

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.

Angiogenic 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 neo-vascularization 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 angiogenic 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. Angiogenic properties appear to be another feature that should be characterized further *in vitro* and *in vivo*.

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

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Abstract

Adrenomedullin (AM) administered intracolonicly 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 intracolonicly 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.

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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-

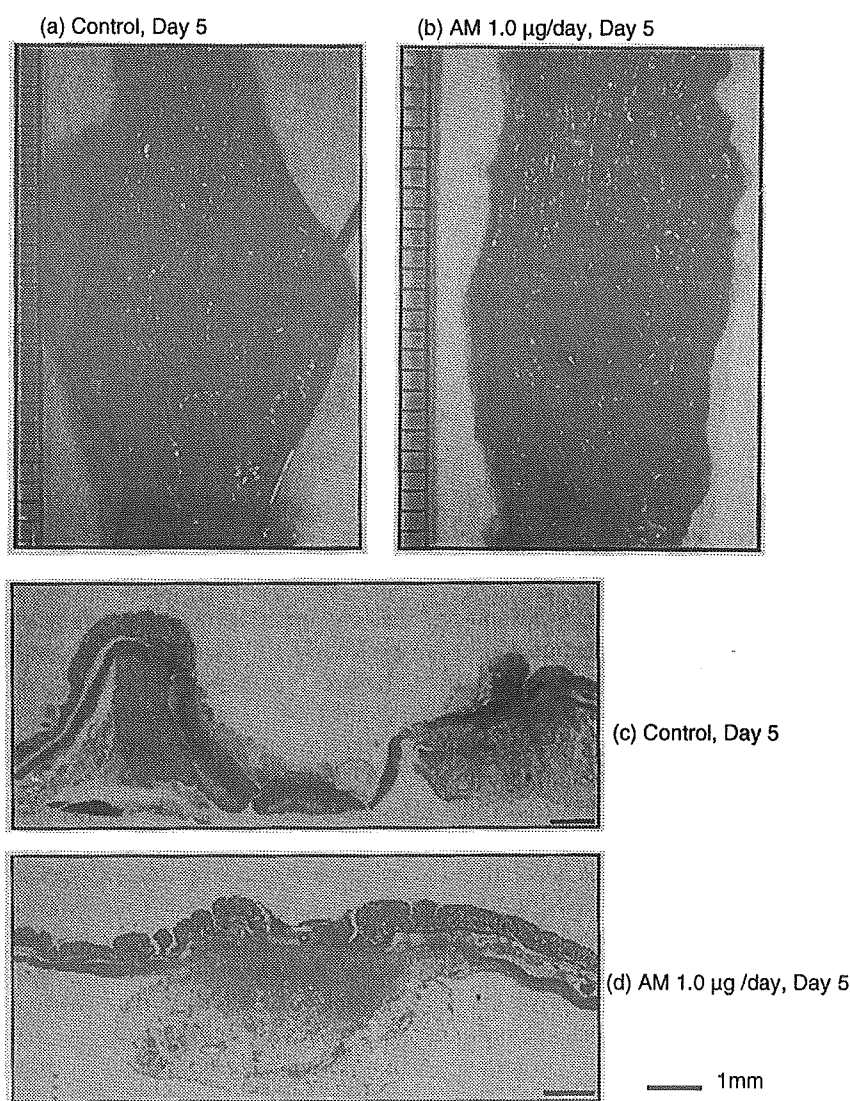


Fig. 1. Macroscopic and histological appearance of colonic ulcers induced by subserosal injection of acetic acid. Rats were treated with AM (1.0 $\mu\text{g}/0.5$ ml saline) or saline alone (0.5 ml) for 5 days. (a) Macroscopic view of the colon of a control animal exhibiting a wide and deep ellipse-shaped 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 \pm 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 $\mu\text{g}/\text{day}$) 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 $\mu\text{g}/\text{day}$ (Fig. 2a).

