibits $O_2^{\cdot -}$ production and restores NOS function, improving endothelial function [30–32]. Our observation that treating DS rats with BH4 for 4 weeks improved endothelial function strongly suggests that BH4 levels are insufficient in the DS rat aorta, which would lead to overproduction of $O_2^{\cdot -}$ and endothelial dysfunction.

Recent studies have shown that transfer of the GTP cyclohydrolase I gene to endothelial cells or deoxycorticosterone acetate (DOCA)-salt rat vessels leads to increases in both BH4 production and NOS activity [33,34], confirming the central role played by GTP cyclohydrolase I in regulating NO production. Our finding that expression of GTP cyclohydrolase I mRNA was downregulated in the genetically hypertensive DS rat model, and that infusion of AM restored the enzyme's expression is consistent with those earlier findings.

The mechanism by which AM upregulates GTP cyclohydrolase I expression may be related to the level of endothelial cAMP. AM was first discovered by monitoring the rise in cAMP elicited in platelets by extracts of pheochromocytoma [1]. Since then, cAMP has been shown to be the major second messenger mediating AM signaling in both cultured cells and blood vessels [35–40]. Although little is known about the regulation of GTP cyclohydrolase I, there is one report showing that the enzyme is upregulated by cAMP [41], which is at least suggestive of the mechanism by which AM stimulates its expression.

It is noteworthy that we also found eNOS expression to be upregulated in DS rats. This is consistent with earlier studies showing endothelial dysfunction to be associated with increases in both eNOS expression and oxidative stress in rat models of diabetes mellitus and angiotensin II-induced rat hypertension [42,43]. As demonstrated by Harrison's group, the increase in eNOS levels could be related to the increase in hydrogen peroxide caused by dismutation of excess $O_2^{\cdot -}$ [44]. Another important consideration is that $O_2^{\cdot -}$ derived from NADPH oxidase causes NOS uncoupling through oxidation of BH4 by peroxynitrite [45]. Indeed, upregulation of p22^{phox} mRNA expression was also observed in the present study, and treatment with AM attenuated that effect, as reported previously [46].

Finally, our finding that the AM-induced reduction in $O_2^{\cdot -}$ production had no effect on blood pressure suggests that the oxidative stress seen in the blood vessels of DS rats is a consequence of their high blood pressure, not the cause. In similar fashion, the angiotensin II type 1 receptor antagonist candesartan reduced $O_2^{\cdot -}$ production without affecting blood pressure in the same model [47]. The AM-induced decrease in HR seen in DS rats could be explained by the amelioration of oxidative stress, which has been shown to have a positive relation with sympathetic nerve activity; indeed, treatment with the SOD mimetic Tempol significantly reduces both $O_2^{\cdot -}$ and HR [48,49].

In summary, DS rats fed a high salt diet exhibited downregulated GTP cyclohydrolase I transcription and upregulated p22^{phox} transcription, which likely contributed to increase in uncoupled NOS activity and in-

creased $O_2^{\cdot-}$ production. Infusion of AM suppressed $O_2^{\cdot-}$ production, increasing both the synthesis and bioavailability of NO—i.e., AM was able to greatly improve endothelial function. This also suggests that AM or a similarly acting analogue may represent as an ideal therapeutic agent with both antihypertensive and antioxidant properties.

Acknowledgments

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Antifibrotic effect of adrenomedullin on coronary adventitia in angiotensin II-induced hypertensive rats

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Abstract

Objective: The extracellular matrix (ECM) determines the structural integrity of the heart and vasculature, participating in cardiovascular remodeling. We previously reported that adrenomedullin (AM) inhibited cellular proliferation and protein synthesis of cardiac fibroblasts; however, the precise mechanisms of AM actions as an antifibrotic factor remain unknown. The purpose of this study was to examine the biological actions of AM against the profibrotic factor angiotensin II (Ang II) in coronary adventitia.
Methods and results: Rats with hypertension induced by Ang II infusion were administered 0.06 μg/kg/min recombinant human AM subcutaneously for 14 days. The AM infusion significantly ($p<0.05$) reduced the Ang II-induced increase of coronary adventitial fibroblasts expressing Ki-67 and α-smooth muscle actin (α-SMA) in the left ventricle, by 65%, and 62%, respectively, without affecting systolic blood pressure, left ventricle/body weight, or cross-sectional area of myocardial fibers. Collagen deposition of coronary arteries was reduced by the AM infusion (−24%, $p<0.01$), and these effects of AM were accompanied by significant reductions in gene expression of type 1 collagen (−49%, $p<0.05$) and transforming growth factor-β1 (TGF-β1) (−55%, $p<0.01$). In cultured cardiac fibroblasts, 10^{-7} mol/L AM exerted an inhibitory effect on TGF-β1-induced α-SMA expression ($p<0.01$) that was mimicked by 8-bromo-cAMP and attenuated by the protein kinase A inhibitor H-89.
Conclusion: AM decreased Ang II-induced collagen deposition surrounding the coronary arteries, inhibiting myofibroblast differentiation and expressions of ECM-related genes in rats. The present findings further support the biological action of AM as an antifibrotic factor in vascular remodeling.
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Keywords: Extracellular matrix; Fibrosis; Hypertension; Peptide hormone; Remodeling

1. Introduction

Cardiac fibrosis is an important clinical disorder leading to deleterious consequences for myocardial function such as systolic and diastolic heart failure [1]. Particularly, thickening of the adventitia surrounding intramyocardial coronary arteries, where extracellular matrix (ECM) first accumulates in response to systemic hypertension, has been thought to reduce oxygen and nutrient supply to the *myocardium*, resulting in deterioration of ventricular function [2]. Emerging concepts of vascular remodeling underline the importance of the ECM scaffold in the vessel wall. The activated adventitial fibroblasts, known as myofibroblasts characterized by α-smooth muscle actin (α-SMA) expression, play important roles in the pathological vascular remodeling [3,4]. Therefore, both understanding of the regulation of fibroblast activation and the

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development of effective pharmacological intervention to manipulate fibroblast function are necessary to attenuate adverse remodeling.

A body of evidence suggests that the renin–angiotensin–aldosterone system is an important factor in progression of myocardial and vascular fibrosis accompanied by upregulation of transforming growth factor-β1 (TGF-β1) [5–7]. TGF-β1 induces a phenotypic change of fibroblasts to myofibroblasts in hypertensive heart disease, coronary restenosis following angioplasty, and in the healing process after myocardial infarction [8,9]. Blockage of TGF-β1 signaling was indeed reported to prevent fibroblast proliferation and diastolic cardiac dysfunction [10].

Adrenomedullin (AM), initially isolated from human pheochromocytoma [11], has been reported to have multiple functions in the cardiovascular system [12]. We and others have previously reported that AM inhibited proliferation and collagen synthesis induced by angiotensin II (Ang II) in cardiac fibroblasts of neonatal rats in vitro [13,14], suggesting a possible role of AM in attenuating cardiovascular remodeling. However, the precise mechanism by which AM acts as an antifibrotic factor in vivo remains to be elucidated.

Based upon previous studies, we hypothesized that activation of adventitial fibroblasts would result in coronary matrix remodeling in rats infused with Ang II and that pharmacological intervention with AM would lead to attenuation of perivascular fibrosis by modulating fibroblast function. Our aim in this study was to examine the biological action of AM against the profibrotic factor Ang II in coronary adventitia of rats.

2. Methods

2.1. Animals experiments

Eight-week-old male Wistar rats (Charles River Japan) weighing 200 to 250 g were housed in a temperature- and light-controlled room (25±1 °C; 12/12-h light/dark cycle) with normal rat chow and water given ad libitum. The rats were divided into three groups: control group (n=5) and two Ang II-infused groups with (n=11) or without (n=15) AM treatment. They were implanted with miniosmotic pumps (Alzet model 2002) under pentobarbital sodium anesthesia, that released either saline or 250 ng/kg/min Ang II for 14 days. In the Ang II-infused groups, another pump was implanted to infuse saline or 0.06 μg/kg/min of recombinant human AM (Shionogi & Co., Japan). The dose of AM used in this study was determined by referring to our previous observation, in which cardiac remodeling after myocardial infarction was significantly inhibited without affecting systemic blood pressure [15]. Blood pressure was measured while awake at least 9 times by tail-cuff plethysmography (Softron, BP-98A), and the mean value was recorded. At day 14, the rats were killed by decapitation and trunk blood was collected for measuring AM concentration. Plasma levels of human and rat AM were determined with commercially available immunoradiometric assay kits (Shionogi & Co., Japan). After removing atria and right ventricle of the heart, left ventricle was frozen in liquid nitrogen or fixed in 10% formalin and was embedded in paraffin wax.

