Receptors Mediating Vascular Actions of AM

AM has been shown to elevate intracellular cAMP levels in not all but many cells and tissues, including blood vessels, where it exerts biological actions, though identification of the AM receptor subtype has been controversial.[3,4] McLatchie et al identified 3 subtypes of receptor activity–modifying protein (RAMP1 to 3), an accessory protein required for the transport of calcitonin-receptor–like receptor (CRLR) to the cell membrane.[29] CRLR can function as either an AM receptor or a CGRP receptor, depending on the subtype of RAMP expressed: CRLR serves as a CGRP receptor when coexpressed with RAMP1, whereas it functions as an AM receptor when coexpressed with either RAMP2 or 3.[29] AM stimulates intracellular cAMP production in cultured vascular endothelial cells and SMCs,[21,30] and indeed, the mRNAs for CRLR and RAMPs have been detected in these cells and in rat aorta.[31,32] Meanwhile, not all the vascular actions of AM can be fully explained by this receptor system linked to adenylate cyclase: some have been shown to be independent of cAMP.[33,34] This raises the possibility of the presence of unknown receptor systems, and further studies are required to clarify the intracellular signaling for AM.

AM Production in Blood Vessels and Atherosclerotic Lesions

AM was initially isolated from pheochromocytoma tissue, but subsequently the AM gene was found to be expressed in various organs and tissues, including the cardiovascular tissues and cells in humans as well as in rats.[3–5,35] Immunohistochemical studies revealed that three layers of the vessel wall were positive for AM peptide,[35,36] and consistent with this, AM was found to be produced and secreted from 3 types of cultured vascular cells: endothelial cells, SMCs, and adventitial fibroblasts.[37–39] According to Marutsuka et al, AM peptide was expressed in the endothelium of rat aortic arch in a site-dependent fashion, ie, intense immunohistochemical staining for AM was observed in the area where branches begin and on the inner side of the curvature.[40] In these areas, shear stress is relatively low, and indeed, production of AM has been found to be modulated by shear stress in cultured vascular endothelial cells.[41,42] Other factors shown to stimulate production of AM in endothelial cells are oxidative stress and hypoxia.[43,44]

Immunoreactivity for AM was reported to be detected in SMCs of the intima and media of human atherosclerotic lesions.[40] Interestingly, its expression in coronary artery plaques obtained by directional atherectomy was augmented in patients with unstable angina in comparison with stable angina.[45] This finding is consistent with cell culture studies showing that AM production and secretion from cultured vascular SMCs were increased by factors, presumably proatherogenic, such as angiotensin II, endothelin-1, aldosterone, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α).[38,46,47] In addition, aldosterone was shown to stimulate AM production in cultured adventitial fibroblasts as well as in vascular SMCs.[38,39] Macrophages play a pivotal role in the progression of atherosclerotic vascular lesions.[1] Production of AM was detected in macrophages not only in a cell culture experiment,[48] but also by immunohistochemical analysis where intense positive staining was found in advanced atherosclerotic vascular lesion of humans.[40]

Circulating AM in the Bloodstream

Radioimmunoassays for AM revealed that AM peptide was circulating in the blood at mean plasma levels ranging from 2.8 to 10 fmol/mL in healthy human subjects.[7,49,50] Immunoreactive AM in plasma or tissues was found to consist of 2 molecular forms, mAM and iAM (Figure 2), with the major molecular type in plasma and tissues being iAM and mAM, respectively.[7,49–51] As described in the next section, plasma levels of immunoreactive AM were found to be higher in patients with arteriosclerotic vascular diseases than controls, although there was no notable difference in the ratio of mAM and iAM.[52] iAM is thought to have no biological effects by itself, but our ex vivo study showed that iAM dilated rat aorta after its conversion to mAM probably in the aortic wall.[53] Meanwhile, very little information is currently available as to the role of iAM, which should be clarified further with experiments in vivo.

To identify the organs or tissues contributing to the plasma AM level, we examined the plasma levels of AM of various sites in blood vessels of patients with ischemic heart disease.[54] What we found was a step-up in plasma AM levels between the femoral artery and vein.[54] Taking the active secretion of AM from cultured vascular cells into account, it seems likely that the vasculature contributes to the plasma AM level, secreting AM into the bloodstream. On the other hand, there was found to be a step-down between the plasma AM levels of the pulmonary artery and capillary.[54] Substantial levels of AM gene expression were detected in the lungs and the presence of AM peptide in the pulmonary vasculature was immunohistochemically proven,[3–5,55] but the lungs appear to be a target organ or a site for the clearance of circulating AM peptide rather than an AM-secreting organ. Consistent with this notion is the report of abundant expression of AM receptors in the lungs.[56]

Plasma Level of AM in Arteriosclerosis

As an approach to clarifying the role of AM in arteriosclerosis, AM levels in plasma of patients with various types or degrees of arteriosclerotic vascular disease were measured, and the relationships between the plasma levels and the other clinical parameters were examined.[52,57,58] In patients with cerebrovascular disease, a possible association was found between plasma AM levels and endothelial damage by comparing the plasma levels of AM with those of endothelin and thrombomodulin, markers of endothelial damage.[57] Similarly in patients with chronic ischemic stroke, increased plasma AM levels were shown to be associated with the degree of carotid atherosclerosis.[58] Recently, Suzuki et al reported that plasma AM concentrations were elevated in patients with peripheral arterial occlusive disease in proportion to its severity.[52] Moreover, they found close associations between the plasma levels of AM and those of such inflammatory parameters as C-reactive protein and IL-6 in the patients.[52] This finding is not only comparable with the increased production of AM in cultured SMCs by inflammatory cytokines,[46] but also of interest in view of the involve-

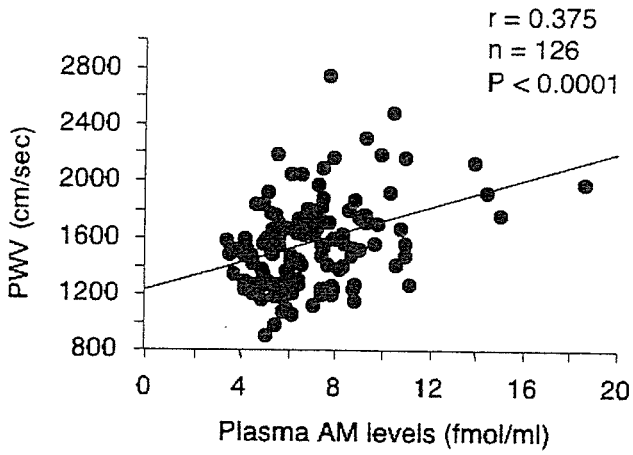

Figure 3. Relationship between the pulse wave velocity (PWV) and plasma AM levels in patients with various degrees of arteriosclerosis. Reprinted from Kita et al [60] with permission from the Japanese Society of Hypertension.

ment of low-grade inflammation in the development and progression of atherosclerotic vascular lesions.[59] Because arterial stiffness is an important cardiovascular risk factor, we measured plasma AM levels in patients with various degrees of atherosclerosis and compared the plasma levels with indirectly measured pulse wave velocity, a parameter used to assess arterial stiffness and sclerosis.[60] As shown in Figure 3, a significant correlation was noted between the plasma AM levels and pulse wave velocity and this relationship was confirmed by multiple regression analysis to be independent of age and blood pressure.[60] These findings are indirect, but indicative of a possible pathophysiological role of AM in arteriosclerotic vascular diseases.

In the latter part of this review, vascular protective effects of AM will be discussed based on the results of cell culture and animal experiments. An important issue we need to mention in this section, therefore, is the significance of the increased plasma AM levels in patients with arteriosclerosis. Because of active production of AM in cultured vascular cells and vessel walls, AM has been assumed to act in a autocrine or paracrine fashion.[3,4,37-40] Indeed, blockade of the actions of endogenous AM with anti-AM antibody or the AM antagonists impaired the vascular protective effects in vitro.[61,62] Meanwhile, according to our experiments in vivo,[63,64] the long-term infusion of AM significantly suppressed neointimal formation and adventitial hyperplasia, raising plasma AM levels by 1 to 2 fmol/mL, an increase within the physiological range. This suggests a possible role for AM, not only as a local modulator, but also as a factor circulating in the blood.

Vascular Protective Effects In Vitro

As discussed in the section Vascular Actions of AM, AM exerts endothelium-dependent vasodilatation, which can be blocked by inhibitors for NO synthase. Consistent with this, in cultured vascular endothelial cells, AM was found to stimulate phospholipase C activation and inositol 1,4,5-triphosphate formation, resulting in an elevation of the intracellular Ca^{2+} level and activation of NO synthase.[65] Kato et al reported that AM inhibited serum deprivation–induced apoptosis of cultured rat vascular endothelial cells.[61] Blockade of the endogenous AM by anti-AM anti-serum impaired the inhibitory effect of the nonimmune serum on apoptosis, suggesting an autocrine or paracrine role for AM.[61] According to the subsequent study by that group, AM upregulated the expression of Max protein, leading endothelial cells to survive.[62] Meanwhile, other adenylate cyclase activators such as PG I_2 and forskolin failed to exert an antiapoptotic effect and a cAMP antagonist was unable to block the effect of AM, therefore a cAMP-independent mechanism seems involved in this action.[61] An antiapoptotic effect of AM was further observed by an independent group. Sata et al found that AM inhibited serum deprivation–induced apoptosis of cultured human umbilical vein endothelial cells.[66] In their experiment, the effect of AM was abrogated by L-NAME, but not by an inhibitor for soluble guanylate cyclase, suggesting an NO-dependent but cGMP-independent mechanism.[66]

Furthermore, AM was shown to cause vascular regeneration by promoting the proliferation and migration of cultured vascular endothelial cells.[67] AM promoted re-endothelialization of wounded human umbilical vein endothelial cells, and this effect was attenuated by inhibitors for protein kinase A and PI3K, suggesting an action mediated by cAMP and the PI3K–Akt pathway.[67] Stimulation of the proliferation and migration of endothelial cells may be involved in the angiogenic action of AM, which will be discussed later in this review. Although the mechanisms of action are still under investigation, these effects of AM on endothelial cells may be protective against vascular damage and arteriosclerosis.

