Protective Effects of Endogenous Adrenomedullin on Cardiac Hypertrophy, Fibrosis, and Renal Damage

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Background—Adrenomedullin (AM) is a novel vasodilating peptide thought to have important effects on cardiovascular function. The aim of this study was to assess the activity of endogenous AM in the cardiovascular system using AM knockout mice.

Methods and Results—Mice heterozygous for an AM-null mutation (AM+/−) and their wild-type littermates were subjected to aortic constriction or angiotensin II (Ang II) infusion. The resultant cardiovascular stress led to increases in heart weight/body weight ratios, left ventricular wall thickness, and perivascular fibrosis, as well as expression of genes encoding angiotensinogen, ACE, transforming growth factor-β, collagen type I, brain natriuretic peptide, and c-fos. In addition, renal damage characterized by decreased creatinine clearance with glomerular sclerosis was noted. In all cases, the effects were significantly more pronounced in AM+/− mice. Hearts from adult mice subjected to aortic constriction showed enhanced extracellular signal-regulated kinase (ERK) activation, as did cardiac myocytes from neonates treated acutely with Ang II. Again the effect was more pronounced in AM+/− mice, which showed increases in cardiac myocyte size, protein synthesis, and fibroblast proliferation. ERK activation was suppressed by protein kinase C inhibition to a greater degree in AM+/− myocytes. In addition, treatment of cardiac myocytes with recombinant AM suppressed Ang II–induced ERK activation via a protein kinase A–dependent pathway.


Key Words: peptides ■ angiotensin ■ cardiovascular diseases ■ hypertrophy ■ kidney

Adrenomedullin (AM) is a vasodilator peptide originally isolated from the extract of human pheochromocytoma. It has also been shown to be produced in vascular endothelial and smooth muscle cells and in cardiac myocytes, and high levels are found in the heart, lung, kidney, and adrenal medulla. In addition to its ability to reduce vascular tone, it stimulates myocardial infarction, and renal dysfunction. This led us to hypothesize that AM serves as a local paracrine or autocrine modulator of cardiac remodeling.

Cardiac hypertrophy, which is recognized in many cardiovascular diseases, is an independent risk factor of cardiac morbidity and mortality and can be induced by mechanical stress and by humoral factors such as angiotensin II (Ang II). Several studies have suggested that application of exogenous AM suppresses the development of cardiac hypertrophy and renal damage. Moreover, mechanical stretch causes AM production in cardiac myocytes, whereas Ang II stimulation causes its production in cardiac fibroblasts, which suggests that AM serves as a local paracrine or autocrine modulator of cardiac remodeling.

To directly evaluate the function of endogenous AM, we recently generated a strain of AM knockout mice. Homozygous mice (AM−/−) died in utero by embryonic day (E) 13.5, but heterozygotes (AM+/−) survived until adulthood. They did exhibit reduced AM expression and elevated blood pressure, however. The aim of the present study was to use this model to examine the effect of endogenous AM expression on cardiac hypertrophy, fibrosis, and renal damage.

Methods

Generation of AM Knockout Mice

AM gene targeting was performed as described previously. At 12 weeks of age, male mice were used in the experiments. All experiments were performed in accordance with the Declaration of
Helsinki and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Pressure Overload Produced by Aortic Constriction

Pressure overload was produced by constriction of the abdominal aorta as described previously.29 Thirty minutes, 2 hours, or 28 days after ligation, the hearts were removed, weighed, and subjected to further analysis.

Ang II Infusion

Ang II was infused for 14 days into mice with a subcutaneously implanted osmotic minipump (Alzet Co). The delivery rate was 3.2 mg · kg⁻¹ · d⁻¹. To control for the effect of the elevated basal blood pressure on cardiac hypertrophy in AM+/+ mice, an antihypertensive drug, hydralazine hydrochloride, was dissolved in the drinking water of some AM+/− mice. This led to a daily intake of 5 mg · kg⁻¹ · d⁻¹, which was sufficient to eliminate the difference in blood pressure between AM+/− and wild-type mice.

In our knockout mice, proadrenomedullin N-terminal 20 peptide (PAMP), another product of the proadrenomedullin gene, is also deleted. To evaluate the effect of that deletion in the present study, in some mice PAMP was also infused at a rate of 20 ng/h along with Ang II.

Cell Culture of Cardiac Myocytes

Cardiac myocytes were harvested from 1-day-old neonatal mice and rats as described previously.20 The cells were cultured for 24 hours, after which the medium was replaced with a complete serum-free medium for 24 hours. The cells were then incubated for an additional 24 hours, with or without 1 µmol/L Ang II. After Ang II treatment, myocyte surface area was measured, and protein synthesis was assayed by measuring ³H-phenylalanine incorporation (see below).