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 (2003-023). This investigation confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Histology and immunohistochemistry

Ventricular tissues, fixed in 10% formalin and embedded in paraffin, were sectioned at 2 μm thickness. After deparaffinization with xylene and graded alcohol, slides were immersed in 3% H_2O_2 in methanol to block endogenous peroxidase activities, thereafter incubated with 5% skim milk to reduce the nonspecific background. The section slides were then stained with either anti-monoclonal α-SMA antibody (Clone 1A4, DAKO) at a dilution of 1:200, or antipolyclonal TGF-β1 antibody (sc-146, Santa Cruze) at 1:100 at 4 °C. After the overnight reaction with antibodies, the slide sections were incubated with EnVision+ (DAKO) for 30 min, visualized with 0.05% 3, 3′-diaminobenzidine containing hydrogen peroxide, and counterstained with hematoxylin. For detection of Ki-67 antigen, a nuclear protein expressed in proliferating cells, tissue sections were autoclaved at 121 °C for 10 min in 10 mmol/L citrate buffer (pH 6.0) prior to incubation with primary antibody (Clone MIB-5, DAKO) at a dilution of 1:25. For the detection of collagen, slides were incubated with 0.1% picrosirius red (Direct Red 80, Sigma) dissolved in saturated picric acid for 10 min as described before [15]. The specificity of the antibody for TGF-β1 was confirmed by substitution of nonimmune rabbit serum and the absorption test as described before [16].

2.3. Morphology and cell counting

Morphological evaluation and cell counting of coronary arteries sectioned at the middle portion in the left ventricle were performed by a single observer in a blind manner. Each section immunostained with the antibody against either Ki-67 or α-SMA was scanned at a magnification of ×200, and the number of positive cells surrounding the coronary artery was determined. At least five images of fibrosis areas surrounding the blood vessel were randomly selected from each slide, and examined using an image

analysis system (Axio Vison 2.05 Carl ZEISS, Munchen, Germany) to calculate ratios of the perivascular fibrosis area to the total vascular area. To evaluate the interstitial fibrosis, collagen volume fraction in the interstitial space between myocardial fibers was determined by calculating the ratio of collagen area to the selected myocardial tissue area as previously described [15]. To measure the *cardiocyte* size, cross-sectional area of *myocardial fiber* was measured at the level of nuclei in at least 10 *cardiocytes* as described before [16]. Longitudinal- or oblique-sectioned *cardiocytes* were excluded for the analysis.

2.4. Gene expression

Gene expressions for TGF-β1 and type 1 collagen in total RNA isolated from left ventricle were measured by using real time-quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems) as previously described [17]. cDNA reverse transcribed from total RNA was amplified with the following oligonucleotide probes labeled with 6-carboxy-fluorescencein as reporter fluorescence and 6-carboxy tetramethyl-rhodamine as quencher fluorescence: TGF-β1 [18], TACGCCTGAGTGGCTGTCTTTTGA (nucleotide 985–1008); type 1 collagen [19], ACTGGAGACAGAGGA-CCGCGTGGAC (nucleotide 103–127); 18*S* ribosomal RNA [20], TGCCGACGGGCGCTGACC (nucleotide 176–193) and with the following pairs of oligonucleotides: TGF-β1 [18], TTCCTGGCGTTACCTTGGT (nucleotide 943–961, forward primer) and GCCACTGCCGGACAACT (nucleotide 1018–1034, reverse primer); type 1 collagen [19], TGCTGCTTGCAGTAACGTCG (nucleotide 32–51, forward primer) and TCAACACCATCTCTGCCTCG (nucleotide 148–167, reverse primer); 18*S* rRNA [20], CTTTGGTCGCTCGCTCCTC (nucleotide 118–136, forward primer) and CTGACCGGGTTGGTTTTGAT (nucleotide 229–248, reverse primer). The PCR products electrophoresed were observed at the expected molecular sizes, and the gene expression levels were normalized relative to that of 18*S* rRNA.

2.5. Cell culture

Cultured cardiac fibroblasts of neonatal rats were prepared as previously described [13]. After achieving confluence in the DMEM/F12 medium with 10% FBS, the cells were incubated with serum-free medium containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite for 24 h. The medium was then exchanged for fresh serum-free medium described above and incubated with or without synthetic rat AM (Peptide Institute, Osaka, Japan), recombinant human TGF-β1 or 8-bromo-cAMP (Sigma, MO, USA). In another series of experiment, H-89 (Seikagaku, Tokyo, Japan), a specific protein kinase A inhibitor, was added to culture medium at least 30 min before the incubation with AM or TGF-β1.

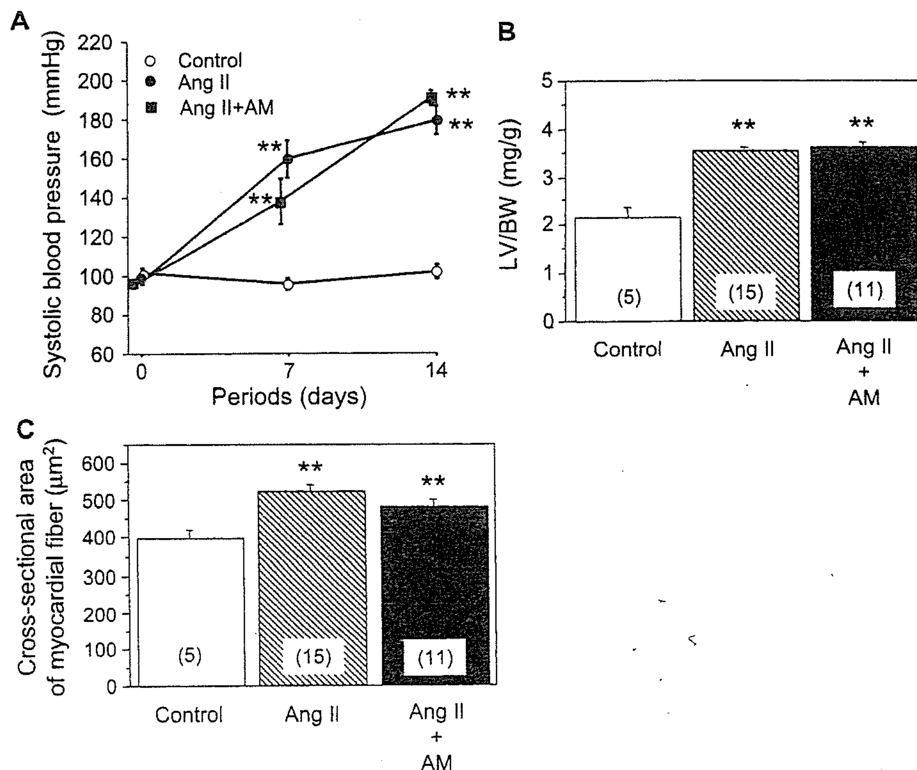

Fig. 1. Effects of Ang II and co-administration of AM on systolic blood pressure (A), left ventricular weight/body weight (LV/BW) (B), and *cross-sectional area of myocardial fiber* (C). Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined. $^{**}p<0.01$, compared to controls.

2.6. Western blot

Denatured protein extract (5 μg) from the cultured cardiac fibroblasts was subjected to sodium dodecyl sulfate–polyacrylamide gel as previously described [21]. The separated proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (BIO-RAD). Equal protein loading was verified by staining the gels with Coomassie brilliant blue. After blocking the non-specific background with 5% skim milk, PVDF membranes were incubated with the anti-α-SMA monoclonal antibody at a dilution of 1:1000, followed by incubation with horseradish peroxidase-coupled second antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and intensities of the bands were analyzed densitometrically (Chemi Doc™ Documentation System, BIO-RAD).

2.7. Statistical analysis

All data are expressed as means±S.E.M. Comparisons between groups were assessed with one-way ANOVA followed by the Fisher's test. A statistical significance was accepted at $p<0.05$.

