Original Article

Accelerated Cardiac Hypertrophy and Renal Damage Induced by Angiotensin II in Adrenomedullin Knockout Mice

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Adrenomedullin (AM) is a potent vasodilating and natriuretic peptide that is thought to play important roles in cardiovascular function. Whether or not AM is involved in the development of cardiac hypertrophy and renal damage remains controversial. In the present study, using heterozygote knockout mice of the AM gene (AM^{+/−}), we analyzed the physiological and pathological roles of the endogenous AM gene. There were no differences in body size or heart and kidney weight compared with wild-type (AM^{+/+}) mice. However, angiotensin II (Ang II) infusion resulted in more severe cardiac hypertrophy in AM^{+/-} mice. The increases in the heart weight-to-body weight ratio and wall thickness of the left ventricle were more prominent in the AM^{+/-} mice. Renal dysfunction characterized by decreased creatinine clearance (Ccr) was more severe in AM^{+/-} after Ang II infusion. These results suggest that AM plays critical roles in the defense mechanism against cardiac hypertrophy and renal dysfunction. An improved understanding of these roles may pave the way to a novel pharmacological approach for the prevention of cardiovascular diseases.

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Key Words: adrenomedullin, knockout mouse, angiotensin II, hypertrophy, renal damage

Introduction

Adrenomedullin (AM) is a potent vasodilator and natriuretic, diuretic peptide that was originally identified from human pheochromocytoma (1). AM belongs to the calcitonin gene-related peptide (CGRP) family, whose other members are also known to be strong vasodilators. High levels of AM are found in the heart, lung, kidney, and vascular endothelial and smooth muscle cells, as well as in the adrenal medulla (2–5). Although AM appears to act in a local paracrine and/or autocrine fashion within the tissue of origin, it also circulates in plasma and has been shown to act as a regulator of the cardiovascular system. AM induces dose-dependent blood pressure reduction, and increases in cardiac index, glomerular filtration rate, and natriuresis (6–8). In addition, plasma levels of AM have been shown to be increased in patients with a number of cardiovascular diseases, including hypertension, chronic renal failure, congestive heart failure, and shock (9–11). Together with the potent biological activity of AM, these findings lead us to speculate that AM participates in the pathophysiology of the development of cardiovascular diseases.

Cardiac hypertrophy and fibrosis, which are recognized in many cardiovascular diseases, together constitute an independent risk factor of cardiac morbidity and mortality (12). Cardiac hypertrophy can be induced by mechanical stress (13) as well as humoral factors such as angiotensin II (Ang
II) (14). Whether or not AM is involved in the development of cardiac hypertrophy and renal damage remains to be clarified.

We recently generated AM knockout mice in order to better understand the physiological roles of AM in vivo. Homozygous mice (AM -/ -) died in utero at embryonic day 13.5 (15). Heterozygotes (AM +/ -), on the other hand, survived until adulthood and were apparently normal and fertile, although they did exhibit elevated blood pressure. We here demonstrate that the AM reduction in AM knockout mice resulted in severe cardiac hypertrophy and further renal damage in response to stressors.

**Methods**

**Animals**

Twelve-week-old male AM +/ + mice (n = 42) and AM +/ - mice (n = 38) from the same genetic background were used in the present study. Mice were housed under climate-controlled conditions with a 12-h light/dark cycle and were provided with standard food and water. All experiments were performed in accordance with the Declaration of Helsinki and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

**Angiotensin II-Infusion Model**

An osmotic minipump (model 2002; Alzet Corp., Palo Alto, USA) containing Ang II dissolved in 0.15 mol/l NaCl and 1 mmol/l acetic acid was implanted subcutaneously into the mice. The delivery rate was 3.2 mg/kg per day for 14 days. After 14 days, the hearts and kidneys were removed, weighed and subjected to further analysis.

**Physiological Studies**

Systolic blood pressure (SBP) and heart rate were determined before and 7 and 14 days after the drug infusion using a programmable sphygmomanometer connected to a cuff probe for mice (MCP-1; Softron, Tokyo, Japan) as described previously (16). Ten repeated values were averaged at each determination. This noninvasive method to measure blood pressure has been validated in mice and correlates well with intra-arterial measurements made in normotensive and hypertensive mice; in addition, it requires minimal warming of the mice (usually less than 15 min) before blood pressure measurement.

Transthoracic echocardiography was performed with an HP Sonos 100 (Hewlett-Packard Co., Palo Alto, USA) using a 12 MHz imaging transducer as described previously (17, 18). After a good-quality two-dimensional image was obtained, M-mode images of the left ventricle were recorded. Intraventricular septum thickness (IVST) and ejection fraction (EF) were also measured.