For Western blot analysis, cardiac myocytes from AM+/− or wild-type mice were incubated for 30 minutes with or without 1 µmol/L H7 (Seikagaku Co), an inhibitor of protein kinase C (PKC), and then treated with 1 µmol/L Ang II for 8 minutes. Cardiac myocytes from rat were incubated with 300 nmol/L human recombinant AM with or without 1 µmol/L H89 (Seikagaku Co), an inhibitor of protein kinase A (PKA), and then treated with Ang II.

Incorporation of ¹⁴C-Phenylalanine

Protein synthesis by cardiac myocytes was assayed by measuring ¹⁴C-phenylalanine incorporation (see below). For Western blot analysis, cardiac myocytes from AM+/− or wild-type mice were incubated with 1 µmol/L Ang II and 0.4 µCi/mL ¹⁴C-Phe for 24 hours, they were fixed with 10% TCA. Radioactivity incorporated into the TCA-precipitated material was determined by liquid scintillation counting after solubilization in 1 N NaOH.

Cell Culture of Cardiac Fibroblasts

Cardiac fibroblasts isolated from neonates were cultured for 24 hours, and the serum was starved for 72 hours, after which 1 µmol/L Ang II was added for 48 hours and cell proliferation was quantified with a cell counter (Coulter).

Echocardiographic Analysis

Echocardiography was performed with an HP Sonos 100 (Hewlett-Packard Co) with a 12-MHz imaging transducer as described previously.20,21 Intraventricular septum thickness, left ventricular diastolic diameter, and left ventricular systolic diameter were measured. Ejection fraction (EF) was calculated by the cubed method with the formula EF=[(LVEDD)³−(LVESD)³]/LVEDD³×100 (%).

Histological Analysis

In the heart, perivascular fibrotic area/vascular area ratios were calculated in the sections of left ventricle. The cross-sectional area of cardiac myocytes was also measured. In the kidney, glomerular sclerosis scores were assigned as follows: 0, no changes; 1, lesions involving <25% of the capillary tuft; 2, lesions involving 25% to 49% of the capillary tuft; 3, lesions involving 50% to 75% of the capillary tuft; 4, lesions involving >75% of the capillary tuft.

Statistics

Data are expressed as mean±SEM. Differences within groups were compared with the Fisher protected least significant difference test and Student t test. Values of P<0.05 were considered significant.

Results

Characterization of AM+/-

Targeted null mutation of the AM gene is lethal in utero.18 AM+/- mice, by contrast, survived until adulthood and were apparently normal, although levels of cardiac and renal AM mRNA and protein were reduced to approximately half that seen in their wild-type (AM+/+) littermates (Figures 1A and 1B). Plasma cAMP levels were also lower in AM+/- (Figure 1C).

Cardiac Hypertrophy Induced by Pressure Overload or Continuous Ang II Infusion

The survival rate after aortic constriction was lower among AM+/- mice (Figure 2A). In particular, the death rate
during the first week after the surgery was markedly higher among AM+/− mice (AM+/− 38% versus AM+/+ 18%, P<0.05). An additional 9% of the AM+/− mice died between weeks 2 and 4 after surgery; no wild-type mice died during this period. Accompanying the higher death rate in AM+/− mice were increased levels of BNP gene expression, which has been positively correlated with cardiac dysfunction22 (Figure 2B).

Transthoracic echocardiography was performed to visualize the changes in cardiac morphology and function. After aortic constriction, the ventricular wall was thicker in AM+/− mice (intraventricular septum thickness: AM+/+ 1.067±0.02 mm versus AM+/− 1.164±0.02 mm; P<0.01; Figures 3A and 3B). Although left ventricular diastolic diameter was wider in AM+/− mice in the sham-operated group, the difference was offset by the wall thickening after aortic constriction. Ejection fraction was smaller in AM+/− mice (intraventricular septum thickness: AM+/+ 0.02 mm versus AM+/− 0.05). An additional 9% of the AM+/− mice showed greater cross-sectional lengths (Figure 4B). Although heart weight/body weight ratios were increased in both strains, the change was more prominent in AM+/− mice (Figure 4C).

Hydralazine treatment did not affect the cardiac hypertrophy observed in Ang II−treated AM+/− mice (Figure 4D). This suggests that the cardiac hypertrophy seen in Ang II−treated AM+/− mice is a direct effect of AM reduction in the heart rather than an indirect effect of hemodynamic changes. PAMP treatment also failed to affect the cardiac hypertrophy seen in Ang II−treated mice, which indicates that PAMP deletion did not play a significant role in the observed responses (Figure 4D).