The proliferation of vascular SMCs in the media and intima of arteries is involved in the progression of vascular remodeling or atherosclerotic lesions. Because AM is produced by SMCs in the media, its effects on the proliferation and migration of this type of cell were tested in vitro; however, there has been some inconsistency regarding the actions of AM. AM was shown to inhibit the proliferation of cultured SMCs via a mechanism mediated by cAMP,[68] whereas Iwasaki et al found that AM stimulated proliferation of the cells in a mitogen-activated protein kinase–dependent manner.[69] Horio et al reported an inhibitory effect of AM on the migration of cultured SMCs, which is presumably mediated by intracellular cAMP.[70] Inhibition of the migration of SMCs by AM was confirmed by an independent group,[34] but according to this report, AM inhibited migration via a cAMP-independent mechanism.[34] These discrepancies may have resulted from differences in the experimental conditions or types of cultured cells used, though there has currently been no clear explanation. Meanwhile, as discussed in the next section, recent studies in vivo suggest that AM inhibits intimal hyperplasia induced by periarterial cuff or by intimal balloon injury.

Another vascular protective action of AM recently reported in SMCs was a reduction in the generation of reactive oxygen species (ROS), a group of molecules involved in vascular damage and the progression of arteriosclerosis. The generation of intracellular ROS induced by angiotensin II was inhibited by AM, in a cAMP- and protein kinase A–dependent manner, in cultured vascular SMCs of rats.[71] Moreover,

AM weakened redox-sensitive cellular responses such as the activation of c-Jun amino-terminal kinase (JNK) and gene expression for plasminogen activator inhibitor (PAI)-1, monocyte chemoattractant protein-1, and Nox-1, a component of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[71]

Not only the intima and media but also the adventitial layer has been recognized to have a significant role in the process of vascular remodeling. Blood vessels would increase their stiffness if an excessive accumulation of extracellular matrix or proliferation of adventitial fibroblast were to occur. The proliferation of adventitial fibroblasts induced by aldosterone, a factor involved in the fibrosis of cardiovascular tissue, was found to be suppressed by AM, with a concomitant reduction in the activity of extracellular signal-related kinase.[39] Additionally in that study, autocrine or paracrine inhibition by AM was proposed, based on the production of AM by the adventitial fibroblasts and on augmented proliferation by the AM receptor antagonists.[39] By synthesizing and degrading matrix proteins, adventitial fibroblasts are known to modulate the formation of the extracellular matrix in the adventitia. Our recent experiments showed that AM upregulated the enzymatic activity and protein expression of matrix metalloproteinase-2 (MMP-2), which degrades collagens and elastin, in cultured adventitial fibroblasts of rat aorta possibly via the cAMP–protein kinase A pathway.[72] Collectively, these findings suggest a role for AM in modulating adventitial proliferation and extracellular matrix formation.

Vascular Protective Effects In Vivo

As discussed above, plasma AM levels are elevated in patients with various arteriosclerotic vascular diseases, and the findings from cell culture studies have implied a role for AM, which is presumably protective of blood vessels. To investigate whether or not AM has protective effects on vascular damage and remodeling in vivo, 3 experimental approaches have so far been taken: long-term administration of AM, virally-mediated overexpression of AM, and genetic manipulation of the AM gene.

Using the first method, we found that prolonged AM infusion for 2 weeks partially inhibited neointimal hyperplasia induced by balloon injury in rat carotid arteries (Figure 4).[63] Meanwhile, somewhat conflicting findings were obtained by Shimizu et al, who showed that chronic infusion of the AM antagonist CGRP(8–37) inhibited neointimal hyperplasia induced by ballooning in rats.[73] CGRP(8–37) is a CGRP receptor antagonist, which has been able to block some, but not all, the actions of AM in relatively short-term experiments.[16,20,30,62] However, it has yet to be clarified whether or not this antagonist can block the action of endogenous AM when infused chronically. It should be noted that in our study mentioned above, the prolonged infusion of AM suppressed not only balloon injury–induced intimal hyperplasia but also the proliferation of fibroblasts and collagen deposition of the adventitia (Figure 4),[63] a finding consistent with the in vitro inhibitory effect of AM on the proliferation of cultured adventitial fibroblasts.[39] Inhibition of adventitial hyperplasia by AM was confirmed by our study in vivo, in which perivascular fibrosis of coronary arteries of

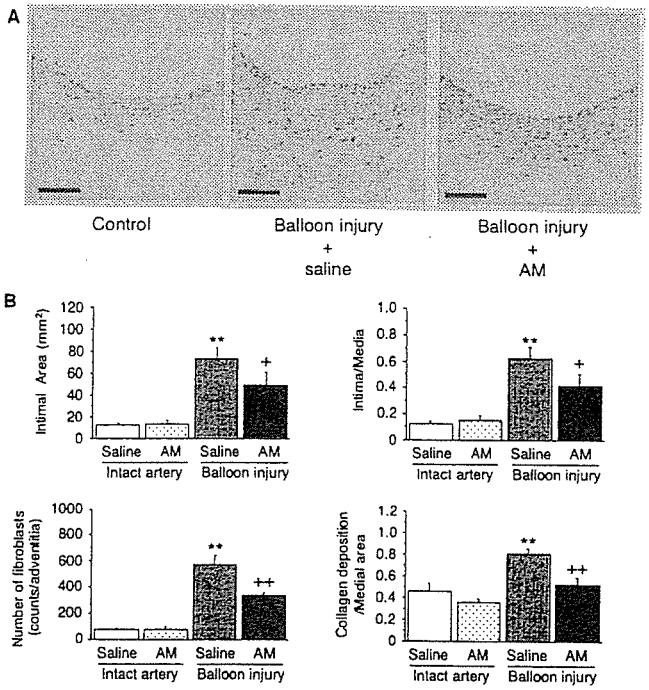

Figure 4. Effects of AM on neointimal and perivascular hyperplasia in rat carotid arteries injured by ballooning. A, Histological findings of the intact and injured arteries of rats infused intravenously with 200 ng/h of AM or saline for 2 weeks. B, Quantitative analyses of intimal and adventitial hyperplasia. Values are the means±SEM; $^{**}P<0.01$ vs intact artery with saline infusion; $^{+}P<0.05$, $^{++}P<0.01$, vs injured artery with saline infusion; bar, 100 μm. Reprinted from Tsuruda et al[63] with permission from Elsevier.

rats infused chronically with angiotensin II was suppressed by coinfusion of AM.[64] This effect was accompanied by the suppression of fibroblast activation and transforming growth factor (TGF)-β1 expression, but not by a significant reduction of blood pressure.[64]

In accord with the effect of prolonged infusion of AM, adenovirus-mediated local delivery of the AM gene was shown to inhibit neointimal hyperplasia of carotid arteries after balloon injury in rats.[74] Interestingly in that study, endothelial regeneration was more pronounced in rats given the AM gene than in the controls,[74] a result consistent with the cell culture experiments, where AM promoted reendothelialization of a wounded monolayer of endothelial cells.[67] The inhibition of neointimal hyperplasia by the AM gene delivery was accompanied by an elevation of tissue cGMP levels, suggesting a mechanism involving the NO–cGMP pathway.[74]

Thirdly, vascular protective effects have been suggested by genetic manipulation of the AM gene in mice. Transgenic mice overexpressing the AM gene (AM-Tg) were found to be resistant to neointimal hyperplasia induced by a periarterial cuff placed on the femoral artery.[75] This resistance seems also to be mediated by the NO–cGMP pathway because it disappeared on administration of L-NAME.[75] Moreover, a protective effect of AM was demonstrated by cross-mating apoE knockout (apoE-KO) mice with AM-Tg. The apoE-KO mice overexpressing AM showed a less extensive hypercholesterolemia-induced fatty streak formation with a greater

endothelium-dependent vasodilatation, compared with the control apoE-KO mice.[75] In contrast to the mice overexpressing AM, heterozygotes of AM knockout mice given angiotensin II and excessive salt showed a more severe perivascular fibrosis and intimal thickening of coronary arteries, compared with their wild-type littermates, despite a similar elevation of blood pressure.[76] Based on increases in the production of ROS and in NADPH oxidase expression in the AM knockout mice, the possibility of augmented oxidative stress was raised as the mechanism responsible for the severe vascular lesions.[76] Periarterial cuff-induced intimal thickening of the femoral artery was also found to be more severe in the knockout mice, compared with the control mice.[77] The enhanced neointimal formation was reversed by delivery of the AM gene and by an NADPH oxidase inhibitor or tempol, a superoxide dismutase mimetic,[77] further suggesting augmented oxidative stress in the AM knockout mice.

Lastly in this section, we should mention the effect of AM on the pulmonary vascular bed as a protective action. In addition to the pulmonary vasodilator effect, prolonged subcutaneous infusion of AM was found to inhibit medial thickening of the pulmonary artery of rats with pulmonary hypertension induced by monocrotaline.[78] However, when infused intravenously, AM lowers not only pulmonary artery pressure but also systemic blood pressure.[24] In an attempt to avoid the effect on the systemic circulation, Nagaya et al administered AM as an aerosol using an ultrasonic nebulizer in rats with pulmonary hypertension.[79] Repeated inhalation effectively inhibited medial thickening of pulmonary arteries, reducing pulmonary artery pressure and total pulmonary resistance, without affecting the systemic arterial pressure or heart rate. Furthermore, the same group reported that in patients with idiopathic pulmonary arterial hypertension, inhalation of AM lowered pulmonary artery pressure and resistance, improving exercise tolerance.[80] Although the long-term effects need to be examined, this novel approach seems promising for using AM in the treatment of primary pulmonary hypertension, for which few effective medical treatments are currently available.