**Histological Analysis**

For histological analysis, hearts and kidneys were fixed with 10% formalin by perfusion fixation. Fixed hearts and kidneys embedded in paraffin were sectioned at 4-µm thickness and Masson trichrome staining was performed. In the kidney, the glomerular injury scores were graded as follows: 0, no changes; 1, lesions involving < 25% of the capillary tuft; 2, lesions involving 25–49% of the capillary tuft; 3, lesions involving 50–75% of the capillary tuft; 4, lesions involving > 75% of the capillary tuft. The resulting index in each animal was expressed as a mean of all scores obtained. All sections used were evaluated under blind conditions without prior knowledge as to which section belonged to which mouse.

**Serum and Urine Laboratory Analyses**

Ten to 14 days after the osmotic minipumps containing Ang II were implanted, mice were housed in metabolic cages for 24-h urine collection. Urine volume and water intake were recorded, and then the urine samples were assayed for creatinine (Cr). At 14 days, blood was collected to measure Cr, and then creatinine clearance (Ccr) was calculated.

**Statistics**

Differences within groups were compared by one-way analysis of variance (ANOVA) and Student’s t-test. Values of p < 0.05 were considered to indicate statistical significance.

**Results**

**Characterization of AM Knockout Mice**

Targeted null mutation of the AM gene in utero is lethal. The mortality rate among AM -/ - embryos was over 80% at embryonic day 13.5. The most apparent abnormality in AM -/ - embryos was severe hemorrhage, readily observable under the skin and in visceral organs (15). In contrast, AM +/ - mice survived until adulthood and were apparently normal and fertile.

**Changes in Blood Pressure by Ang II Infusion**

We have reported that arterial blood pressure, directly measured by means of a catheter inserted into the femoral artery, was about 10 mmHg higher in AM +/ - mice compared with their wild-type littermates (15). Before treatment in the present study, AM +/ - mice also tended to have higher blood pressure as measured by a programmable sphygmomanometer, but the difference was not significant. Nearly the same degree of blood pressure elevation was observed in both AM +/ - and wild-type mice, and no significant difference in blood pressure was observed between the two groups.
at the 14th day, the end point of the study (Fig. 1).

Severe Cardiac Hypertrophic Changes in AM<sup>+</sup>/<sup>-</sup> Mice

At 14 days after Ang II infusion, the heart weight-to-body weight ratio increased significantly in both AM<sup>+</sup>/<sup>-</sup> and AM<sup>+</sup>/<sup>+</sup> mice, although the increase was more prominent in the AM<sup>+</sup>/<sup>-</sup> mice (Fig. 2). We also evaluated morphometrical and functional changes after Ang II infusion using echocardiography (Fig. 3). IVST was significantly thicker in AM<sup>+</sup>/<sup>-</sup> mice than in AM<sup>+</sup>/<sup>+</sup> mice after Ang II infusion (AM<sup>+</sup>/<sup>-</sup>, 1.06 ± 0.14 mm; AM<sup>+</sup>/<sup>+</sup>, 1.16 ± 0.09 mm; \( p < 0.01; n = 18 \)) (Fig. 3A). In contrast, EF was significantly lower in AM<sup>+</sup>/<sup>-</sup> mice both before and after Ang II infusion (before: AM<sup>+</sup>/<sup>-</sup>, 0.94 ± 0.11; AM<sup>+</sup>/<sup>+</sup>, 0.89 ± 0.05; \( p < 0.05 \); after: AM<sup>+</sup>/<sup>-</sup>, 0.93 ± 0.05; AM<sup>+</sup>/<sup>+</sup>, 0.87 ± 0.06; \( p < 0.05; n = 18 \)) (Fig. 3B).

Severe Renal Damage in AM<sup>+</sup>/<sup>-</sup> Mice

Urine volume was significantly lower in AM<sup>+</sup>/<sup>-</sup> mice compared with their wild-type littermates. Continuous infusion of Ang II markedly increased urine volume and water intake in both strains of mice. However, the increases in both urine volume and water intake were significantly lower in the AM<sup>+</sup>/<sup>-</sup> mice compared with their wild-type littermates (Fig. 4).

Glomerular sclerosis was more severe and glomerular injury score was significantly higher in AM<sup>+</sup>/<sup>-</sup> mice compared with their wild-type mice (Fig. 5A, B). \( C_v \) was significantly lower in AM<sup>+</sup>/<sup>-</sup> mice (Fig. 5C).