Histological examination of samples of colonic mucosa from control animals revealed deep ulceration, severe 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)

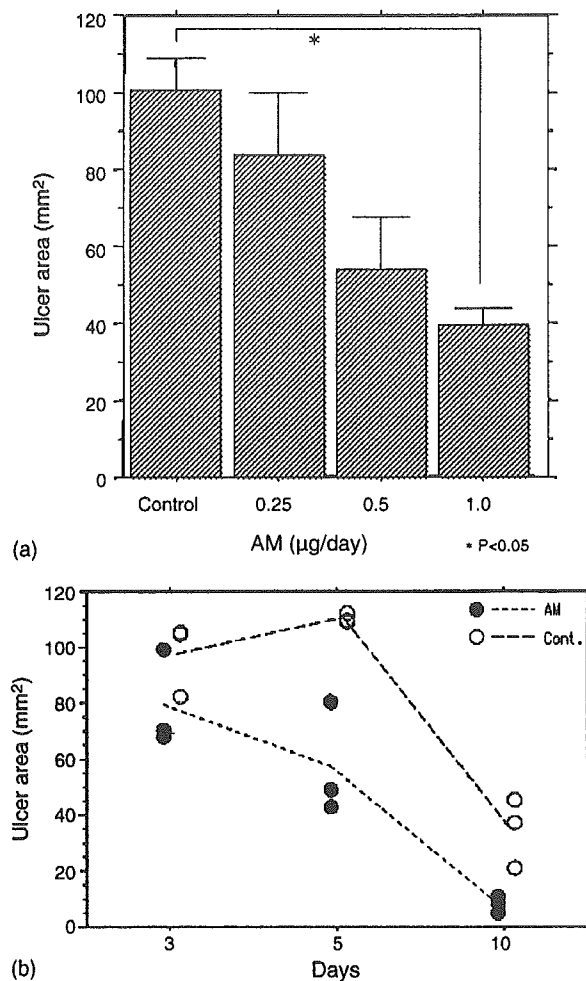


Fig. 2. (a) Effects of AM on the size of acetic acid-induced colonic ulcers observed 5 days after induction ($n = 5$ for each group). AM (0.25–1.0 $\mu\text{g}/\text{day}$) dose-dependently reduced ulcer area, with a significant decline being achieved at a dose of 1.0 $\mu\text{g}/\text{day}$. Bars represent the means \pm 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 $\mu\text{g}/\text{day}$) 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 $\mu\text{g}/\text{day}$)-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).

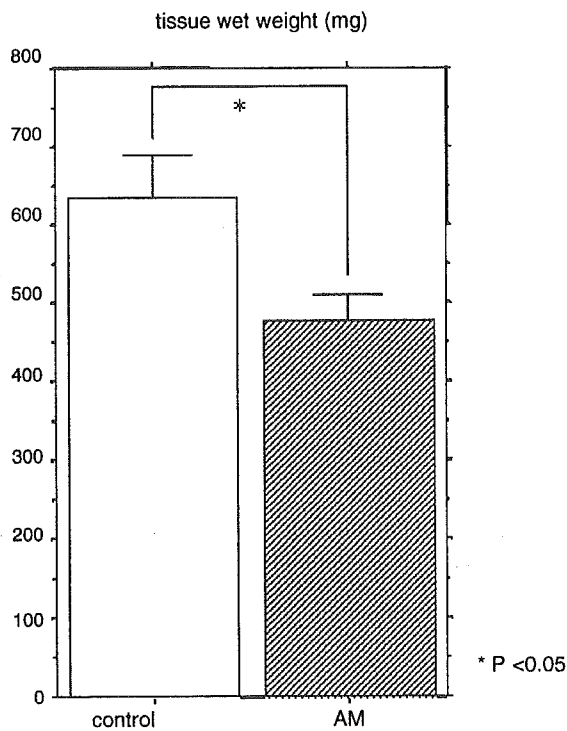


Fig. 3. Wet weights of the tissue specimens (Day 5). Wet weights of 3 cm colonic tissue with the ulcerative lesion at their centre were significantly reduced in AM-treated group ($n=5$ for each group). Bar represents the mean \pm S.E.; * $P < 0.05$ vs. control.

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].