Angiogenetic Effect of AM

A novel action of AM only recently discovered is angiogenesis, an effect implied by experiments with cultured vascular endothelial cells.[67] By subcutaneously injecting gel plugs containing AM into mice, AM was found to promote neovascularization in a protein kinase A- and PI3K-dependent manner.[67] Consistent results were obtained by Iimuro et al, who showed that AM increased collateral capillary density in ischemic limbs of mice, augmenting the expression of vascular endothelial growth factor (VEGF) and activating Akt.[81] Conversely, heterozygotes of AM gene knockout mice showed less capillary development and VEGF expression compared with their wild-type littermates, suggesting a role for endogenous AM.[81] In addition to augmented VEGF expression and activations of protein kinase A and Akt, mitogen-activated protein kinase/extracellular signal-regulated kinase1/2 (ERK1/2) and focal adhesion kinase were proposed as the intracellular mediators responsible for AM-induced endothelial proliferation.[82] Hypoxia was reported to increase not only expression of AM but also of CRLR, a component of AM receptor, in cultured endothelial cells.[44,82] This suggests significance of the AM signaling system in angiogenesis under hypoxic conditions. It would also be of interest to compare the angiogenetic effects of AM with the findings from homozygotes of AM gene knockout mice, which died in the uterus because of insufficient development of blood vessels.[83]

Very recently, AM infusion was shown to enhance the angiogenic potency of implanted bone marrow–derived cells by inhibiting apoptosis of the cells. Angiogenesis after transplantation of bone marrow–derived mononuclear cells was augmented by AM in rats with hind limb ischemia.[84] Similarly in a rat model of cerebral infarction, the angiogenic effect of transplanted mesenchymal stem cells was enhanced by AM infusion in ischemic penumbra of the brain, improving neurological deficits.[85] Collectively, it seems likely that AM possesses angiogenic properties, suggesting its potential as a therapeutic tool in the treatment of organ or tissue ischemia.

Conclusion and Perspective

Since the discovery of the novel vasodilator peptide AM, much research, basic and clinical, has been done to clarify the vascular actions of AM and its role in modulating vascular remodeling and atherosclerosis. As discussed in this review, a substantial amount of data accumulated in this field suggests that AM functions as a protective factor for blood vessel, exerting various vascular actions, mostly inhibitory, against vascular damage and remodeling. Research on AM now seems to be entering a new phase, with clinical benefits to be examined and specified. AM itself is orally inactive, but the development of either analogues of AM or drugs inhibiting the degradation of AM would provide us a new therapeutic tool to inhibit the progression of vascular damage and remodeling. They would, in particular, be beneficial for patients with primary pulmonary hypertension, for which therapeutic methods one can choose are currently very limited. Meanwhile, there is no doubt that more basic studies are necessary to resolve issues such as the receptor system and the intracellular mechanisms mediating the vascular actions of AM. Angiogenetic properties appear to be another feature that should be characterized further in vitro and in vivo.

Acknowledgments

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Effect of adrenomedullin administration on acetic acid-induced colitis in rats

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Abstract

Adrenomedullin (AM) administered intracolonically ameliorated the severity of acetic acid-induced colonic ulceration in rats. Ulcers were induced by subserosal injection of acetic acid into the colon. AM-treated group was administered 0.25–1.0 μg of AM in 0.5 ml of saline intracolonically once a day; the control group received only saline. AM administration dose-dependently and significantly reduced the size of the ulcerative lesions, the associated edema, and the infiltration of the affected area by inflammatory cells. AM also reduced tissue levels of interleukin-6, but not interferon-γ. AM reduces the severity of acetic acid-induced colitis in rats, probably by inhibiting the production and/or release of Th-2 cell-derived factors such as interleukin-6.

Keywords: Acetic acid; Adrenomedullin; Colitis; Inflammatory bowel disease; T helper 2 cells

1. Introduction

Although originally isolated from human pheochromocytoma as a vasodilatory peptide [12], adrenomedullin (AM) is now known to have a wide spectrum of other actions, including stimulation of diuresis and natriuresis and several gastrointestinal functions [11,29]. Among the last, it reportedly exerts a protective effect against experimentally-induced damage to the gastric mucosa [2,9,30,31].

Inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease are now recognized to be caused by crosstalk between a variety of factors. Although the exact nature of its pathogenesis is still uncertain, it has been suggested that a focal immunoreactive disorder and/or dysfunction of the mucosal defence system is involved [27]. Moreover, it is now known that inflammatory cytokines such as interleukin (IL)-1, IL-6, and interferon gamma (IFN-γ) are up-regulated at focal lesions induced by dietary antigen and/or intestinal bacteria [5,8,17,24,28]. Consequently, the therapeutic strategy for treating IBD now focuses on the use of anti-inflammatory agents [33].

The cytokines found at sites of inflammation are released by T helper (Th) cells that can be divided into two distinct subsets, Th-1 and Th-2, based on the cytokines they express [23]. Characteristically, Th-1 cells produce IL-2 and IFN-γ, whereas Th-2 cells produce IL-4, -5, -6, -9, and -10. In previous studies, the role of Th cell cytokines in the pathogenesis of IBD has been examined using experimental models of colitis induced by intracolonic application of trinitrobenzene sulfonic acid (TNBS) or acetic acid [4,6,18,21,22]. Our aim in the present study was to investigate the extent to which intracolonic administration of AM would protect the colon in the acetic acid model. We assessed the efficacy of AM by examining its effects on the macroscopic and histological characteristics of the induced lesions, as well as its effects

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on the levels of Th-1 and Th-2 cytokines in the affected tissues.

2. Materials and methods

2.1. Animals and peptide

Male Wistar rats (6–7 weeks old) were used throughout these experiments. The rats were housed under specific pyrogen-free conditions and maintained on standard pellet chow and tap water ad libitum. The recombinant human AM used in this study was provided by Shionogi & Co., Ltd. (Osaka, Japan). All experiments were carried out in accordance with the regulations of the Animal Research Committee of Miyazaki Medical College (no. 2004-077).

2.2. Induction of colitis

For 24–36 h prior to inducing colitis, rats were allowed tap water but were deprived of food. Colitis was then induced according to the method of Kojima et al. [13]. A small midline incision was made under light anaesthesia with ether, and acetic acid (20%, 0.02 ml) was injected into the subserosa of the colon 5–6 cm from the anal verge.

2.3. Treatment with AM

AM (0.25–1.0 μg of AM diluted in 0.5 ml of saline) was delivered into the lumen of the colon using a 20-gauge animal feeding tube (8 cm long) inserted into the rectum to a depth of approximately 5–6 cm from the anal verge. Control rats were administered 0.5 ml of saline with-

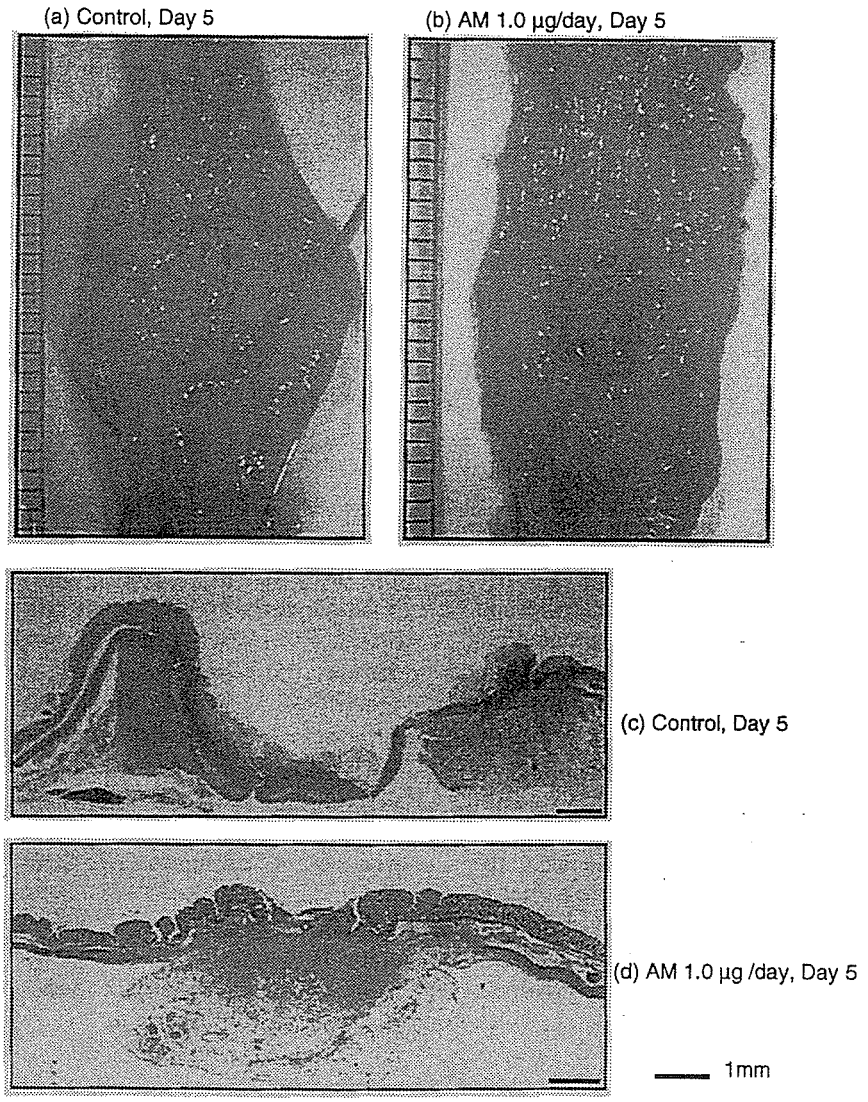

Fig. 1. Macroscopic and histological appearance of colonic ulcers induced by subserosal injection of acetic acid. Rats ware treated with AM (1.0 μg/0.5 ml saline) or saline alone (0.5 ml) for 5 days. (a) Macroscopic view of the colon of a control animal exhibiting a wide and deep ellipse-shaped ulcer with severe edema; (b) the ulcerated area and the associated edema were both reduced in AM-treated animals; (c and d) histological examination of colonic mucosal tissue from a control and an AM-treated rat: the control animal showed severe ulceration, edematous changes and infiltration of the lamina propria by inflammatory cells (c); the AM-treated animal showed less ulceration and edema (d).

out AM. AM and saline were administrated once a day for 3–10 days.