Perivascular fibrosis was also more severe in AM+/− mice (Figure 4E). In both banded and Ang II−infused mice, perivascular fibrotic area/vascular area ratios were significantly higher in AM+/− mice (Figure 4F), which indicates that stress−induced proliferation of fibroblasts was enhanced.

**Histological Analysis of the Heart After Aortic Constriction and Ang II Infusion**

Cardiac hypertrophy induced by aortic constriction or Ang II infusion was exacerbated in AM+/− mice (Figure 4A). In addition, cardiac myocytes from AM+/− mice showed significantly higher ratios after aortic constriction or Ang II infusion. D, Heart weight/body weight ratios in AM+/− mice after 14 days of Ang II infusion. Both PAMP and hydralazine hydrochloride (HYD) treatment failed to reduce cardiac hypertrophy in Ang II−infused AM+/− mice. E, Accumulation of perivascular collagen (blue stained in Masson trichrome staining) in heart. F, Perivascular fibrotic area (PVF)/vascular area (VA) ratios in mice subjected to aortic constriction or Ang II infusion. B, C, and D, Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively. **P<0.01.

**Figure 3.** M-mode echocardiograms. A, Analysis of cardiac morphology and function after 28 days of aortic constriction in AM+/− (n=14) and AM+/+ mice (n=18). Fisher protected least significant difference was used for statistical analysis. IVST indicates intraventricular septum thickness (mm); LVDd, left ventricular diastolic diameter (mm); LVDs, left ventricular systolic diameter (mm); and EF, ejection fraction (%). Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively. *P<0.05; **P<0.01. B, Representative transthoracic M-mode echocardiograms after aortic constriction. Top panels show recordings from sham-operated animals, bottom panels show recordings from treated animals.

**Figure 4.** Histological examination of heart. A, Transverse sections of heart at papillary muscle level after 28 days of aortic constriction or 14 days of Ang II infusion. B, Cross-sectional lengths of cardiac myocytes. C, Heart weight/body weight ratios. AM+/− mice showed significantly higher ratios after aortic constriction or Ang II infusion. D, Heart weight/body weight ratios in AM+/− mice after 14 days of Ang II infusion. Both PAMP and hydralazine hydrochloride (HYD) treatment failed to reduce cardiac hypertrophy in Ang II−infused AM+/− mice. E, Accumulation of perivascular collagen (blue stained in Masson trichrome staining) in heart. F, Perivascular fibrotic area (PVF)/vascular area (VA) ratios in mice subjected to aortic constriction or Ang II infusion. B, C, and F, Open and filled columns represent mean±SEM of AM+/+ and AM+/− mice, respectively. **P<0.01.
Stress-Induced Changes in Cardiac Gene Expression

Hypertrophic responses are thought to involve programmed expression of specific sets of genes. After 28 days of aortic constriction, expression of angiotensinogen, ACE, transforming growth factor-β (TGF-β), and collagen type I genes was upregulated in the heart, and the effect was more pronounced in AM+/− mice (Figure 5A). At the same time, expression of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA)-2 was downregulated to a greater degree in AM+/− mice (Figure 5B). Thus, pressure overload caused upregulation of the angiotensin system and other growth factors, which in turn led to cardiac hypertrophy and fibrosis and was associated with cardiac dysfunction in AM+/− mice.

We next analyzed the expression of c-fos, an early-response gene, the upregulation of which is closely associated with cardiac hypertrophy. As expected, expression of c-fos was upregulated to a greater degree in AM+/− mice than in wild-type mice within 120 minutes (Figure 5C).

AM Suppresses Cardiac Myocyte Hypertrophy and Fibroblast Growth

When Ang II was administered to the cardiac myocytes from AM+/− mice, we observed enlargement of cardiac myocytes (Figures 6A and 6B) that was accompanied by increases in protein synthesis (Figure 6C). Ang II stimulation also enhanced proliferation of fibroblasts isolated from AM+/− mice (Figure 6D) and enhanced collagen type I gene expression (Figure 6E).