Discussion

It has been reported that AM plays important roles in cardiac and renal function in addition to blood pressure regulation (7, 8). Plasma levels of AM peptide are increased in hypertensive animal models, which may indicate that AM partici-

pates in a mechanism to counteract high blood pressure (19). In addition, increased levels of locally synthesized and systemic plasma AM may also compensate for or prevent damage in target tissues, such as the heart and kidney, as well as in the vasculature (20–22). We previously showed that transgenic mice overexpressing AM developed significantly lower blood pressure compared with their wild-type littermates (23). After induction of lipopolysaccharide shock, AM transgenic mice exhibited a smaller decline in blood pressure, less severe organ damage, and higher 24-h survival rates. Taken together, these results indicate that the enhancement of systemic and/or local AM systems may play an important role in the attenuation of cardiovascular and renal damage.

We applied an Ang II-infusion model using AM knockout mice to further evaluate in vivo function in the presence of cardiorenal diseases. Continuous Ang II infusion has been shown to cause cardiac hypertrophy and renal dysfunction in animal models (24, 25). In the basal state in the present study, there was no significant difference in the ratios of heart and kidney weight-to-body weight between AM<sup>+</sup>/<sup>-</sup> mice and their wild-type littermates. In contrast, Ang II infusion induced more marked hypertrophic responses, including increases in heart weight and left ventricle wall thickness in
AM+/+ mice compared with wild-type mice. This result clearly indicates that AM is critically involved in the development of cardiac hypertrophy.

However, the mechanism by which AM is involved in the prevention of cardiac hypertrophy is still unclear. We have reported that AM protects renal tissues from ischemia/reperfusion injury through its nitric oxide (NO)-releasing activity, based on the finding that the increase in severity of renal damage observed in AM+/− mice relative to wild-type mice was canceled when they were pretreated with Nω-nitro-l-arginine methyl ester (L-NAME), an inhibitor of NO synthase (20). In contrast, the preventive effect of AM against cardiac hypertrophy was not likely to have been mediated by NO in this study. In our preliminary study, treatment of L-NAME (dissolved in the drinking water at a concentration of 200 mg/l) could not cancel the difference of

Fig. 3. Comparison of (A) intraventricular septum thickness (IVST) and (B) ejection fraction (EF). Data are shown as the mean ± SD; n = 18 for each group. Open column, AM+/+; closed column, AM+/−. * p < 0.05; ** p < 0.01; n.s., not significant. (C) Representative charts of transthoracic M-mode echocardiograms of Ang II-infused mice.

Fig. 4. Effects on (A) urine volume and (B) water intake by Ang II infusion. Data are shown as the mean ± SD. Open column, AM+/+; closed column, AM+/−. * p < 0.05; ** p < 0.01; n.s., not significant.
AM gene delivery improved renal function (studies have also reported that exogenous AM infusion or flow, urine flow, and urinary sodium excretion of AM induced dose-dependent increases in renal blood flow and water intake were significantly lower in the AM +/+ mice compared with the wild-type mice after Ang II infusion. Furthermore, Ccr was significantly lower, and glomerular injury score was significantly higher, in AM +/+ mice. It has been reported that intrarenal infusion of AM induced dose-dependent increases in renal blood flow, urine flow, and urinary sodium excretion (29). Previous studies have also reported that exogenous AM infusion or AM gene delivery improved renal function (30–32). Our present results demonstrate that endogenous AM actually plays critical roles in the prevention of renal damage. In addition, AM may have beneficial hemodynamic effects and may promote maintenance or improvement in renal function.

Proadrenomedullin N-terminal 20 peptide (PAMP) has been shown to inhibit catecholamine release from synthetic nerve endings and adrenal medullary cells (34). However, the role of PAMP in the heart and kidney remains unclear and should be further investigated. CGRP and AM share common structural characteristics and receptors and belong to the same peptide family. We have also established CGRP -/- mice (35). While AM -/- mice did not survive beyond midgestation, CGRP -/- mice survived until adulthood and demonstrated increased sympathetic activity. Thus, targeted disruption of AM and CGRP genes reveals their distinct biological roles (36).

In this study, we have demonstrated that AM reduction induced severe cardiac hypertrophy and renal damage under stress, suggesting that AM may play important roles in safeguarding against organ damage, and providing evidence for the potential use of AM as a candidate for the treatment of cardiovascular and renal diseases.

**Fig. 5.** (A) Pathological changes of the glomerulus after Ang II infusion. (B) Glomerular injury score and (C) creatinine clearance (Ccr) after Ang II infusion. Open column, AM +/+ ; closed column, AM +/-. Data are shown as the mean ± SD. *p < 0.05.

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**References**