3. Results

3.1. Systolic blood pressure, left ventricle/body weight, and cardiocyte size

Fig. 1A illustrates the effects of Ang II and AM on systolic blood pressure. Continuous, subcutaneous Ang II infusion significantly ($p<0.01$) increased systolic blood pressure at days 7 and 14, and the co-administration of AM

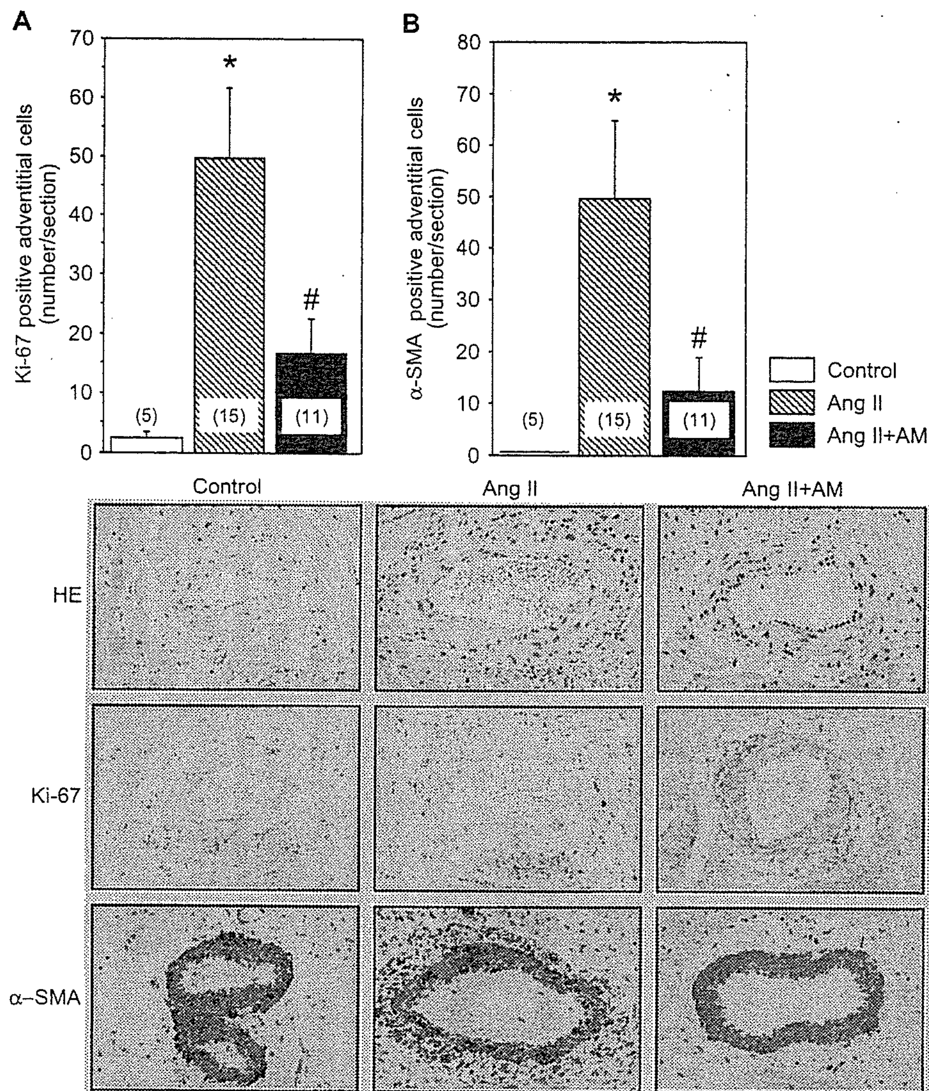

Fig. 2. Effects of Ang II and AM on the number of adventitial fibroblasts expressing Ki-67 antigen (A) and of those positive for α-SMA (B). The bottom panels show the representative histological sections stained with hematoxylin eosin (HE), anti-Ki-67, and α-SMA antibodies. Values are shown as means±S.E.M. Parentheses indicate the number of rats examined. *$p<0.05$, compared to controls; $^{\#}p<0.05$, compared to Ang II group.

with Ang II did not affect systolic blood pressure significantly. In addition, Ang II significantly ($p<0.01$) increased the left ventricle/body weight (LW/BW) and *cross-sectional area of myocardial fiber*, compared to control at day 14, without a significant difference in LV/BW or *cardiocyte* size between the Ang II and Ang II+AM groups (Fig. 1B and C).

3.2. Fibroblast proliferation and myofibroblast differentiation

Fig. 2A and B illustrate the effects of Ang II and AM on staining for Ki-67 antigen and α-SMA in the perivascular area of coronary arteries. Ang II significantly ($p<0.01$) increased the number of fibroblasts expressing Ki-67 antigen, a marker for proliferating fibroblasts, and this increase was significantly ($p<0.05$) inhibited by the co-administration of AM at day 14 (Fig. 2A). Similarly, the Ang II-induced increase in number of the fibroblasts expressing α-SMA, a marker for myofibroblast differentiation, was significantly ($p<0.05$) reduced by AM (Fig. 2B).

3.3. Type 1 collagen gene expression and adventitial area

Fig. 3A illustrates the effects of Ang II and AM on type 1 collagen mRNA expression. The Ang II infusion significantly ($p<0.05$) increased type 1 collagen expression in the left ventricle, and the co-administration of AM significantly ($p<0.05$) attenuated its expression by 49% at day 14. The effects of Ang II and AM on the adventitial area surrounding the coronary arteries are shown in Fig. 3B as composite data and in Fig. 3C as representative pictures. Ang II significantly ($p<0.01$) increased perivascular fibrosis at day 14, and the co-administration of AM significantly ($p<0.01$) decreased it. Similarly, the Ang II infusion significantly increased interstitial fibrosis of the left ventricular myocardium (+130%, $p<0.01$), while AM inhibited this Ang II effect (−54%, $p<0.01$).

3.4. TGF-β1 expression

As shown in Fig. 4A, Ang II significantly ($p<0.01$) increased TGF-β1 gene expression in the left ventricle, while the co-administration of AM significantly ($p<0.01$) attenuated its expression by 55%. Fig. 4B illustrates the distribution of TGF-β1 immunoreactivity in the coronary arteries. TGF-β1 immunoreactivity was intensely stained in the adventitial fibroblasts, as well as in vascular smooth muscle cells and *myocardial fibers* of the Ang II-treated rats, while those cells were faintly stained in the control and AM-treated rats.

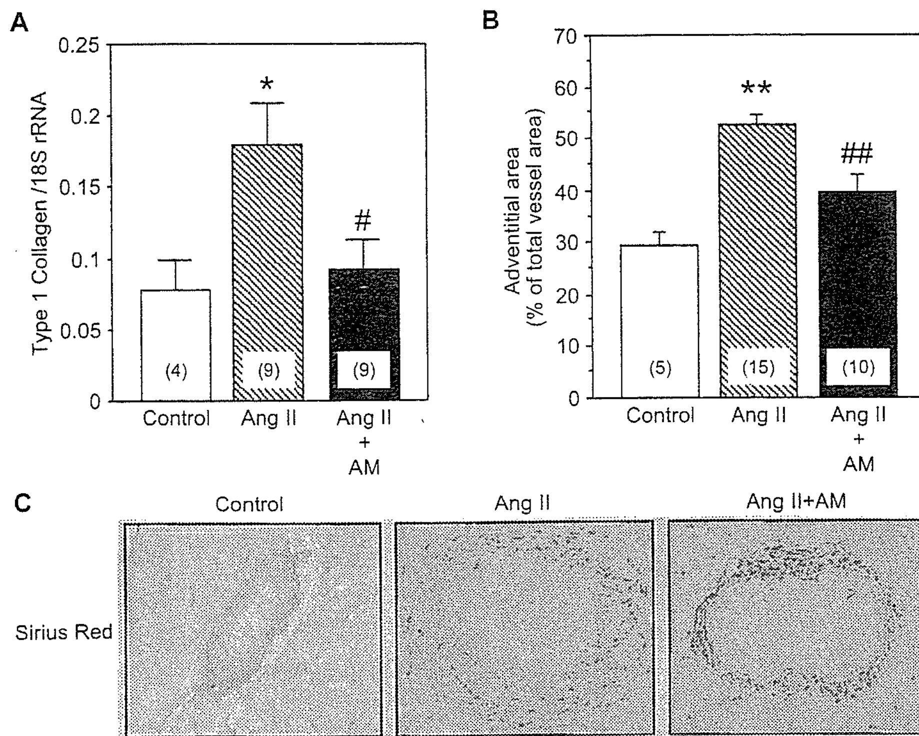

Fig. 3. Effects of Ang II and AM on gene expression of type 1 collagen (A) and on adventitial area determined by sirius red staining (B). The bottom panels (C) show the representative pictures for sirius red staining. Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined. *$p<0.05$, **$p<0.01$, compared to controls; #$p<0.05$, ##$p<0.01$, compared to Ang II.