AM-Induced Changes in Extracellular Signal-Regulated Kinase Activation

The Ras-extracellular signal-regulated kinase (ERK) pathway plays a key role in the induction of early-response genes like c-fos during pressure overload. We found ERKs to be activated within 30 minutes after the onset of aortic constriction, and the level of activation was much greater in AM+/− mice (Figure 7A). Treatment with 1 μmol/L Ang II increased ERK phosphorylation in both strains, but the effect was greater in AM+/− mice (Figure 7B). By contrast, the PKC inhibitor H7 suppressed ERK activation in both strains, and the inhibitory effect was more pronounced in AM+/− mice (Figure 7C), which suggests PKC activity is enhanced by Ang II in AM+/− mice.

Using rat cardiac myocytes, we confirmed the ability of Ang II to elicit prompt ERK activation and found the effect to be antagonized by exogenous administration of recombinant AM (Figure 7D). Moreover, the inhibitory effect of AM was attenuated by H89, an inhibitor of PKA (Figure 7D). Taken together, these findings suggest that ERK is activated in Ang II–stimulated cardiac myocytes, that AM attenuates this activation, and that the effect of AM is mediated by both activation of PKA and suppression of PKC.
Renal Damage Induced by Continuous Ang II Infusion

After continuous infusion of Ang II, creatinine clearance was lower in AM+/− mice (Figure 8D), and morphological examination revealed much greater damage to the kidneys of AM+/− mice (Figure 8A), which had significantly higher glomerular injury scores (Figure 8B). Increased expression of TGF-β was also more prominent in AM+/− mice (Figure 8C).

Discussion

Using AM knockout mice, we have been able to make 3 key observations: (1) that reduced expression of endogenous AM exacerbates the cardiac hypertrophy and fibrosis caused by pressure overload (aortic constriction) or Ang II infusion, leading to diminished cardiac function; (2) that reduced AM expression exacerbates the renal damage caused by Ang II infusion; and (3) that AM suppresses development of cardiac hypertrophy via suppression of ERK activation.

In the present study, we found that although the respective heart, kidney, and body weights were similar in AM+/− and wild-type mice, cardiac and renal expression of AM in AM+/− mice was only approximately half that seen in wild-type mice. Moreover, AM+/− mice exhibited wider left ventricular diastolic diameter than wild-type mice, even under basal conditions, which suggests that AM is necessary for proper development of the heart.

The differences between AM+/− and wild-type mice became much more apparent when stress was applied to the cardiovascular system. Compared with wild-type mice, AM+/− mice exhibited lower survival rates, which were accompanied by higher cardiac brain natriuretic peptide levels; higher heart weight/body weight ratios; greater left ventricular wall thickening; and reprogramming of specific cardiac genes. In particular, downregulation of the SERCA2 gene, which is associated with cardiac dysfunction, was more apparent in the hearts of AM+/− mice. Using cultured cardiac myocytes from AM+/− mice, we confirmed directly that these cells show more pronounced hypertrophic responses to stressful stimuli. AM+/− mice also exhibited more extensive perivascular fibrosis, and fibroblasts isolated from AM+/− mice showed faster proliferation and enhanced collagen type I gene expression. Taken together, these results suggest that endogenous AM serves as an autocrine or paracrine factor to exert a number of crucial protective effects when the cardiovascular system comes under stress. Consistent with our findings, Caron et al recently reported hypertrophic ventricular trabeculae in AM−/− mice, whereas Shimozawa et al reported that administration of Ang II with a high-salt diet produced marked perivascular fibrosis in AM−/− mice.

The findings of the present study are also suggestive of the mechanism by which AM exerts its antihypertrophic effects. Because ERKs are known to play a key role in the development of cardiac hypertrophy, we examined the extent to which AM-mediated intracellular signaling affected transduction in the ERK. In the hearts of adult mice subjected to pressure overload, as well as in neonatal cardiac myocytes isolated from mice that received acute infusion of Ang II, activation of ERKs was more pronounced in AM+/− mice. AM was originally identified as a peptide that increases cAMP levels in platelets. Subsequent experiments in mesangial cells yielded results similar to those reported herein; AM increased cAMP levels, which led to a PKA-dependent decrease in cell proliferation that was associated with decreased ERK activity. It was also shown that delivery of the AM gene attenuates cardiac hypertrophy via production of cAMP and that AM cAMP-dependently inhibited DNA synthesis and collagen production in cardiac fibroblasts.
the present study, PKC inhibition suppressed ERK activation to a greater degree in AM+/− cardiac myocytes. Thus, AM appears to inhibit the development of cardiac hypertrophy, at least in part, by suppressing ERK activation via activation of PKA and inhibition of PKC.

In conclusion, using AM knockout mice, we showed that endogenous AM plays an important role in guarding against cardiovascular damage. We also provide evidences of the potential utility of AM in the treatment of cardiovascular and renal diseases.

References

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