2.4. Assessment of severity of colonic ulcer

Groups of rats were killed with an overdose of ether on the designated days, after which the distal colon was removed and opened longitudinally. The length (mm) and width (mm) of the ulcerated area were measured using a calliper gauge, and their product was taken as the ulcer area [13].

2.5. General histology

Samples of colonic tissue were fixed in buffered 10% paraformaldehyde (pH 7.4), processed for histological examination using standard techniques, and stained with hematoxylin and eosin.

2.6. Evaluation of cytokine production

Three-centimetre samples of colonic tissue with the ulcerative lesion at their centre were homogenized in 100 ml/g tissue PBS, after which the homogenates were centrifuged at 15,000 rpm for 10 min. Cytokines present in the supernatant were measured using a sandwich ELISA (Endogen®, USA). IFN-γ and IL-6 served as candidate Th-1 and Th-2 cytokines, respectively.

2.7. Statistical analysis

Data are presented as means ± S.E. Unpaired *t*-test was used to compare two groups, and multiple comparisons were evaluated by 1-way ANOVA, followed by the Scheffe *F*-test. Values of $P < 0.05$ were considered significant.

3. Results

We first evaluated the effect of intracolonic administration of AM on day 5 after application of acetic acid by estimating the mucosal damage in the colon both macroscopically and histologically. We found that the colons of rats in the control group were severely ulcerated by day 5 (Fig. 1a), but that administration of AM (0.25–1.0 μg/day) reduced the size of the lesion in a dose-dependent manner (Figs. 1b and 2). AM significantly ($P < 0.05$) reduced the severity of the mucosal damage at a dose of 1.0 μg/day (Fig. 2a).

Histological examination of samples of colonic mucosa from control animals revealed deep ulceration, sever edematous changes and infiltration of the lamina propria by inflammatory cells (Fig. 1c). By contrast, AM-treated rats showed less severe ulceration and less edema (Fig. 1d), which is consistent with the reduction in the wet weight of the tissue observed in AM-treated rats (Fig. 3)

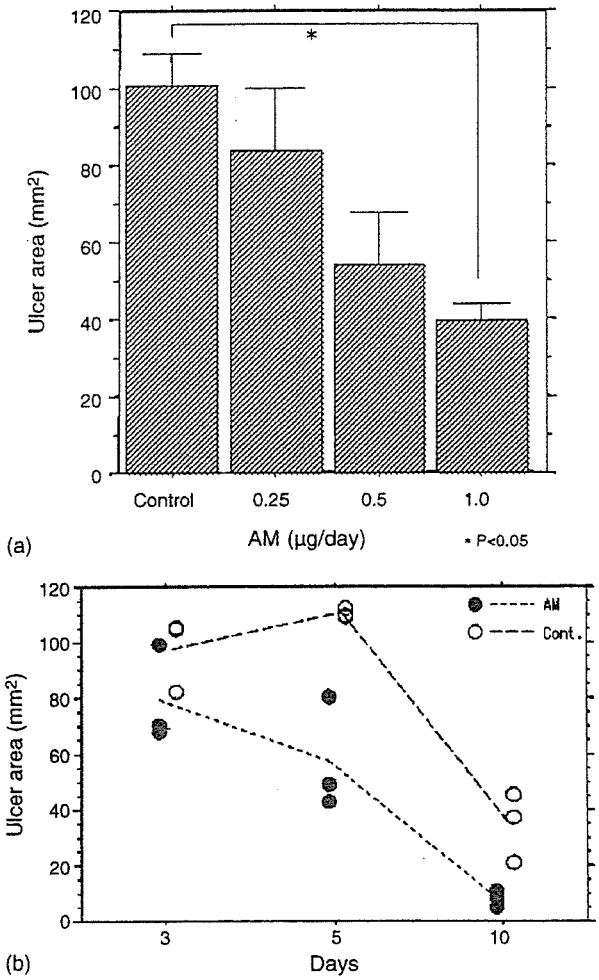

Fig. 2. (a) Effects of AM on the size of acetic acid-induced colonic ulcers observed 5 days after induction ($n = 5$ for each group). AM (0.25–1.0 μg/day) dose-dependently reduced ulcer area, with a significant decline being achieved at a dose of 1.0 μg/day. Bars represent the means ± S.E.; $^*P < 0.05$ vs. control. (b) Time-dependent changes in the size of acetic acid-induced colonic ulcers. Rats were administered AM (1.0 μg/day) or saline for 3, 5, or 10 days after ulcer induction ($n = 3$ for each group). The size of the ulcerative lesions in the AM-treated group was reduced in a time-dependent manner, while that of control group increased at days 3 and 5.

We next assessed the time course of ulcer development and the effects of AM by examining the colons of rats on days 3, 5, and 10 after application of acetic acid. We found that AM reduced the severity of the ulcerative lesions in a time-dependent manner and that, as compared to the controls, AM (1.0 μg/day)-treated rats showed reduced ulceration on days 5 and 10 (Fig. 2b).

Finally, we investigated the role of Th cells in acetic acid-induced ulcer formation by measuring the tissue levels of IFN-γ and IL-6, which respectively served as candidate Th-1 and Th-2 cytokines. On day 5 after application of acetic acid, we found levels of IL-6 in the affected tissue to be significantly lower in AM-treated than control rats (Fig. 4a). On the other hand, no significant difference in the levels of IFN-γ was observed (Fig. 4b).

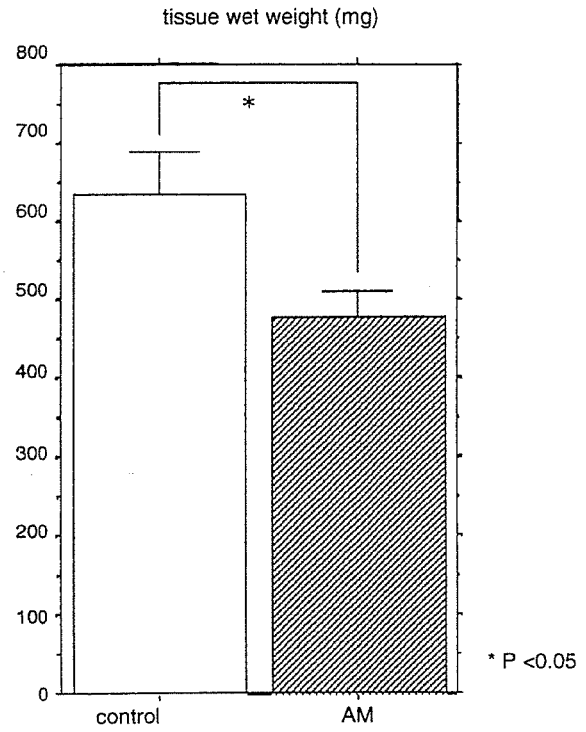

Fig. 3. Wet weights of the tissue specimens (Day 5). Wet weights of 3 cm colonic tissue with the ulcerative lesion at their centre were significantly reduced in AM-treated group ($n=5$ for each group). Bar represents the mean ± S.E.; $^{*}P<0.05$ vs. control.

4. Discussion

Induction of colitis in rats using acetic acid is a classical method used to produce an experimental model of human IBD [7,18,20,32]. A variety of factors involved in the pathogenesis of human IBD, including excessive oxidative stress, enhanced vasopermeability, prolonged neutrophil infiltration, and increased production of inflammatory mediators and cytokines, are also involved in the induction of this animal model [4,7,13,18]. It is presently accepted, therefore, that this model is suitable for investigating the pathogenesis of IBD and for evaluating potential therapeutic agents to be used in treating the disease.

First isolated from human pheochromocytoma as a potent vasodilatory peptide [12], AM is now known to exert effects in a variety of organs or tissues [16]. In that regard, AM immunoreactivity has been detected throughout the gastrointestinal tract, with comparably high concentrations in the stomach, cecum, and colon [29]. Although little is known about the physiological function of AM in the gastrointestinal tract, this suggests that endogenous AM may play an important protective role in the colon. Consistent with that idea, AM was recently reported to inhibit secretion of histamine and acid in the stomach of rats subjected to ethanol-induced gastric injury [9]. AM also inhibits contraction of the gastric artery, perhaps thereby protecting the mucosa by improving blood flow to damaged areas [30], and relaxes intestinal smooth muscle [14,26].