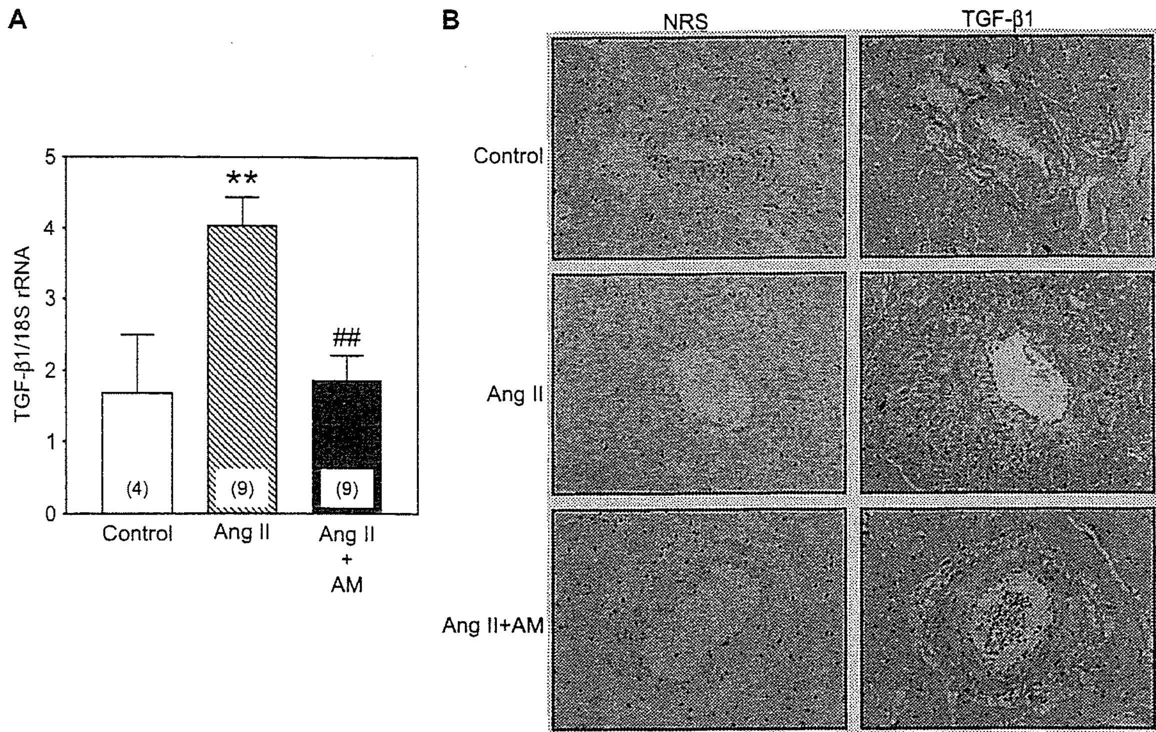

Fig. 4. Effects of Ang II and AM on the gene expression of TGF-β1 in LV (A) and representative pictures for the distribution of TGF-β1 immunoreactivity (B). Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined and NRS denotes nonimmune rabbit serum. $^{**}p<0.01$, compared to controls; $^{\#\#}p<0.01$, compared to Ang II.

3.5. Plasma levels of rat and human AM

The Ang II infusion had no significant effect on the plasma levels of endogenous rat AM at day 14 (control, 4.7±0.5; Ang II, 5.0±0.3 fmol/mL). Human AM immunoreactivity was detectable only in the plasma of recombinant AM-treated rats at 0.7±0.4 fmol/mL at day 14.

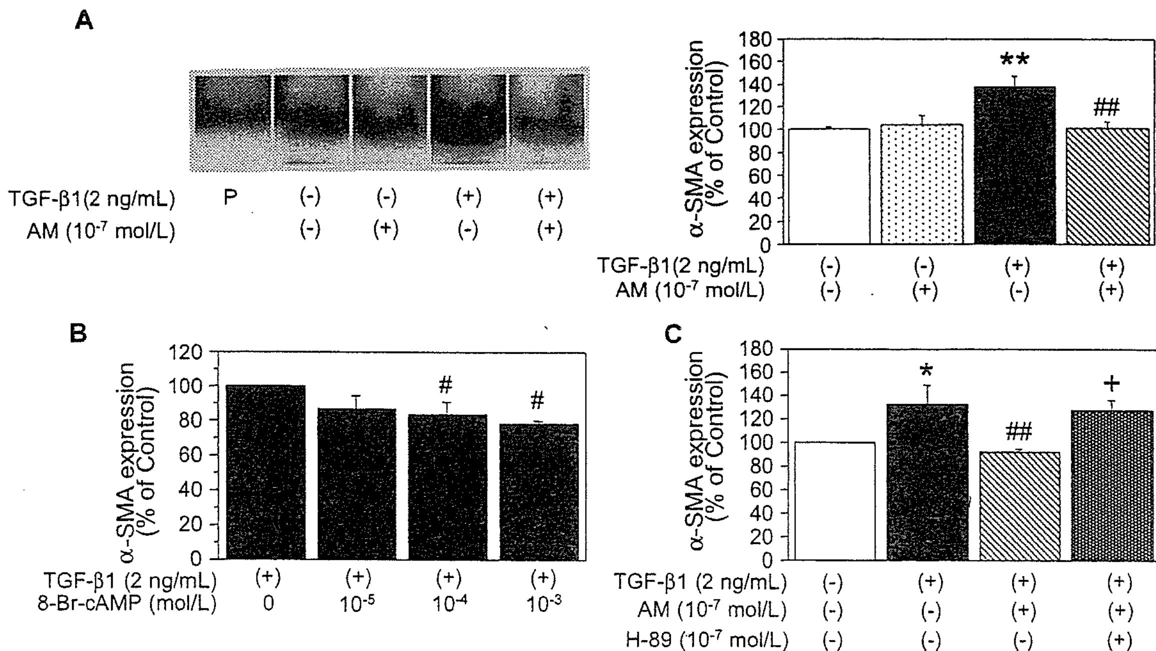

Fig. 5. Effects of AM (A), 8-bromo-cAMP (8-Br-cAMP; B), and H-89, a protein kinase A inhibitor (C) on the α-SMA expression stimulated by TGF-β1 in cultured cardiac fibroblasts. Values are shown as the means±S.E.M. of 5 to 7 (A), 3 (B), and 5 (C) samples examined. $^{*}p<0.05$, $^{**}p<0.01$, compared to controls; $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to 2 ng/mL TGF-β1; $^{+}p<0.05$, compared to TGF-β1 plus AM. P: human aorta.

3.6. α-SMA expression in vitro

To further clarify the direct action of AM on myofibroblast phenotypic change, cultured cardiac fibroblasts were treated with TGF-β1 and/or AM to look at the expression level of α-SMA. Fig. 5A illustrates a representative Western blot and the composite data. Two ng/mL TGF-β1 significantly ($p<0.01$) increased the α-SMA expression by 38% in these cells. Treatment with 10^{-7} mol/L AM significantly ($p<0.01$) inhibited the TGF-β1-induced α-SMA expression by 27%. Similarly, 8-bromo-cAMP, an analogue of cyclic AMP (cAMP), inhibited the α-SMA expression induced by TGF-β1 (Fig. 5B); while pretreatment with 10^{-7} mol/L H-89, a specific protein kinase A inhibitor, significantly ($p<0.05$) attenuated the action of AM (Fig. 5C).

4. Discussion

In this study, we report that AM attenuates the Ang II-induced perivascular fibrosis of coronary arteries, suppressing myofibroblast differentiation and expressions of TGF-β1 and type 1 collagen, without affecting blood pressure, left ventricular weight, and cross-sectional area of myocardial fiber. Ventricular remodeling characterized by *myocardial* hypertrophy and fibrosis results in serious consequences for cardiac function. Remodeling of the myocardium involves alteration of the function of fibroblasts, the major cells making up two-thirds of the total cell number in the heart [22]. Fibroblasts change their phenotype to myofibroblasts capable of producing ECM proteins, and this was reported to be a critical step for progression of the fibrosis [3]. The ECM initially accumulates around coronary arteries in response to systemic hypertension and then expands into the interstitial space between *myocardial fibers* [2], therefore suppressing the activation of perivascular fibroblasts might be important to attenuate the adverse remodeling. Using an Ang II-induced hypertensive model, our study supports the previous report by Campbell et al. [7] that Ang II temporally induces the phenotypic change of fibroblasts in the rat heart.