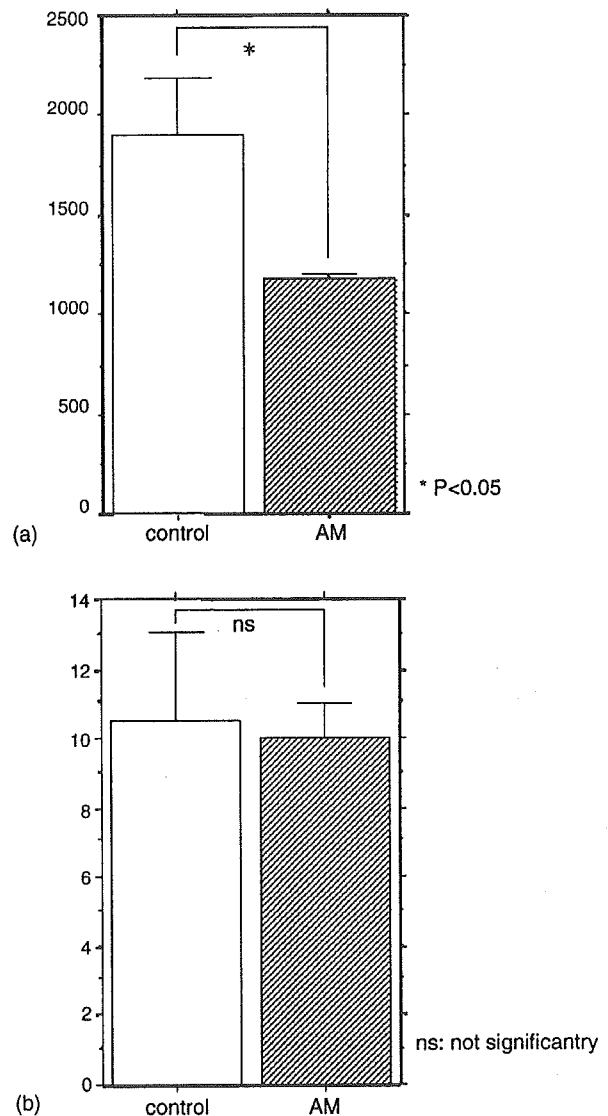


Fig. 4. Effect of AM on the IL-6 (a) and IFN- γ (b) contents of 3 cm samples of colonic tissue affected by application of acetic acid. The cytokine levels were measured on day 5 after induction of colitis ($n=5$ for each group). AM significantly reduced IL-6 levels and wet weights, but had no effect on IFN- γ . Bars represent the means \pm 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 adre-

nomedullin 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 $18,000 \times g$ 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% NaN_3). 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

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.

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.

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).

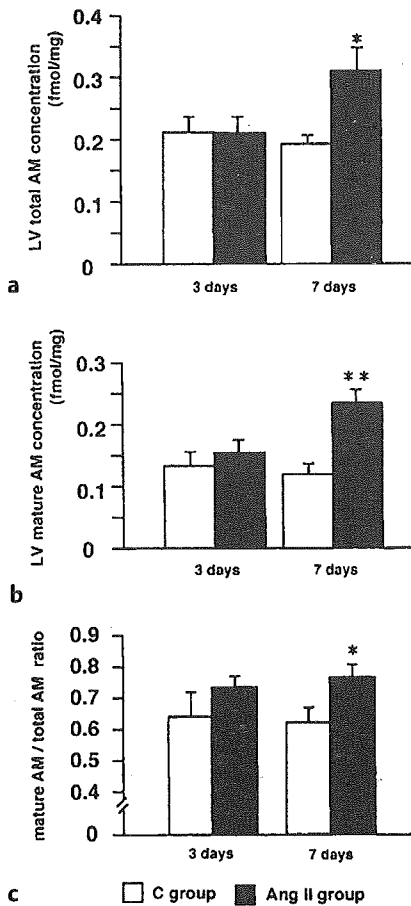


Fig. 1 Total and mature AM concentrations in LV and mature AM/total AM ratio in LV of Ang II and C groups at days 3 and 7. Total (a) and mature (b) AM concentrations in LV are significantly increased in Ang II group at day 7. Tissue mature AM/total AM ratio in LV is also increased in Ang II group at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