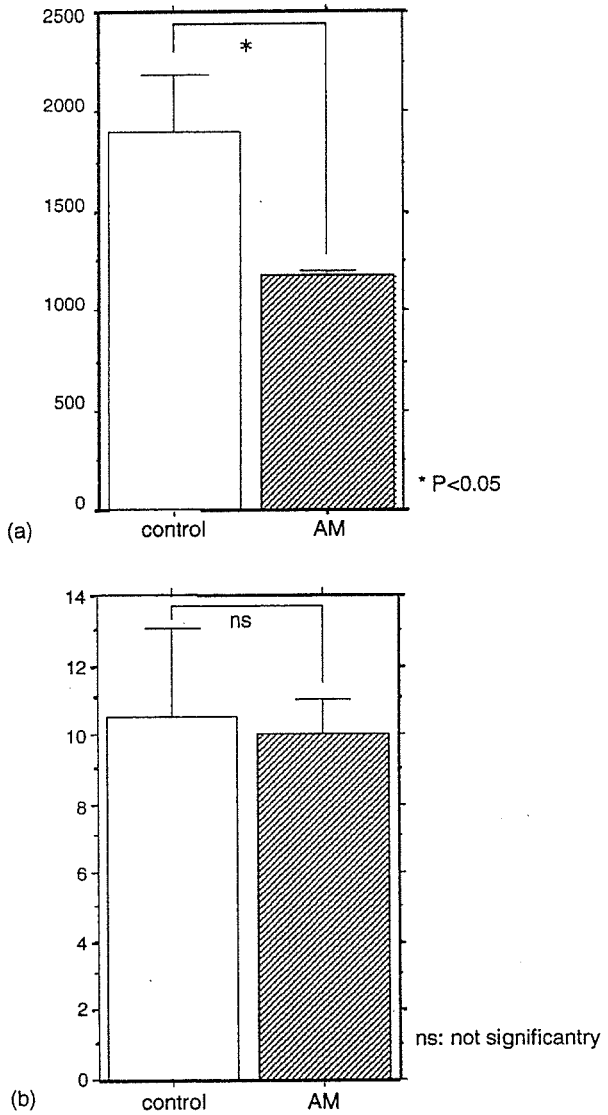

Fig. 4. Effect of AM on the IL-6 (a) and IFN-γ (b) contents of 3 cm samples of colonic tissue affected by application of acetic acid. The cytokine levels were measured on day 5 after induction of colitis ($n=5$ for each group). AM significantly reduced IL-6 levels and wet weights, but had no effect on IFN-γ. Bars represent the means ± S.E.; $^{*}P<0.05$ vs. control.

Against that background, we evaluated the effect of AM on acetic acid-induced colitis and production of Th-1 (IFN-γ) and Th-2 (IL-6) cytokines. We found that the peptide significantly reduces the severity of the ulcerative lesions and the production of IL-6, but has no effect on production of IFN-γ. This finding is consistent with recent studies showing that AM reduces plasma IL-6 levels in sepsis [35] and inhibits IL-6 production by synoviocytes collected from patients with rheumatoid arthritis [25]. Kubo et al. showed that AM suppressed secretion of IL-6 from a murine macrophage cell line, reducing mRNA level for IL-6 [15]. Their in vitro study suggests a direct inhibitory action of AM on production of IL-6 in cell types capable of producing it, though further studies are needed to clarify the mechanism by which AM

lowered the tissue IL-6 level in the present study. IL-6 is secreted from monocytes, macrophages, lymphocytes (Th-2 cells), and epithelial cells, including intestinal epithelial cells, and its strong expression has been observed in colonic mucosa affected by IBD [10]. Moreover, recent studies using anti-soluble IL-6 receptor antibodies further confirmed the pivotal role played by IL-6 in the development chronic colitis, and suggested that prevention of its secretion may be of benefit in the treatment of IBD [1,3,19,34]. Our results suggest that acetic acid-induced colitis requires the development of a Th-2 response in the intestinal mucosa and that AM lessens the severity of the colitis by inhibiting that response.

In summary, we have shown that AM reduces the severity of acetic acid-induced colitis in rat, probably by inhibiting production and/or release of IL-6 by Th-2 cells infiltrating the region. Further analysis of the phenotype shift in TNBS colitis using normal and cytokine-deficient mice should contribute further to our understanding of the complex pathogenesis of IBD and may reveal the underlying immunologic circumstances that give rise to the ailment. Finally, our results suggest that AM may be of benefit in the treatment of the mucosal damage caused by IBD and experimental colitis.

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Angiotensin II Stimulates Cardiac Adrenomedullin Production and Causes Accumulation of Mature Adrenomedullin Independently of Hemodynamic Stress *in Vivo*

Abstract

Adrenomedullin is a potent hypotensive peptide that may act on myocytes to inhibit hypertrophy and on fibroblasts to inhibit growth *in vitro* induced by mechanical stretching and angiotensin II. Adrenomedullin is processed from the inactive intermediate adrenomedullin precursor with a glycine extension, which is subsequently converted to biologically active mature adrenomedullin by enzymatic amidation. Total adrenomedullin is the sum of intermediate and mature adrenomedullin. We examined the effect of a subpressor dose of angiotensin II on the production of left ventricular adrenomedullin and on protein levels of mature adrenomedullin in the left ventricle *in vivo*. We also investigated whether the effect is mediated by the angiotensin II type 1 receptor. Concentrations of total and mature adrenomedullin in the left ventricle and mature adrenomedullin-to-intermediate adrenomedullin ratio were significantly increased by angiotensin II infusion, regardless of pressure overload. Total and mature adrenomedullin concentrations significantly correlated with the weight of the left ventricle. Furthermore, increased adrenomedullin gene expression and protein levels were completely suppressed by a subdepressor dose of angiotensin II type 1 receptor blocker. In conclusion, angiotensin II stimulates the production of cardiac adrenomedullin and accumulates mature adrenomedullin in the left ventricle independently of hemodynamic stress. These processes are partially regulated through the angiotensin II type 1 receptor *in vivo*.

Key words
Amidation · Angiotensin II type 1 receptor · Hypertrophy · Pressure overload · Renin-angiotensin system

Introduction

Adrenomedullin (AM) is a potent hypotensive peptide [1], whose gene is expressed in various organs including the heart and vascular tissues [2]. The cardiac AM level is elevated by pressure overload concomitant with the extent of left ventricular (LV) hypertrophy [3]. Hemodynamic stress [4] and humoral factors such as angiotensin II (Ang II) [5] or endothelin [6] play important roles in the hypertrophic process during pressure overload. Additionally, a growing body of evidence indicates that local Ang II plays a critical role in cardiac hypertrophy induced by pressure overload [7]. We have demonstrated that mechanical stretching as well as Ang II increases AM production via the Ang II type 1 (AT1) receptor in cultured myocytes [8], and that Ang II can stimulate AM gene expression irrespective of hemodynamic stress *in vivo* [9]. AM might function as an autocrine or paracrine factor in myocytes and fibroblasts, and it might inhibit myocyte hypertrophy as well as fibroblast growth *in vitro* [10,11].

AM is processed from an inactive precursor with a glycine extension known as intermediate AM that is subsequently converted by enzymatic amidation to biologically active, mature AM [12]. Total AM is the sum of the intermediate and mature forms of AM. Plasma AM consists of both of these forms and their levels have been

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examined in patients with heart failure [13] and hypertension [14]. The concentration of the two forms has also been investigated in the hypertrophied left ventricle of the rat [15]. However, whether hemodynamic stress or humoral factors affect mature and intermediate forms of AM in cardiac tissue remains obscure. In this study, we have examined whether a subpressor dose of Ang II affects the LV AM peptide concentration (total AM and mature AM) independently of hemodynamic stress *in vivo*, and if so, whether the process is mediated by the AT1 receptor.

Material and Methods

Experiment 1

Animals and Surgery: An Alzet osmotic minipump (model 1003D or 2001, DURECT Corp., Cupertino, CA) containing Ang II in saline or saline alone was subcutaneously implanted into eight-week-old male Wistar rats (280 – 310 g) under ether anesthesia. Ang II (0.4 mg/kg/day, Ang II group) [16] or saline (control: C group) was continuously infused into the rats, which were studied 3 (Ang II group, n = 5; C group, n = 6) and 7 (Ang II group: n = 7, C group: n = 5) days after implantation.

Hemodynamic measurements: The rats were anesthetized with pentobarbital sodium (50 mg/kg ip). A 2F catheter tipped with a micromanometer (Model SPC-320, Millar Instruments, Houston, TX) was then inserted into the ascending aorta via the right carotid artery. Heart rate (HR) and the systolic blood pressure (SBP) of the ascending aorta were measured using a transducer control unit (Model TCB-500, Millar Instruments, Houston, TX) connected to a polygraph (Model 141 – 6, San-Ei, Tokyo), and the chest was opened. The right ventricle was dissected along the septal insertion, and the ventricular septum was considered to be part of the LV. Each portion was weighed, immediately frozen in liquid nitrogen, and stored at – 80 °C.

Assays of tissue levels of total AM and mature AM : The LVs were boiled in 10 volumes of 1 mol/l acetic acid for 10 min to inactivate intrinsic proteases. The tissue was cooled and disrupted using a Polytron for 1 min. The homogenate was centrifuged at 18,000 × *g* for 30 min, and the supernatant was lyophilized and dissolved in assay buffer (100 mM sodium phosphate buffer; pH 7.4 containing 5 mM EDTA, 80 mM NaCl, 0.05 % Triton X-100 and 0.05 % NaN3). Mature AM from the tissues and total AM were measured by immunoradiometry using specific kits (AM mature RIA SHIONOGI and AM RIA SHIONOGI, Shionogi Co., Ltd., Osaka, Japan).

Experiment 2

We investigated the effects of a subdepressor dose of an AT1 receptor antagonist (candesartan) on LV AM gene expression and mature and total AM concentrations in the LV of rats infused with a subpressor dose of Ang II. The rats were randomized into the following groups: Ang II subpressor dose (n = 5), Ang II subpressor dose + candesartan (0.7 mg/kg/day) (n = 5), saline alone (n = 5) and saline + candesartan (0.7 mg/kg/day) (n = 5). Candesartan was suspended in 5 % gum arabic and administered daily through the stomach by gastric gavage starting 5 days before minipump implantation, and continued for 7 days. Hemodynamics, LV AM gene expression and levels of mature and total AM in the LV were examined 7 days after implantation. All experiments, handling and care of the animals were conducted according to the guidelines for Institutional Animal Care and Use of Laboratory Animals of Miyazaki Medical College.