We previously showed that synthetic AM inhibited the Ang II-induced cellular proliferation and growth of cultured cardiac fibroblasts [13]. Consistent with our previous in vitro study [13], we observed in the present study that AM exerted an antiproliferative effect on fibroblasts as determined by the number of Ki-67-positive cells, counteracting the effect of Ang II. In addition, we demonstrated for the first time that the number of adventitial fibroblasts expressing α-SMA, a marker for fibroblast activation, significantly decreased following the AM administration. It should be noted that these AM effects were observed with little change in blood pressure and in left ventricle/body weight and *size of myocardial fiber*. Accordant with the in vitro study by Tomoda et al. [23], cardiac fibroblasts may be more sensitive to AM than *cardiocytes*. Meanwhile, we recently reported using a rat model of myocardial infarction, that AM infusion in an acute phase of the infarction inhibited not only chronic progression of interstitial fibrosis but also of *myocardial* hypertrophy [15]. This seems inconsistent with the present study in terms of alleviation of cardiac hypertrophy; however, the model differs from each other and left ventricular end-diastolic pressure was lowered by the AM infusion in our myocardial infarction experiment. This difference may support the hypothesis for differential regulation of *myocardial* hypertrophy and fibrosis; inappropriate humoral activations stimulate myocardial fibrosis, while hemodynamic factors regulate *growth of myocardial fibers* [2,10,24]. Another possible explanation for the inconsistency may be a difference in the experimental periods of 2 vs. 9 weeks. Because humoral factors including endothelin-1 and TGF-β1 produced by cardiac fibroblasts have been reported to be involved in the *cardiocyte* growth in vitro [25,26], AM treatment for longer periods of time would reduce *growth of myocardial fibers* by modulating fibroblast function.

TGF-β1 plays an important role in myocardial and vascular fibrosis by stimulating the phenotypic change of fibroblasts to myofibroblasts [3] capable of producing matrix proteins. Indeed, blockage of the TGF-β1 action produced the beneficial effect on fibrosis in pressure-overloaded heart [10]. This is comparable with the report by Jesmin et al. [27] showing that the TGF-β1 immunoreactivity is intensely stained in the perivascular area as well as in the vascular wall, concomitantly with TGF-β1 gene up-regulation, in the process of vascular remodeling. In the present study, the reductions of TGF-β1 and type 1 collagen expression with reduced collagen deposition were observed in the AM-treated rats. Both TGF-β1 and AM have been reported to be expressed in a similar pattern during the development of embryonic mouse heart [28] and, in addition, von der Hardt et al. [29] reported that aerosolized AM inhibited TGF-β1 gene expression in the porcine lung. Thus, there seems to be interaction between these two growth-regulatory factors in the process of vascular remodeling.

Many of the AM actions have been shown to be mediated by accumulation of intracellular cyclic AMP (cAMP) [12] and consistent with this, significance of cAMP signaling in attenuating the myofibroblastic change was reported in lung fibroblasts [30] and in hepatic stellate cells [31]. Our in vitro experiments of this study showed that both AM and the cAMP analogue inhibited protein expression of α-SMA induced by TGF-β1 in cultured cardiac fibroblasts; while the protein kinase A inhibition reversed the action of AM. In comparison with the in vivo experiments, the much higher concentration of AM was required to see the clear suppression of α-SMA levels in cultured cardiac fibroblasts; although the present findings suggest possible involvement of the cAMP-protein kinase A pathway in attenuation of the myofibroblast differentiation by AM.

According to the recent reports, heterozygotes of AM knockout mice have shown augmented responses of interstitial or perivascular fibrosis in the myocardium of pressure overload [32] and Ang II/salt-loading hypertension [33] and of intimal hyperplasia in cuff-induced vascular injury [34], compared to their littermates, suggesting cardiovascular protective effects of AM. The proposed mechanisms for such AM effects protective against cardiovascular remodeling are suppression of the renin–angiotensin–aldosterone system and reductions of oxidative stress and protein kinase C activity [15,32–34]. Our present study suggests the profile of AM as an antifibrotic factor counteracting TGF-β1 action by modulating myofibroblast differentiation in the process of vascular remodeling. Meanwhile, Ang II was used to induce hypertension and coronary perivascular fibrosis in the present study, but we are unable to attribute the beneficial effects of AM to specific inhibition of the action of Ang II. These effects may be expected in other forms of hypertension; although further studies are necessary to clarify this point.

In summary, AM infusion for 2 weeks attenuated the Ang II-induced coronary matrix remodeling, suppressing fibroblast activation and expression of TGF-β1 in rats. Because AM is produced in the myocardium and vascular wall, these findings further support the notion that AM is a modulator of cardiovascular remodeling via modulation of fibroblast function.

Acknowledgements

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Adrenomedullin alleviates not only neointimal formation but also perivascular hyperplasia following arterial injury in rats

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Abstract

Producing components of the extracellular matrix, the vascular adventitia has been recognized as an important modulator of the vascular remodeling process, which determines the vessel architecture. In this study, we examined the effect of the vasodilator peptide adrenomedullin on vascular remodeling induced by balloon injury of rat carotid arteries. Endothelial denudation with wall stretch by ballooning not only induced neointimal formation accompanied with a reduced ratio of the lumen to vessel area, but also increased the fibroblast number and collagen deposition in the adventitial layer. When compared with the saline infusion, intravenous adrenomedullin infusion at 200 ng/h for 14 days suppressed the neointimal formation (−33%, P=0.033), reversing the ratio of lumen to vessel ratio (P=0.030), without affecting systolic blood pressure. Moreover, the adrenomedullin infusion decreased the number of adventitial fibroblasts (−41%, P<0.001) and the collagen deposition (−36%, P=0.006) in the adventitial layer of the injured artery. In conclusion, the intravenous adrenomedullin infusion effectively attenuates vascular remodeling following the arterial injury via suppression of hyperplasia in the intima and adventitia, suggesting a potential of adrenomedullin as a therapeutic tool against vascular remodeling.
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Keywords: Remodeling; Extracellular matrix; Adventitia

1. Introduction

Arterial remodeling is a physiological and pathological reaction in response to hemodynamic, immunologic, and biochemical stimuli (Pasterkamp et al., 2004). Medial hypertrophy and neointimal lesion were focused on as important features; however recent studies have concentrated on reorganization of the entire vessel architecture as vascular remodeling (Strauss and Rabinovitch, 2000; Ward et al., 2000). Accumulating evidence suggests an importance for the adventitial layer, which modulate the remodeling process through regulation of the extracellular matrix formation (Sartore et al., 2001; Strauss and Rabinovitch, 2000). A rodent model of arterial balloon injury is widely used to examine the remodeling process due to its similarity to restenotic vascular lesions seen after angioplasty in humans (De Meyer and Bult, 1997). In this model, the vascular injuries cause proliferation and migration of vascular smooth muscle cells (VSMC) into the intima, and fibroblasts increase in cell number, along with an increase in extracellular matrix deposition in the adventitial layer, further aggravating vascular remodeling (Sartore et al., 2001; Ryan et al., 2003). Various humoral interactions between growth factors, inflammatory cytokines or vasoactive peptides have been reported to be involved in the remodeling process (Sartore et al., 2001). Adrenomedullin, initially isolated from human pheochromocytoma (Kitamura et al., 1993), has been shown to have multiple functions in the cardiovascular system (Kitamura

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et al., 2002). Adrenomedullin was shown to inhibit the migration and proliferation of VSMC in vitro (Kano et al., 1996; Kohno et al., 1997), and Agata et al. (2003) reported that adrenomedullin gene delivery produced an inhibitory action on neointima formation after balloon injury, suggesting an important role for this bioactive peptide in vascular remodeling. However, it remains unknown whether the adrenomedullin actions are observed only in the vascular intimal layer or in the whole vascular structure in the remodeling process. The aim of the present study was to examine the biological actions of adrenomedullin on vascular remodeling, which includes not only the neointima formation but also the adventitia hyperplasia in balloon-injured carotid arteries of rats.

2. Materials and methods

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 (2003-023).