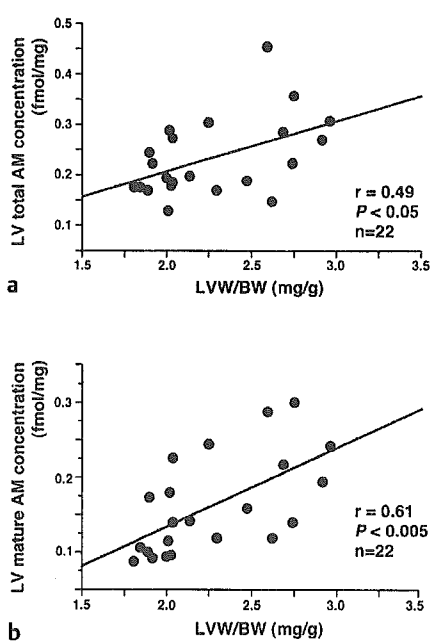


Fig. 2 Relationship between total and mature AM concentrations in LV and LVW/BW in rats infused with Ang II. Data are from Ang II and C groups at days 3 and 7. Total (a) and mature (b) AM concentrations in LV significantly correlated with LVW/BW ($r = 0.49$, $p < 0.05$ and $r = 0.61$, $p < 0.005$, respectively).

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 subde-

Tab. 2 Physiological changes 7 days after candesartan treatment

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

Values are mean \pm 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. saline-infused rats, ## $p < 0.005$ vs. saline-infused rats; * $p < 0.05$ vs. C group, ** $p < 0.01$ vs. C group, § $p < 0.05$ vs. saline-infused rats.

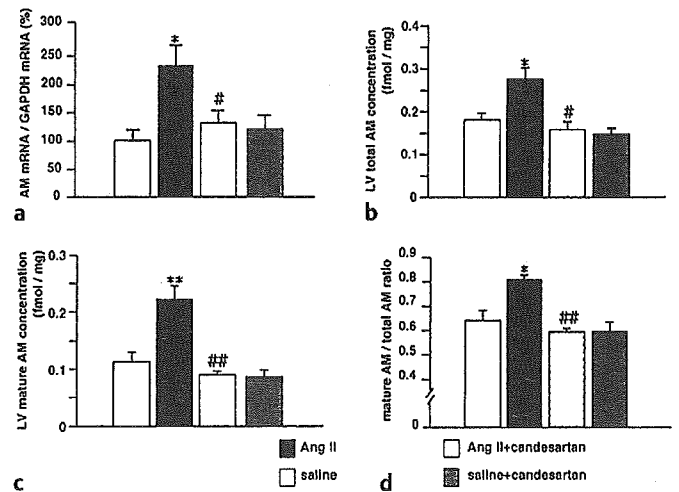


Fig. 3 Adrenomedullin mRNA levels, total and mature AM concentrations in LV and mature AM/total AM ratio in rats infused with Ang II, saline, Ang II + candesartan and saline + candesartan at day 7. All levels were augmented in rats infused with Ang II and suppressed by candesartan. Values are means \pm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

pressor 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 up-regulated 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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Dopaminergic Influence on Thyrotropin Secretion in Primary Hypothyroidism

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Abstract

This study was conducted to assess the influence of dopamine on thyrotropin secretion in patients with primary hypothyroidism before and after optimized L-thyroxin replacement therapy. Thyrotropin responses to dopamine infusion (4 µg/kg/min over 3 hours) and IV metoclopramide (10 mg bolus), a dopamine receptor blocker were studied in 25 consecutive patients with primary hypothyroidism before and after achieving stable euthyroid state and compared with 15 normal age-matched controls. Thyrotropin response to both dopamine infusion (decremental) and IV metoclopramide bolus (incremental) was greater in patients with primary hypothyroidism than that in the control subjects. Thyrotropin response was greater in women than in men. The magnitude of decremental thyrotropin response to dopa-

mine infusion and the incremental response to IV metoclopramide bolus significantly correlated with the basal T₃ and T₄ levels. Thyrotropin response was blunted to dopamine infusion but not to metoclopramide at follow-up after six-month replacement with L-thyroxin, and both the responses were comparable in women and men in patient group. We conclude that modulation of dopaminergic system by dopamine or by dopamine receptor blocker has a greater influence on thyrotropin secretion in patients with primary hypothyroidism than euthyroid normal subjects.