Tab. 1 Physiological changes in saline- and Ang II-infused rats

		3 days	*7 days*
BW (g)	C group	336 ± 10	335 ± 4
	Ang II	311 ± 2	271 ± 8##
LVW (mg)	C group	664 ± 22	642 ± 19
	Ang II	744 ± 54	723 ± 7#
LVW/BW (mg/g)	C group	1.97 ± 0.03	1.91 ± 0.05
	Ang II	2.39 ± 0.18	2.68 ± 0.06##
HR (bpm)	C group	40 ± 17	439 ± 13
	Ang II	413 ± 22	432 ± 26
SBP (mm Hg)	C group	121 ± 4.7	120 ± 3.3
	Ang II	119 ± 6.1	124 ± 6.4

Values are means ± SEM. (n = 4 – 6 per group). C group, saline-infused rats; Ang II, angiotensin II-infused rats; BW, body weight; LVW, left ventricular weight; LVW/BW, left ventricular weight/body weight ratio; HR, heart rate; SBP, systolic blood pressure; *p < 0.05 *vs.* C group; #p < 0.005 *vs.* C group; ##p < 0.0001 *vs.* C group.

RNA isolation and real-time quantitative polymerase chain reaction (PCR): Total RNA isolated from LV tissues using TRIzol (Invitrogen, Carlsbad, CA) was reverse-transcribed into cDNA using Super Script reverse transcriptase (Invitrogen, Carlsbad, CA) into cDNA. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and AM mRNAs were determined using real time-quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA) and oligonucleotide primers and probes as described [8]. PCR products were used as standards, and mRNA levels were compared after normalization relative to those of GAPDH.

Statistical analysis

All results are expressed as means ± SEM. Differences among the three or four groups in each experiment were evaluated using the one-way analysis of variance followed by Scheffe's test. Linear regression analysis revealed relationships between variables. Differences were considered significant at values of $p < 0.05$.

Results

Experiment 1

Table **1** shows changes in body weight (BW), LV weight (LVW), LVW/BW, HR and SBP. The SBP values in the Ang II group did not change over 7 days compared to the control group. In contrast, the LVW/BW ratio in the Ang II group was significantly increased compared to the control group. The LV total AM concentration in the Ang II group at day 7 was significantly increased ($p < 0.05$) compared to the control group (Fig. **1a**). Likewise, the concentration of mature AM in the LV ($p < 0.01$), and the mature AM/ total AM ratio ($p < 0.05$) was significantly increased at day 7 compared to the control group (Fig. **1b** and **c**). Correlations between LVW/BW and either of the total or mature AM concentrations in the LV were significantly positive (Fig. **2**).

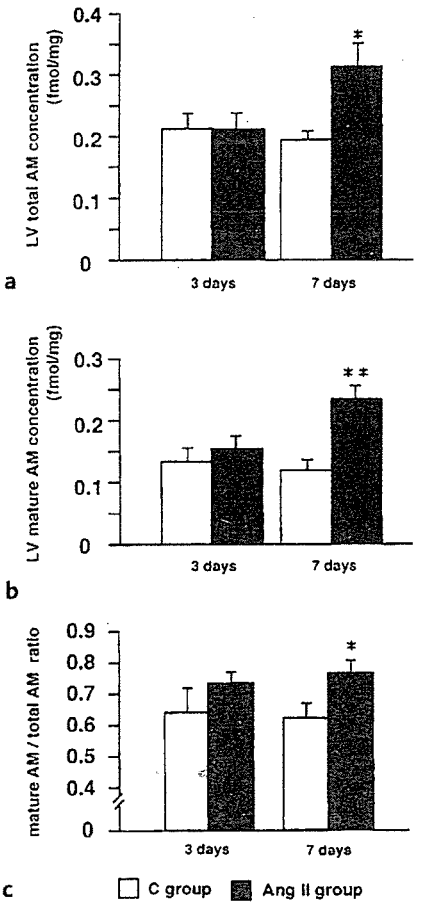

Fig. 1 Total and mature AM concentrations in LV and mature AM/total AM ratio in LV of Ang II and C groups at days 3 and 7. Total (**a**) and mature (**b**) AM concentrations in LV are significantly increased in Ang II group at day 7. Tissue mature AM/total AM ratio in LV is also increased in Ang II group at day 7 (**c**). Values are means ± SEM. $^{*}p < 0.05$ *vs.* C group, $^{**}p < 0.01$ *vs.* C group.

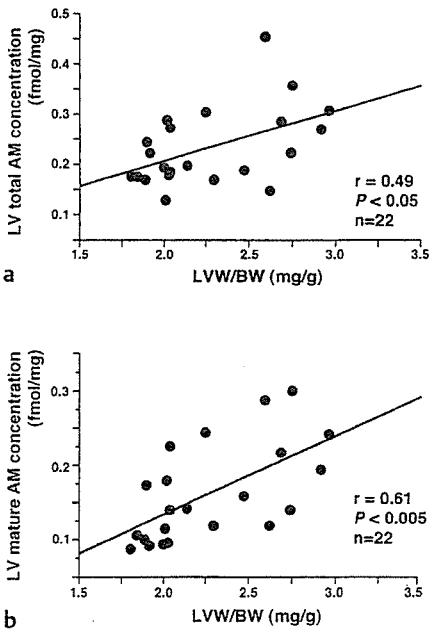

Fig. 2 Relationship between total and mature AM concentrations in LV and LVW/BW in rats infused with Ang II. Data are from Ang II and C groups at days 3 and 7. Total (**a**) and mature (**b**) AM concentrations in LV significantly correlated with LVW/BW ($r = 0.49$, $p < 0.05$ and $r = 0.61$, $p < 0.005$, respectively).

Tab. 2 Physiological changes 7days after candesartan treatment

	Ang II	*Ang II candesartan*	*Saline*	*Saline candesartan*
BW (g)	$265 \pm 10^{\#\#}$	$302 \pm 3^{*}$	335 ± 4	$314 \pm 3^{\S}$
LVW (mg)	$728 \pm 6^{\#}$	$606 \pm 10^{*}$	633 ± 18	$580 \pm 13^{\S}$
LVW/BW (mg/g)	$2.7 \pm 0.05^{\#\#}$	$2.01 \pm 0.02^{**}$	1.89 ± 0.04	1.85 ± 0.04
HR (bpm)	408 ± 25	371 ± 15	425 ± 10	$363 \pm 14^{\S}$
SBP (mm Hg)	115 ± 6	115 ± 5	128 ± 5	113 ± 5.3

Values are mean ± SEM. (n = 4 – 6 per group). C group, saline-infused rats; Ang II, angiotensin II-infused; rats BW, body weight; LVW, left ventricular weight; LVW/BW, left ventricular weight/body weight ratio; HR, heart rate; SBP, systolic blood pressure; $^{\#}p < 0$; 005 *vs.* saline-infused rats, $^{\#\#}p < 0$; 0005 *vs.* saline-infused rats; $^{*}p < 0$; 0001 *vs.* Ang II-infused rats; $^{**}p < 0$; 0001 *vs.* Ang II-infused rats; $^{\S}p < 0$; 05 *vs.* saline-infused rats.

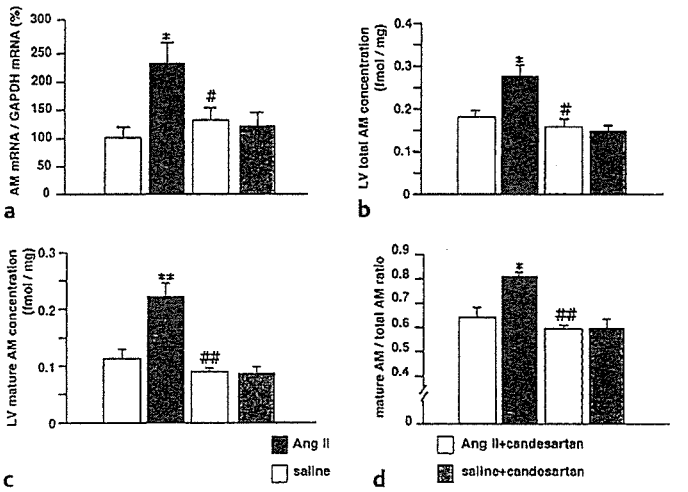

Fig. 3 Adrenomedullin mRNA levels, total and mature AM concentrations in LV and mature AM/total AM ratio in rats infused with Ang II, saline, Ang II + candesartan and saline + candesartan at day 7. All levels were augmented in rats infused with Ang II and suppressed by candesartan. Values are means ± SEM. $^{*}p < 0.05$ *vs.* saline, $^{\#}p < 0.05$ *vs.* Ang II; $^{**}p < 0.01$ *vs.* saline; $^{\#\#}p < 0.001$ *vs.* Ang II.

Experiment 2

Table **2** shows the physiological and hemodynamic effects of Ang II, saline and candesartan in four groups of rats. The augmented LV AM gene expression in the rats infused with Ang II was completely suppressed by the subdepressor dose of candesartan (Fig. **3a**). In contrast, AM mRNA levels were not affected by candesartan in the rats infused with saline (Fig. **3a**). Thus, a subdepressor dose of candesartan did not affect basal AM gene expression, suggesting that a nonspecific effect of candesartan on AM transcription can be ruled out. The total AM concentration in the LV also increased in rats infused with Ang II, which was suppressed by candesartan (Fig. **3b**). Likewise, mature AM concentrations in the LV and mature AM/total AM ratio increased in rats infused with Ang II; these were suppressed by candesartan (Fig. **3c, d**). Total AM in the LV, the mature AM concentration and the mature /total AM ratio in rats infused with saline were not affected by candesartan alone (Fig. **3b, c** and **d**).

Discussion

The production of AM in myocytes is augmented not only by mechanical stretching, but also by Ang II stimulation and partially through AT1 receptors, which suggests local interaction between AM and Ang II in stretched myocytes [8]. Likewise, *in vivo* studies have indicated that ventricular AM production might be partly

affected by Ang II in the pressure-overloaded rat model produced by aortic banding [17]. This is because although candesartan, quinapril (an angiotensin-converting inhibitor), and manidipine (a calcium channel blocker) decrease blood pressure to comparable levels, candesartan and quinapril completely suppress ventricular AM expression, whereas manidipine does not. We recently demonstrated that Ang II stimulates LV AM gene expression independently of pressure overload *in vivo* [9]. The present study revealed that Ang II increases the concentrations of total and mature AM as well as the mature AM/total AM ratio in the LV. This indicates that Ang II stimulates the production of LV AM and the accumulation of mature, rather than intermediate AM in the LV. Furthermore, the enhancement is at least in part mediated through the AT1 receptor.