2.1. Experimental protocol

Ten- to eleven-week-old male Sprague-Dawley rats (CLEA, Japan, Inc.) weighing 350–400 g were housed in a temperature- and light-controlled room (25±1 °C; 12/12-h light/dark cycle) with normal rat chow and water given ad libitum. After the rats were anesthetized with 40 mg/kg i.p. of pentobarbital sodium, endothelial denudation and wall stretch of the left common carotid artery were carried out by three passages of a Fogarty 2F balloon catheter (Baxter International, Deerfield, IL, USA). Then, the rats were randomly divided into two groups infused with saline (n=9) or with synthetic rat adrenomedullin (Peptide Institute, Osaka, Japan) at 200 ng/h (n=6) over 14 days. Immediately after the balloon injury, miniosmotic pumps (Alzet model 2002) were implanted subcutaneously to release either saline or adrenomedullin into the right external jugular vein. Blood pressure was monitored by tail-cuff plethysmography during the experimental period. At day 14, the rats were anesthetized with 40 mg/kg i.p. of pentobarbital sodium and blood samples were collected from the inferior vena cava. Both the injured left common carotid artery and non-injured contralateral were perfused via the left ventricle with phosphate buffer-saline, followed by perfusion fixation with 4% paraformaldehyde, at the physiological constant pressure of about 100 mm Hg, and were then immediately excised.

2.2. Histology and morphological evaluation

The carotid arteries embedded in paraffin were sectioned at 2 μm thickness. After deparaffinization with xylene and graded alcohol, slides were incubated with 0.1% picrosirius red (Direct Red 80, Sigma) dissolved in saturated picric acid for 10 min. Morphological evaluation of the injured and contralateral uninjured carotid arteries was performed at the middle portion of the artery by a single observer in a blind manner. Two samples were too disfigured to be precisely quantified: one was an injured artery of the control and the other was an intact artery of the adrenomedullin group. Therefore, these two samples were excluded from the analysis. The cross-sectional areas of the lumen and those circumscribed by the internal or external elastic lamina were determined by computerized measurement (Axio Vison 2.05 Carl ZEISS, Munchen, Germany), and the areas of the media and intima were calculated by subtraction. The vessel area was defined as the area surrounded by the external elastic lamina. The number of fibroblasts showing a typical spindle shape in the adventitia was determined at a magnification of ×400. To quantify collagen deposition in the vascular wall, sections stained with picrosirius red were scanned by Mac Scope (v. 2.3.2) software under polarized light. The tightly packed collagen surrounding the carotid artery was defined as the collagen deposition in this study.

2.3. Assay for adrenomedullin

Plasma concentrations of rat adrenomedullin were measured with a specific radioimmunoassay, which detects the C-terminal amide structure of adrenomedullin, an essential portion for the biological activity, as previously described (Tsuruda et al., 1999).

2.4. Statistical analysis

All data are expressed as means±S.E.M. Comparisons between groups were made with one-way analysis of variance followed by the Fisher's test, and statistical significance was accepted at P<0.05.

3. Results

3.1. Plasma level of rat adrenomedullin and blood pressure

The adrenomedullin-supplemented rats showed significantly higher rat adrenomedullin levels in the plasma compared with those administered with saline at day 14 (adrenomedullin group, 4.9±0.5; saline group, 3.3±0.2 fmol/ml; P=0.004). Meanwhile, no significant difference in systolic blood pressure was noted before and during the experiment period (data not shown).

3.2. Effects adrenomedullin on neointimal formation and adventitia hyperplasia

Fig. 1 illustrates the hematoxylin-eosin stainings of the intact and balloon-injured carotid arteries at day 14. In the injured artery (B), neointima formation occurred and the

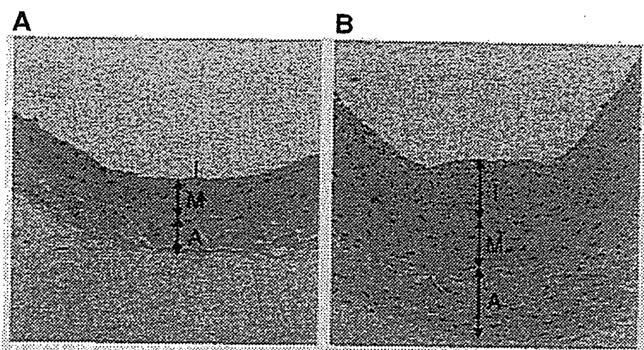

Fig. 1. Histological findings of the intact (A) and injured (B) arteries. I, intima; M, media; A, adventitia. Original magnification, ×200.

adventitial layer thickened with high cellularity, compared with the intact artery (A).

In the quantitative analysis (Fig. 2), the injured arteries showed significant neointimal formation ($P<0.001$) with little influence on the medial area (A) resulting in a significant increase of the intima to media ratio (B). As shown, the adrenomedullin infusion for 14 days significantly attenuated the neointimal formation by 33% ($P=0.033$) and the intima to media ratio by 34% ($P=0.025$), respectively, compared with the saline infusion, while adrenomedullin had no effect on these parameters in the contralateral, intact artery.

Fig. 3A illustrates the effect of adrenomedullin on cell number of fibroblasts in the adventitial layer. The arterial injury increased the number of fibroblasts ($P<0.001$), but this increase was suppressed by the adrenomedullin infusion by 41% ($P<0.001$). Fig. 3B shows the effect of adrenomedullin on the ratio of collagen deposition to the medial areas in the intact and injured arteries. The balloon injury enlarged the collagen deposition area mainly in the adventitia ($P<0.001$); however, the adrenomedullin infusion reduced it by 38% ($P=0.006$).

3.3. Effect of adrenomedullin on geometrical changes in the carotid arteries

Fig. 4A and B illustrate the effect of adrenomedullin on the lumen and vessel areas, respectively. The balloon injury slightly reduced the lumen area of rats infused with saline,

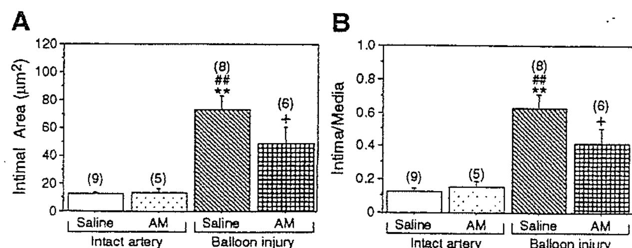

Fig. 2. Effect of adrenomedullin on intimal area (A) and ratio of intima to media (B) in the intact and injured arteries. Values are means±S.E.M., (n). $^{**}P<0.01$ vs. intact artery with saline infusion; $^{\#\#}P<0.01$ vs. intact artery with adrenomedullin infusion; $^{+}P<0.05$ vs. injured artery with saline infusion.

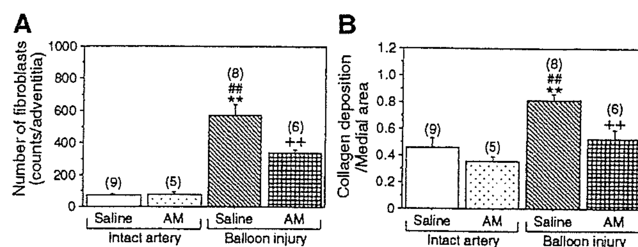

Fig. 3. Effect of adrenomedullin on cell number in the adventitia (A) and collagen deposition/medial area (B). Values are means±S.E.M., (n). $^{**}P<0.01$ vs. intact artery with saline infusion; $^{\#\#}P<0.01$ vs. intact artery with adrenomedullin infusion; $^{++}P<0.01$ vs. injured artery with saline infusion.

but this reduction was statistically insignificant (Fig. 4A), and no significant differences were noted in the vessel area of four study groups (Fig. 4B). As shown in Fig. 4C, the ratio of the lumen to vessel area was significantly ($P<0.001$) reduced by the balloon injury in the saline group, compared with those of the intact arteries. The adrenomedullin supplement significantly ($P=0.030$) reversed this geometrical change toward those of the intact arteries.