Key words

Dopamine · Metoclopramide · Primary hypothyroidism · Thyrotropin

Introduction

The regulation of thyrotropin (TSH) is largely regulated by hypothalamic thyrotropin-releasing hormone (TRH), and thyroxin (T₄) through its intrapituitary conversion to triiodothyronine (T₃) [1,2]. In addition, a variety of other modulators such as somatostatin, dopamine, α-adrenergic agonists, glucocorticoids, leptin, and various cytokines exert influence on TSH secretion [3–5].

Several studies have been carried out to define the influence of dopaminergic system on regulation of TSH both *in vitro* as well

as *in vivo* (animals and euthyroid/hypothyroid humans) [6–9]. The dopaminergic system seems to exert an inhibitory influence on TSH secretion. The influence of dopamine (DA) on TSH secretion has been documented by administration of dopamine or a dopamine agonist and dopamine receptor blocking drugs [10–13].

The data available on dopaminergic influence on TSH secretion in primary hypothyroidism are scarce and conflicting; the present study was designed to investigate the role of dopaminergic control mechanism in patients with primary hypothyroidism, at presentation and after achieving euthyroid state.

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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 O₂^{•-} 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 O₂^{•-} 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.
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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 (O₂^{•-}), 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^- 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.

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 $\mu\text{g}/\text{h}$; 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^- 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 $\mu\text{mol}/\text{L}$) 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

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'-TACTACTCTGTCTCAGCTCAGCAC-3'
Antisense	5'-TCTCCTGCCTTGAGTTGGCT-3'
Probe	5'-TCCACCTCACTGTAGCTGTGCTGGCATAACA-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'

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 $\mu\text{mol}/\text{L}$). 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^- , 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 \pm 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 $\mu\text{g}/\text{h}$ 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

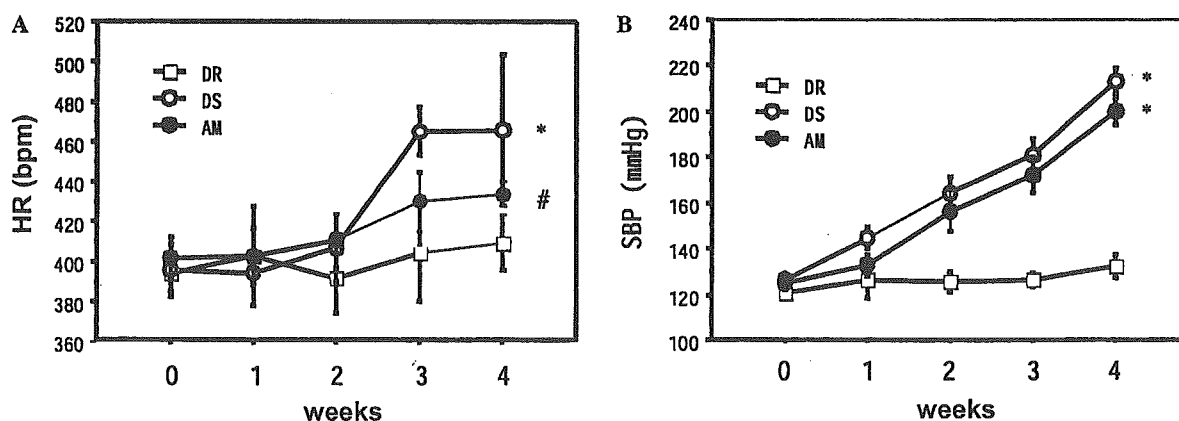


Fig. 1. Time-dependent changes in heart rate (HR) (A) and systolic blood pressure (SBP) (B) in the three rat groups. Data were collected using the tail cuff method and are expressed as means \pm SEM; $n = 12$ in each group; * $P < 0.05$ vs. DR, # $P < 0.05$ vs. DS.