Total AM consists of mature and intermediate forms of AM that are converted to mature AM by enzymatic amidation [12]. Here, we measured total AM in rat LV tissue, as intermediate AM could not be directly measured. The concentrations of both LV total AM and mature AM were concomitantly increased with cardiac hypertrophy induced by Ang II infusion. The tissue mature AM/total AM ratio was increased in the Ang II group, suggesting that Ang II stimulates the accumulation of active mature, rather than inactive intermediate AM in LV tissue. Candesartan suppressed the mature AM/total AM ratio to the control level. We and others have reported that the plasma mature AM/total AM ratio does not change in patients with congestive heart failure or with hypertension compared with normal individuals [13,14]. Additionally, the mature AM/total AM ratio is higher in the urine than in the plasma of patients with chronic renal failure compared with hypertensive and normal individuals [14]. LV mature AM, total AM levels and the mature AM/total AM ratio are significantly higher in deoxycorticosterone acetate-salt spontaneously hypertensive (SHR) rats than in SHR and Wistar Kyoto rats, suggesting that AM production and the amidating enzyme activity are upregulated in certain types of severe hypertension [15]. The gene for the amidating enzyme is expressed in the atria and ventricles [18]. However, whether tissue mature AM or total AM in the ventricle is affected by hemodynamic stress or humoral factors has not been clarified. The present study has demonstrated that Ang II causes the biologically active form of AM to accumulate in the hypertrophied myocardium irrespective of hemodynamic stress. Based on these findings, we speculate that Ang II as well as mechanical stress *per se* plays an important role in the accumulation of biologically active AM in the LV of the pressure-overloaded heart.

The increased LV AM gene expression and LV AM concentrations in rats infused with Ang II were completely suppressed by the subdepressor dose of AT1 receptor blocker, suggesting that ventricular AM production is at least partly regulated through the AT1 receptor. This is compatible with the results of our previous study *in vitro* [8]. The present study did not define any pathophysiological role of cardiac AM during a continuous infusion of Ang II. However, we have suggested that AM, as an autocrine or paracrine factor, acts on cardiomyocytes and fibroblasts to modulate cardiac hypertrophy and growth [10,11]. The present study has found a significantly positive correlation between LVW/BW and either of the LV total AM or mature AM concentrations. These findings suggest that increased AM, especially in its biologically active form in the LV, constitutes an autocrine or paracrine mechanism that offsets cardiac hypertrophy against Ang II stimulation. Our notion is also supported by the finding that AM gene delivery significantly increases cardiac AM concentrations and decreases cardiac hypertrophy induced by hypertension in rats [19]. Ang II at a subpressor dose still sufficient to induce AM production also induced LV hypertrophy, and the total and mature AM concentrations were positively correlated to LVW/BW in the present study. According to the results, we believe that cardiac hypertrophy is necessary for AM production. In other words, Ang II stimulates AM production through LV hypertrophy rather than through direct action. This conclusion tallies with our previous observation that Ang II stimulates AM production in association with increased phenylalanine incorporation (protein synthesis) and increased atrial natriuretic peptide (ANP) mRNA expression in cardiac myocytes *in vitro*. Low concentrations of Ang II insufficient to increase protein synthesis and ANP gene expression cannot induce AM production (unpublished data). The conclusion also tallies with the finding that insufficient pressure overload can induce mild LV hypertrophy, but not AM gene expression, in rats with aortic banding *in vivo* [20]. However, sufficient pressure overload can induce more severe LV hypertrophy as well as AM gene expression in banded rats [3,17].

The molecular or cellular mechanism of AM promotion by Ang II is unknown. However, we have reported that the stimulation of AM secretion by Ang II is significantly reduced by inhibiting or downregulating protein kinase C (PKC) activity, a blockade of L-type Ca^{2+}, and by inhibitors of calmodulin and calmodulin kinase II [21]. These *in vitro* findings suggest that PKC and the Ca^{2+}/calmodulin signaling system are at least partly involved in Ang II induced AM production *in vitro* [21]. In addition, Niu et al. reported that AM might inhibit the development of Ang II induced cardiac hypertrophy at least partly by suppressing extracellular signal-regulated kinase (ERK) activation via protein kinase A activation and PKC inhibition. Thus, ERKs are apparently involved in the link between Ang II and AM [22].

In summary, Ang II stimulates cardiac AM production and increases the LV AM peptide level independently of hemodynamic stress *in vivo*. The peptide level of AM in the LV increased in parallel with LV hypertrophy. In addition, this stimulation was mediated at least partly by the AT1 receptor. We have also demonstrated that levels of the biologically active form of AM increase concomitantly with LV hypertrophy, but the precise mechanism of the increase in mature AM in the LV remains unknown. Activation of the amidation enzyme could be involved in AM maturation induced by Ang II. However, the levels of gene expression of amidation enzyme and/or amidation enzyme activity in the LV should be determined to clarify whether Ang II directly stimulates AM maturation. Intermediate AM could not be directly measured in the present study. A new methodology is required to accomplish this before the mechanism of AM activation can be determined. Further studies are required to define the relationship between AM activation and cardiac hypertrophy or between AM activation and humoral factors such as Ang II.

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Beyond vasodilation: The antioxidant effect of adrenomedullin in Dahl salt-sensitive rat aorta ☆

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Abstract

We have investigated the antioxidant effect of adrenomedullin (AM) on endothelial function in the Dahl salt-sensitive (DS) rat hypertension model. Dahl salt-resistant (DR) and DS rats were fed an 8% NaCl diet. In addition, the DS rats were subcutaneously infused with either saline or recombinant human AM for 4 weeks. Although systolic blood pressures measured weekly in AM- and saline-infused rats did not significantly differ, aortic $O_2^{\cdot-}$ levels were significantly ($P < 0.01$) higher in the latter. Likewise, both endothelial nitric oxide synthase (eNOS) mRNA and protein were significantly higher in saline-infused DS rats. Infusion of AM reduced both O_2^{-} and eNOS expression to levels comparable to those seen in DR rats. AM infusion also upregulated the gene expression of guanosine-5′-triphosphate cyclohydrolase I and downregulated the expression of $p22^{phox}$, suggesting that AM increased the NOS coupling and bioavailability of NO. AM possesses significant antioxidant properties that improve endothelial function.

Keywords: Adrenomedullin; Vasodilator; Nitric oxide synthase; Endothelium; Aorta

Adrenomedullin (AM) is a potent vasodilator peptide first isolated from human pheochromocytoma but later found to be ubiquitously expressed [1,2]. For instance, endothelial cells are a major site of AM synthesis and secretion, suggesting that it may serve an autocrine/paracrine function in blood vessels [3]. When administered intravenously, AM exerts a hypotensive effect in both humans and animals [4–6], which results obtained from isolated rat aortic rings suggest is due to nitric oxide (NO)-induced vasodilation that can be blocked by nitric oxide synthase (NOS) inhibitors [7,8]. In addition, in many pathophysiological conditions associated increased oxidative stress (e.g., hypertension, atherosclerosis, diabetes, and heart failure [11–15]) plasma AM concentrations are increased, perhaps to exert a compensatory effect [9,10].

Oxidative stress is a condition in which cells are exposed to excessive levels of oxygen-free radicals. One such free radical, superoxide ($O_2^{\cdot-}$), is mainly produced by NAD(P)H oxidase; however, recent studies have shown that in the absence of sufficient levels of its essential cofactor, tetrahydrobiopterin (BH4), NOS exists in what is described as an "uncoupled" state and mainly

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produces O_2^- instead of NO [16–18]. That overexpression of AM in the aortic endothelium increases basal NO release in AM transgenic mice is indicative of endogenous AM's involvement in the regulation of aortic tone [19]. On the other hand, previous studies on AM knockout mice showed increased isoprostane production and insulin resistance [20,21], suggesting that AM exerts an antioxidant effect. As yet, however, there is no direct evidence of an antioxidant effect of AM in blood vessels, and the underlying mechanisms are still poorly defined. In the present study, therefore, we evaluated the effect of AM on endothelial function and $O_2^{\cdot-}$ production in aorta by infusing recombinant human AM into Dahl salt-sensitive rats, an established model of genetic hypertension that also exhibits increased oxidative stress.

Table 1
Primers and probes used in the experiment

GAPDH	
Sense	5′-TCCTGCACCACCAACTGCTTAG-3′
Antisense	5′-CACAGCCTTGGCAGCACCAGT-3′
Probe	5′-TGACCACAGTCCATGCCATCACTGCCACTC-3′
eNOS	
Sense	5′-TACTACTCTGTCAGCTCAGCAC-3′
Antisense	5′-TCTCCTGCCTTGAGTTGGCT-3′
Probe	5′-TCCACCTCACTGTAGCTGTGCTGGCATACA-3′
$p22^{phox}$	
Sense	5′-GCTCATCTGTCTGCTGGAGTA-3′
Antisense	5′-ACGACCTCATCTGTCACTGGA-3′
Probe	5′-TGTCCTCCACTTACTGCTGTCCGTGCCTGC-3′
GTPCH I	
Sense	5′-ATTTGTGGGAAGGGTCCA-3′
Antisense	5′-CAGATAACGCTGGCCTCA-3′

Materials and methods

Animals. Five-week-old Dahl salt-sensitive/Jr. Sea (DS) and salt-resistant (DR) rats were purchased from Seac Yoshitomi (Japan). After 1 week on a diet of normal chow, the rats were changed to a high salt (8% NaCl) diet and maintained for an additional 4 weeks. Before being switched to high salt diet, the DS rats were divided into three groups: one infused with AM, a second infused with saline, and a third orally administered tetrahydrobiopterin (BH4; 10 mg/kg/day). Using osmotic minipumps, rats in the AM group ($n = 12$) were subcutaneously infused with recombinant human AM in saline for 4 weeks at a rate of 1.0 μg/h; those in the saline group ($n = 12$) were infused with saline alone. During the infusion period, systolic blood pressure (SBP) and heart rate (HR) were measured weekly, and blood was sampled from the tail to assay the human AM concentration. After 4 weeks, the rats were sacrificed after being anesthetized with sodium phenobarbital, and the thoracic aortas were excised. Part of each aorta was used for ex vivo tension experiments, while the remaining portion was used for $O_2^{\cdot-}$ assays and quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analyses.