4. Discussion

We report here that intravenous adrenomedullin infusion not only attenuated neointima formation but also inhibited fibroblast proliferation and collagen deposition of the adventitia, reducing the ratio of lumen to vessel area, in the balloon-injured carotid arteries of rats. The three layers of the vascular wall, intima, media and adventitia, contribute to inward or outward remodeling which occurs following arterial injury (Ward et al., 2000). Although neointimal formation and medial hypertrophy have been focused on as targets in preventing adverse remodeling, recent reports have

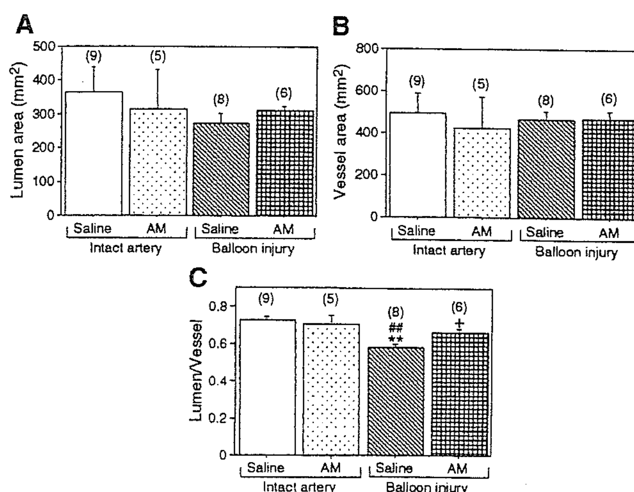

Fig. 4. Effect of adrenomedullin on lumen area (A), vessel area (B) and ratio of lumen to vessel area (C). Values are means±S.E.M., (n). $^{**}P<0.01$ vs. intact artery with saline infusion; $^{\#\#}P<0.01$ vs. intact artery with adrenomedullin infusion; $^{+}P<0.05$ vs. injured artery with saline infusion.

referred more to the role of the adventitial layer (Ryan et al., 2003; Sartore et al., 2001; Strauss and Rabinovitch, 2000).

In a model of arterial injury, endothelial denudation induces VSMC proliferation and migration, making up the neointima formation. Our present data supports previous studies showing that adrenomedullin attenuated the neointima formation induced by arterial injuries in rats (Agata et al., 2003; Yamasaki et al., 2003) and in mice (Imai et al., 2002; Kawai et al., 2004). On the other hand, extracellular matrix deposition in the adventitia with cellular hyperplasia appears to be the major phenomena responsible for adventitial thickening that would subsequently increase stiffness of the vascular walls and peripheral arterial resistance (Intengan and Schiffrin, 2001; Sartore et al., 2001). Importantly, we found that the adrenomedullin administration significantly decreased the number of fibroblasts in the adventitia following the arterial injury in this study. In addition, the adrenomedullin-treated rats showed a significant reduction of collagen deposition in the entire vessel wall, mainly in the adventitia. Considering the importance of extracellular matrix formation in determining stiffness of the vascular wall (Intengan and Schiffrin, 2001), adrenomedullin may exert a beneficial action alleviating vascular stiffness.

In this study, the beneficial effects of adrenomedullin following arterial injury were observed without a significant effect on blood pressure, suggesting a direct action of adrenomedullin on the vascular remodeling. Adrenomedullin has been shown to directly inhibit proliferation and migration of cultured VSMC (Kano et al., 1996; Kohno et al., 1997), and according to our previous report (Tsuruda et al., 1999), adrenomedullin inhibited proliferation of cultured fibroblasts isolated from rat cardiac ventricle. Recently, we reported that adrenomedullin induced matrix metalloproteinase-2 activity in cultured adventitial fibroblasts isolated from rat aorta (Tsuruda et al., 2004). Collagen accumulation is responsible for constrictive remodeling following balloon injury (Ryan et al., 2003). Proteolytic activity induced by adrenomedullin may have contributed to attenuating collagen deposition, however these hypotheses for possible, direct actions of adrenomedullin should be tested in vivo by future experiments.

In summary, the intravenous adrenomedullin infusion effectively improves the vascular geometry of the balloon-injured rat carotid artery, suppressing neointima formation, adventitial fibroblast proliferation and collagen deposition. This study implies a possible utility of adrenomedullin for inhibition of vascular remodeling, where both neointimal formation and adventitial hyperplasia are targeted.

Acknowledgements

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CHAPTER

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Adrenomedullin and Its Related Peptides

KAZUO KITAMURA AND JOHJI KATO

ABSTRACT

Adrenomedullin (AM) is a potent vasodilator peptide that exerts major effects on cardiovascular function. AM, initially isolated from human pheochromocytoma tissue, is biosynthesized in a wide variety of organs and cells. In addition to AM, proadrenomedullin N-terminal 20 peptide (PAMP) is found to be processed from the AM precursor. Both AM and PAMP show hypotensive effects in anesthetized rats but exhibit different hypotensive mechanisms. Further, AM possesses multiple biological effects closely related to cardiovascular homeostasis. Plasma AM concentration is increased in patients with several cardiovascular diseases such as hypertension, congestive heart failure, renal failure, and septic shock. It has been recognized that AM is one of the important vasoactive peptides involved in the physiology and pathophysiology of circulation and body fluid control.

DISCOVERY AND STRUCTURE OF AM

We have been searching for peptides that may be relevant to circulation control, using an assay system that monitors the elevating activity of rat platelet cAMP. By isolating and sequencing all of the bioactive peaks in high-performance liquid chromatography (HPLC) analysis of a human pheochromocytoma tissue extract, we were able to discover the novel biologically active peptide. Because this peptide is also abundant in normal adrenal medulla, it was designated adrenomedullin (AM) [1, 7].

Human AM consists of 52 amino acids and has one intramolecular disulfide bond [1, 7]. In addition, the C-terminal Tyr is amidated, which has been observed in a number of other biologically active peptides, including calcitonin gene–related peptide (CGRP) and amylin, with which AM shares some structural homology. As shown in Fig. 1, the sequence homology of AM with human CGRP and amylin is not high, although they share the C-terminal amide and a six-residue ring structure formed by the intramolecular disulfide linkage. Nevertheless, given the slight sequence homology and pharmacological activities that are similar to those of CGRP, it is likely that AM belongs to the CGRP superfamily. In addition to the human peptide, the amino acid sequences of AM from murine, canine, porcine, and bovine species have now been determined. Porcine AM is nearly identical to the human peptide, with a single substitution (Gly for Asn) at position 40. Rat AM has 50 amino acids, with two deletions and six substitutions, as compared with the human peptide. Notably, among all these species, the ring structure and C-terminal amide, both of which are essential for biological activity, are well conserved.

Very recently a new member of AM family, adrenomedullin 2 (AM2)/intermedin was identified by two groups [18, 23]. Although the sequence identity between AM2/intermedin and AM is relatively low (approximately 30%), as shown in Fig. 1, the pharmacological activities are similar. One of the discoverers, Takei, discusses this peptide in the Renal Peptides Section of this Handbook.

STRUCTURE OF THE PRECURSOR mRNA/GENE

The precursor for human AM (human preproAM) consists of 185 amino acid residues, including the AM sequence [9]. The predicted sequence of proAM contains a Gly-Lys-Arg segment immediately adjacent to the C-terminal tyrosine residue of AM. Gly-X-Y, where X and Y are basic residues, can serve as signals for C-terminal amidation, a process in which the glycine

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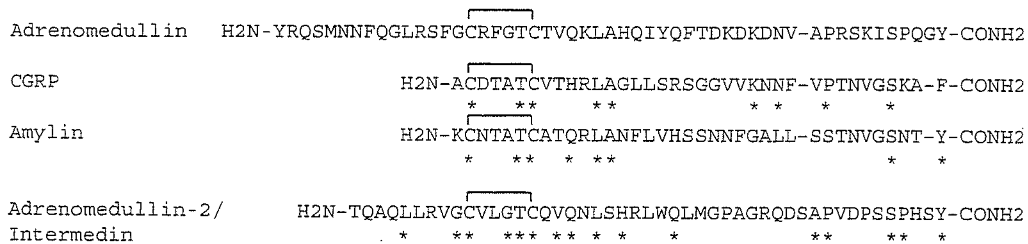

FIGURE 1. Comparison of amino acid sequence of human adrenomedullin with human CGRP, amylin, and adrenomedullin 2/intermedin.

residue donates an amide moiety to the free carboxylic acid group in a reaction catalyzed by the enzyme peptidylglycine α-amidating monooxygenase (PAM; EC 1.14.17.3).

In addition to AM, proadrenomedullin (proAM) contains a unique 20-amino-acid sequence followed by Gly-Lys-Arg, a typical amidation signal, in the N-terminal region. It is possible that a novel 20-residue peptide, termed proadrenomedullin N-terminal 20 peptide (PAMP), whose carboxy terminus is Arg-$CONH_2$, is processed from the AM precursor. We have clarified that PAMP exists in vivo and elicits a potent hypotensive activity in anesthetized rats.