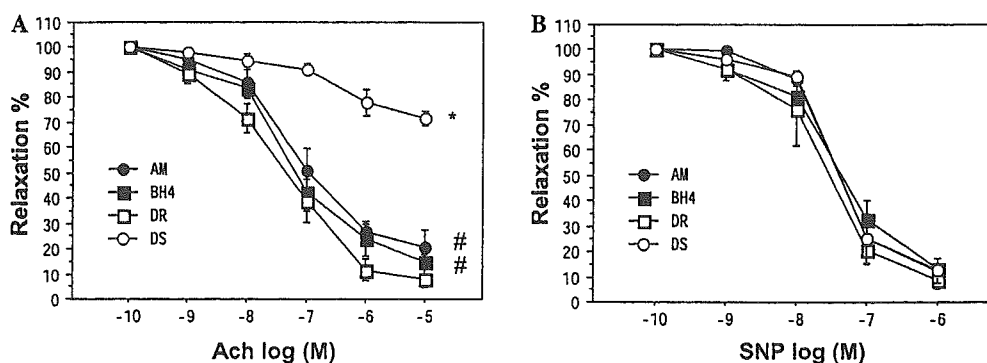


Fig. 2. Vascular responses to ACh (10^{-10} to 10^{-5} mol/L) and SNP (10^{-10} to 10^{-6} mol/L) in phenylephrine-precontracted aortic rings. Data are presented as percentage relaxation and are expressed as means \pm SEM; $n = 8$; * $P < 0.05$ vs. DR, # $P < 0.05$ vs. DS.

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, sup-

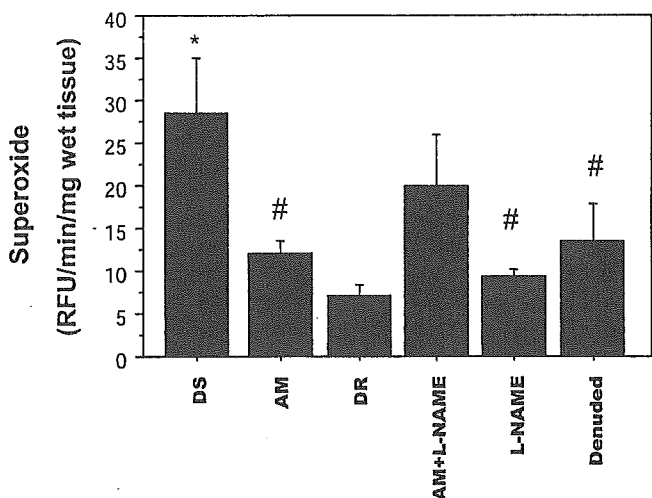


Fig. 3. Superoxide production measured as a function of lucigenin-enhanced chemiluminescence. Aortic rings were incubated with lucigenin (5 $\mu\text{mol/L}$), after which chemiluminescence was measured using a luminometer. Data are presented as relative light units (RLU) per milligram of wet tissue;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

pressing 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-

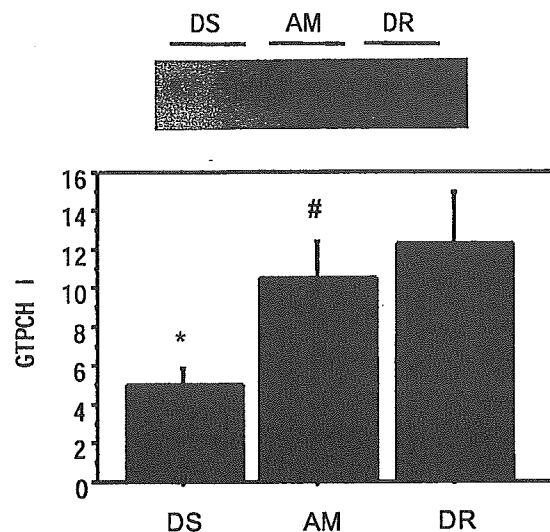


Fig. 6. Expression of mRNAs encoding GTP cyclohydrolase I (GTPCH I). Target genes were amplified by direct RT-PCR, after which the densities of the amplified products on agarose gels were scanned and normalized to GAPDH mRNA, which had been quantified using real-time RT-PCR. Data are expressed as means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