All experiments were carried out according to the regulations of the Animal Research Committee of Miyazaki Medical College. This investigation also conformed to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (1996).

Organ chamber experiments. Isometric tension was measured in aortic rings as described previously [8]. Briefly, aortic rings were mounted horizontally between two stirrups in organ chambers filled with 4 ml of oxygenated modified Krebs–Ringer bicarbonate solution (in mmol/L: NaCl 118.6, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, K_2PO_4 1.2, $NaHCO_3$ 25.1, glucose 10.1, and EDTA 0.026) at 37 °C. One stirrup was connected to an anchor and the other to a force transducer for recording isometric tension. Relaxations elicited by acetylcholine (ACh; 10^{-10} to 10^{-5} mol/L) and sodium nitroprusside (SNP; 10^{-10} to 10^{-6} mol/L) were studied in rings precontracted to 50% of the maximum elicited by phenylephrine.

Superoxide assays. Superoxide levels were assayed using the lucigenin-enhanced chemiluminescence method as described previously [22,23]. Briefly, 5-mm-wide aortic rings were incubated with lucigenin (5 μmol/L) at 37 °C for 10 min in Krebs buffer. After then dark adapting for 5 min, relative light units (RLU) from each aortic ring were recorded using a Lumat LB9506 luminometer (EG&G BERTHOLD, Germany). Ten measurements were made at 30-s intervals and then averaged. Blank values were subtracted and data were expressed as RLU/mg wet tissue. In some cases, vessels were incubated with NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) for 30 min before chemiluminescence measurement.

Vessels were also cut into 20-μm frozen sections, washed three times in phosphate-buffered saline (PBS), dark adapted for 30 min at room temperature, and then incubated with dihydroethidine (1 μmol/L). The sections were then washed again with PBS, covered, and examined under an Olympus FV300 (Tokyo, Japan) confocal laser scanning microscope. In the presence of $O_2^{\cdot-}$, dihydroethidine is oxidized to ethidium bromide and emits a red fluorescence.

Western blot analysis. Aortas were homogenized in sample buffer containing 2% SDS, 2-mercaptoethanol, and glycerol, after which protein concentrations were assayed using a Non-Interfering Protein Assay Kit (Geno Technology, St. Louis, MO. USA). Equal aliquots of protein were then subjected to 7.5% SDS gel electrophoresis and transferred to a Hybond-P (Amersham) membrane. The membrane was then blocked with 5% Block Reagent (Amersham), washed and incubated for 1 h at 25 °C, first with anti-eNOS antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and then with secondary antibody. eNOS protein was detected using an ECL Plus chemiluminescence kit (Amersham), after which it was quantified by densitometry using Image Gauge (LAS-1000, FUJIFILM).

Reverse transcription-polymerase chain reaction. Total RNA was isolated as described previously [24], after which 2-μg samples were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen), and the resultant cDNAs were subjected to semi-quantitative PCR or real-time quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA). The primers and probes used in this experiment were as shown in the Table 1.

Human AM assay. Levels of human AM in blood samples were assayed as previously described [24] using an enzymatic immunoassay that does not cross-react with rat AM.

Calculations and statistics. Results are expressed as means ± SEM. Analysis of variance and Student's t tests were used when appropriate to assess the significance of differences between groups. Values of $P < 0.05$ were considered significant.

Results

Effective rise in plasma hAM levels does not affect blood pressure

Weekly measurement of human AM in rat plasma showed that infusion at a rate of 1.0 μg/h elicited an

effective rise of plasma hAM levels from 1 to 2 fmol/ml (data not shown). This increase was not sufficient to affect blood pressure, although a decline in the heart rates of AM-infused rats was noted (Fig. 1).

Endothelial function and $O_2^{\cdot-}$ production in aorta

Evaluation of the effect of ACh in phenylephrine-precontracted aortic rings revealed the endothelial function of DS rats to be significantly impaired. Among saline-infused animals, the pD_2 value ($-\log EC_{50}$) for ACh was 6.6, and the maximal ACh-evoked relaxation was $28 \pm 2\%$. Infusion of AM largely preserved endothelial function, so that the ACh pD_2 was 7.4 and the maximal evoked relaxation was $76 \pm 11\%$, which were comparable to the values obtained from DR rats fed a high salt diet (pD_2, 7.8; maximal relaxation, $86 \pm 6\%$) (Fig. 2A). DS rats treated with BH4 (10 mg/kg/day for 4 weeks) also showed significantly improved endothelial function (pD_2, 7.5; maximal relaxation, $81 \pm 4\%$). By contrast, endothelium-independent function, as indicated by the response to exogenous NO liberated from sodium nitroprusside (SNP), did not differ among the three groups (Fig. 2B).

Consistent with earlier studies [25,26], we found that $O_2^{\cdot-}$ production was greatly increased in DS rats, but this effect was diminished by 70% ($P < 0.01$) in rats infused with AM (Fig. 3). Removing the endothelium or incubation with the NOS inhibitor L-NAME for 30 min also significantly reduced the production of $O_2^{\cdot-}$ in DS rat aortic rings, indicating that the excess $O_2^{\cdot-}$ was produced in the endothelium by NOS. Of note, L-NAME failed to suppress but increased $O_2^{\cdot-}$ production instead in AM-treated DS rat vessels, suggesting the possible improved eNOS coupling status. Similar findings were obtained when dihydroethidine-labeled aortic sections were examined under a confocal laser scanning microscope. In Fig. 4, the relative levels of $O_2^{\cdot-}$ are indicated by the intensity of the red fluorescence produced when dihydroethidine is oxidized to ethidium bromide. Taken together with the increased responsiveness to ACh, these findings are indicative of a substantial increase in the bioavailability of NO after AM treatment.

mRNA and protein expression in the aorta

Real-time quantitative PCR showed that levels of eNOS mRNA expression in the aortas of DS rats were

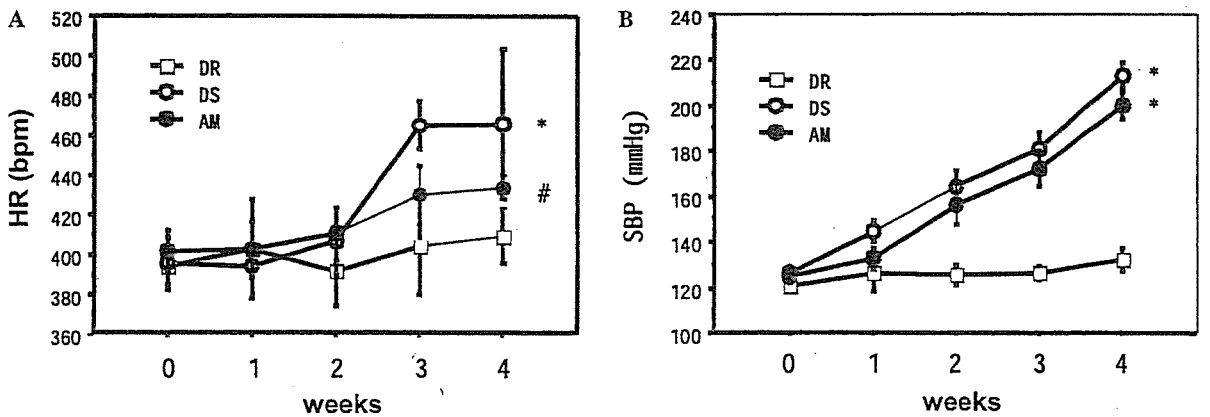

Fig. 1. Time-dependent changes in heart rate (HR) (A) and systolic blood pressure (SBP) (B) in the three rat groups. Data were collected using the tail cuff method and are expressed as means ± SEM; $n = 12$ in each group; $^*P < 0.05$ vs. DR, $^{\#}P < 0.05$ vs. DS.

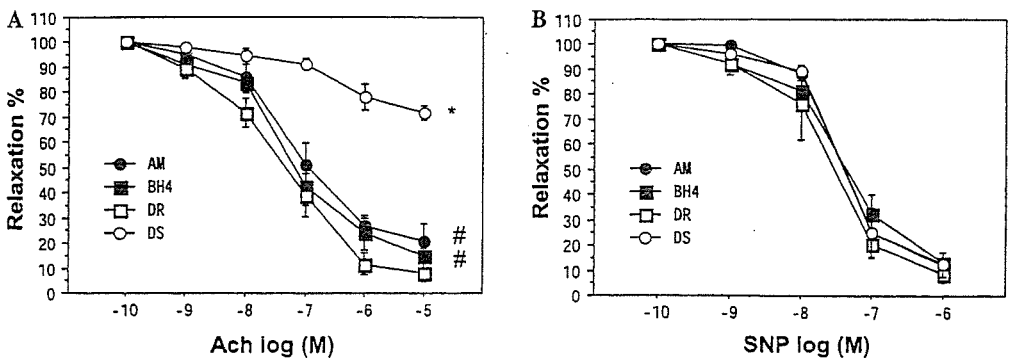

Fig. 2. Vascular responses to ACh (10^{-10} to 10^{-5} mol/L) and SNP (10^{-10} to 10^{-6} mol/L) in phenylephrine-precontracted aortic rings. Data are presented as percentage relaxation and are expressed as means ± SEM; $n = 8$; $^*P < 0.05$ vs. DR, $^{\#}P < 0.05$ vs. DS.