The genes for human and mouse AM were isolated and its structure was determined [3, 16]. The genomic DNA of human AM consists of four exons and three introns, as shown in Fig. 2. The mature AM peptide is coded in the fourth exon, whereas PAMP is interposed by the second intron. In addition, the AM gene is found to be situated in a single locus of chromosome 11. The 5′ flanking region of the gene contains TATA, CAAT, and GC boxes, and there are multiple binding sites for activator protein 2, a cAMP-regulated enhancer element, nuclear factor-kappa (κ) B, and hypoxia response elements. These indicate that the human AM gene contains components for its functional expression and that the expression may be subject to the activity of protein kinase C and feedback from cAMP levels.

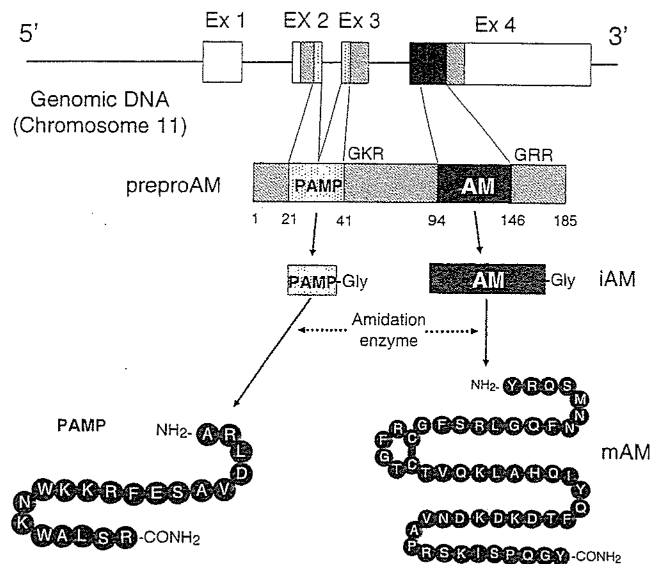

FIGURE 2. The schematic presentation of adrenomedullin (AM) gene and precursor, structures and biosynthesis of AM and proadrenomedullin N-terminal 20 peptide (PAMP).

DISTRIBUTION OF THE mRNA AND PEPTIDE IN THE CARDIOVASCULAR SYSTEM

Although AM was discovered from pheochromocytoma tissue arising from adrenal medulla, AM has been shown to be widely distributed in tissue, including cardiovascular organs. Figure 3 summarizes the distribution of AM mRNA and immunoreactivity in rat tissue. A high level of AM mRNA was found in cardiovascular tissues such as atrium, aorta, kidney, and lung as well as in adrenal gland. A high concentration of immunoreactive AM was observed in lung and atrium as well as adrenal gland. Immunoreactive AM was found ubiquitously in all tissue examined. The concentration of immunoreactive AM in aorta, ventricle, and kidney was less than 5% of that in adrenal gland, yet high levels of AM mRNA were found in these tissues [5]. This discrepancy may be explained by the possibility that AM biosynthesized in these tissues may be rapidly and constitutively secreted into the blood or function as an autocrine or paracrine regulator. In contrast, AM synthesized in adrenal medulla is thought to be stored in the granules and secreted in a regulatory pathway. Therefore, the biosynthetic and excretion systems of AM may be different from tissue to tissue.

Many different cultured cell lines produce AM. The reverse transcription polymerase chain reaction (RT-PCR) revealed the presence of AM in a variety of cells and tissues such as human pulmonary cells, pancreatic islet cells, cardiac myocytes, and vascular endothelial and smooth muscle cells [5]. Endothelial cells (ECs) actively synthesize and secrete AM [22]. In the cul-

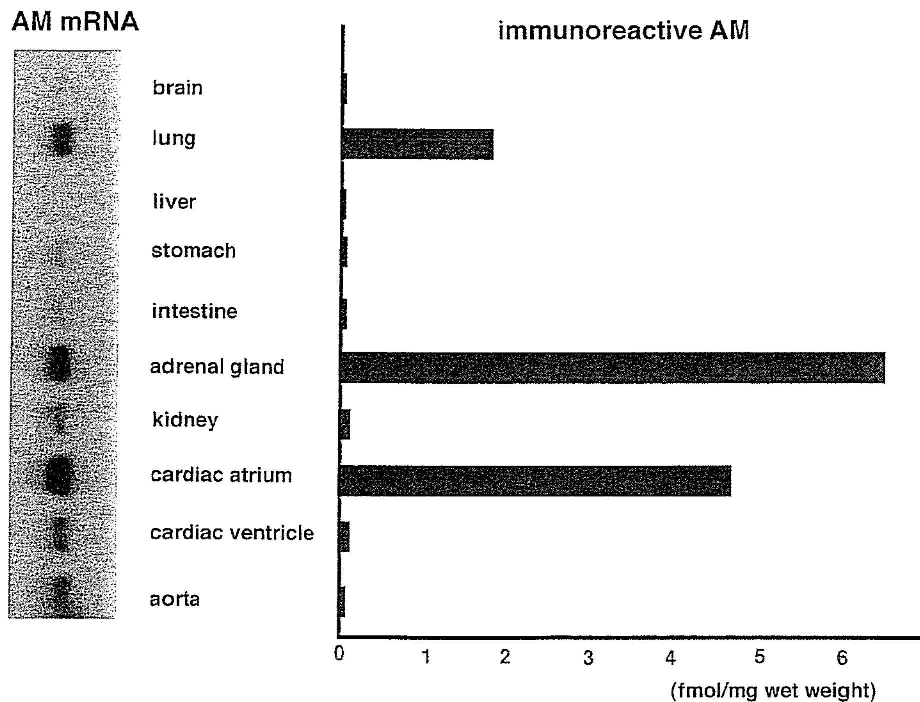

FIGURE 3. Distribution of AM mRNA and immunoreactive AM in rat tissue.

ture medium of rat ECs, the secretion rate of AM was almost comparable to that of endothelin-1. In addition to ECs, vascular smooth muscle cells (VSMCs) were found to produce AM. The presence of specific AM receptors on VSMCs and ECs is consistent with the notion that AM secreted from ECs and VSMCs functions as an autocrine or paracrine regulator in vascular cell communication.

RECEPTORS AND THEIR DISTRIBUTION IN THE CARDIOVASCULAR SYSTEM

AM has been shown to elevate intracellular cAMP levels in many but not all cells and tissues, including blood vessels, where it exerts biological actions, although identification of the AM receptor subtype has been controversial. McLatchie et al. identified three subtypes of receptor-activity-modifying protein (RAMP1–3), an accessory protein required for the transport of calcitonin-receptorlike receptor (CRLR) to the cell membrane [13]. CRLR was originally discovered as an orphan receptor that shows a 55% identity with calcitonin receptor. CRLR can function as either an AM receptor or a CGRP receptor, depending on the subtype of RAMP expressed [10]. RAMP2 enables CRLR to form an adrenomedullin (AM)-specific receptor that is sensitive to AM(22–52) (AM1 receptor). RAMP3 enables CRLR to form an AM receptor sensitive to both CGRP(8–37) and AM(22–52) (AM2 receptor), although rat and mouse AM2 receptors show a clear preference for CGRP(8–37) over AM(22–52). RAMP1 enables CRLR to form the CGRP(8–37)-sensitive CGRP1 receptor, which can also be activated by higher concentrations of AM.

CRLR mRNA is extremely abundant in the rat lung and is expressed in blood vessels by in situ hybridization studies. CRLR protein was also shown to be expressed in vascular endothelial cells by immunocytochemistry. As to the distributions of RAMPs mRNA expression in the human, rat, and mouse, RAMP1 is abundantly expressed in the brain, fat, thymus, and spleen and RAMP2 in the lung, spleen, fat, and aorta; RAMP3 is most abundant in the kidney and lung and is expressed ubiquitously [13]. Recently Kuwasako et al. clearly demonstrated that CRLR is endocytosed together with RAMPs via clathrin-coated vesicles, and both the internalized molecules are targeted to the degradative pathway [11].

BIOLOGICAL ACTIONS IN THE CARDIOVASCULAR SYSTEM

AM was discovered in human pheochromocytoma extract by monitoring activity that elevated rat platelet cAMP [7]. To date, however, it is known that AM is really multifunctional peptide. As summarized in Table 1, several biological effects of AM have been described