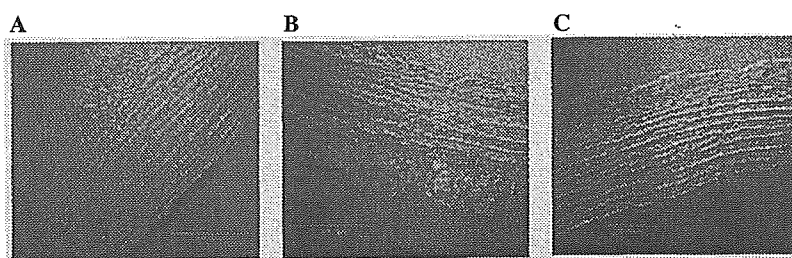


Fig. 4. Confocal images depicting $\text{O}_2^{\cdot-}$ production in representative vessels from the rats in DS (A), AM (B), and DR (C) groups. Frozen sections cut from aortic rings were labeled with dihydroethidine and examined under a confocal laser scanning microscope. The red signal reflects production of $\text{O}_2^{\cdot-}$. Scale bars: (A) and (B), 100 μm ; (C) 50 μm .

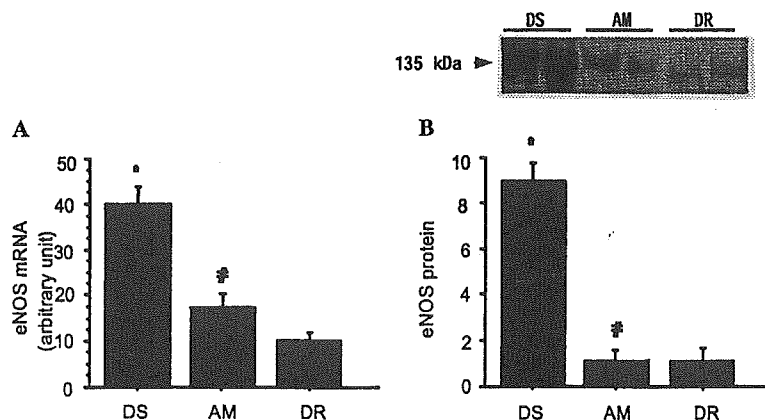


Fig. 5. Endothelial NO synthase (eNOS) mRNA and protein expression. eNOS mRNA (A) and protein (B) expression were evaluated using real-time RT-PCR and Western blot analysis, respectively. Target mRNA expression was normalized by the level of GAPDH mRNA. Data are expressed as means \pm SEM; $n = 6$ in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

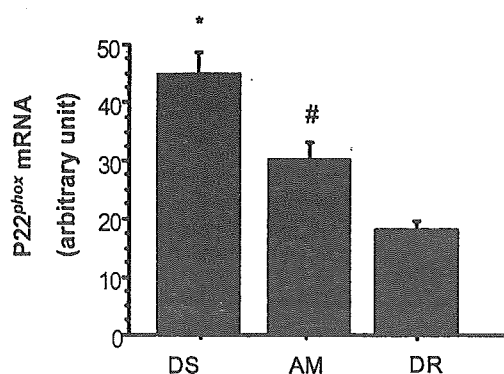


Fig. 7. Expression of mRNA encoding NADPH oxidase subunit p22^{phox} using real-time RT-PCR. Expression of the target mRNA was normalized to the level of GAPDH mRNA. Data are expressed as means \pm SEM; $n = 6$ in each group; * $P < 0.05$ vs. DR, # $P < 0.05$ vs. DS.

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 O₂^{•-}. 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 O₂^{•-} 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 O₂^{•-} 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 O₂^{•-} instead through “uncoupled” electron transfer from its reductase domain to O₂ to form O₂^{•-} [27–29], which in turn leads to endothelial dysfunction. Conversely, exogenous administration of BH₄, or the BH₄ precursor sepiapterin, inhibits O₂^{•-} 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 O₂^{•-} 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 O₂^{•-} [44]. Another important consideration is that O₂^{•-} 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 O₂^{•-} 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 O₂^{•-} 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 O₂^{•-} 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-