Adrenomedullin, an Endogenous Peptide, Counteracts Cardiovascular Damage

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Background—Adrenomedullin (AM), a potent vasodilator peptide, is produced by posttranslational splicing of proadrenomedullin together with proadrenomedullin N-terminal 20 peptide (PAMP), another hypotensive peptide. Although both AM and PAMP have the potential not only to decrease blood pressure but also to protect organs from damage, there is no direct evidence for their individual physiological roles in vivo.

Methods and Results—Using knockout mice with the disruption of AM peptide alone, we investigated the organ-protective effect of AM. Although the AM−/− mutation in mice was embryonic lethal without any apparent phenotypic changes, AM+/− mice were viable and fertile; plasma and organ AM concentrations were almost half of those in AM+/+ mice. With the administration of angiotensin II (Ang II) on a high-salt diet for 12 days, marked perivascular fibrosis and intimal hyperplasia were found in coronary arteries of Ang II/salt–treated AM+/− mice, without the AM upregulation that was observed in Ang II/salt–treated AM+/+ mice. In AM−/− mice, Ang II/salt loading increased both urinary excretion of 8-hydroxydeoxyguanosine and isoprostane, markers of oxidative stress. Consistently, immunostaining of both p67phox and gp91phox, subunits of NAD(P)H oxidase and 3-nitrotyrosine, the metabolites of reactive oxygen species (ROS), and the generation of ROS measured by electron spin resonance spectroscopy apparently increased in the Ang II/salt–treated heart. These data suggested that the overproduction of oxidative stress might be involved in the cardiovascular changes induced by Ang II/salt loading.

Conclusions—The evidence presented supports the hypothesis that endogenous AM possesses a protective action against cardiovascular damage, possibly through the inhibition of oxidative stress production. (Circulation. 2002;105:106-111.)

Key Words: peptides ■ angiotensin ■ genes ■ oxygen ■ stress

Adrenomedullin (AM) was originally isolated from pheochromocytoma cells, but it is also produced in and secreted by vascular endothelial cells.1 The vasodilator action of AM, which causes hypotension, is evident from its ability not only to elevate intracellular cAMP in vascular smooth muscle cells but also to increase nitric oxide production in endothelial cells.2–4 The DNA sequence encoding the precursor of AM, proadrenomedullin, has been identified in human as well as rat and mouse tissue.5,6 The first paired basic amino acid of this precursor (Lys43-Arg44) is a representative site for proteolytic cleavage, yielding the other product now called proadrenomedullin N-terminal 20 peptide (PAMP), which also has a hypotensive effect;7 however, these 2 peptides from the same precursor seem to control blood pressure through different mechanisms. Our previous studies suggested that PAMP reduces norepinephrine overflow from peripheral sympathetic nerve endings of mesenteric arteries, which in turn decreases blood pressure.8,9

In addition to controlling blood pressure, a growing body of evidence indicates that AM possesses many important physiological and pathophysiological properties.3,4 A recent study showed that both PAMP and AM gene disruption caused embryonic lethality in midgestation due to hydrops fetalis and cardiovascular defects.10 This finding suggests that either AM, PAMP, or both play a pivotal role in fetus development. In the clinical setting, plasma AM concentration was increased in patients with cardiovascular diseases, such as congestive heart failure, hypertension, septic shock, and preeclampsia, which are often associated with increased plasma concentrations of renin and angiotensin II (Ang II).3 AM seems to have the potential to protect organs from damage, because AM supplementation was effective in im-

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proving hemodynamics in patients with congestive heart failure and hypertension.\textsuperscript{11,12}

To elucidate the plausible role of AM as an endogenous organ-protective agent, in the present study we generated target-gene–disrupted mice of AM peptide alone. Supplementation with Ang II on a high-salt diet has been reported as an animal model of severe organ damage, possibly through increased oxidative stress.\textsuperscript{13–15} In contrast to Ang II, AM is reported to inhibit not only smooth muscle cell proliferation and migration\textsuperscript{16,17} but also oxidative stress formation.\textsuperscript{18} We have studied the effect of Ang II/salt loading on cardiovascular changes and oxidative stress production in AM-deficient mice. The results of the present study showed that AM deficiency could aggravate cardiovascular damage induced by oxidative stress. It also suggests that AM may be an endogenous organ-protective substance counteracting oxidative stress on the cardiovascular system.

**Methods**

**Generation of AM-Null Mice**

Mouse AM genomic DNA was isolated by screening a TT2 ES cell genomic library with an AM complementary DNA probe. The targeting vector was generated by insertion of a PGK-Neo selection marker in conjunction with loxP in intron 2 of the AM genome and a stop mutation at the beginning of AM coding region. TT2 ES cells were electroporated with the linearized vector and selected with neomycin. Targeted clones were confirmed by Southern blot analysis of 3 independent ES clones. Genomic DNA from wild-type TT2 ES cells (WT) and homologous targeted clones was digested with EcoRI and probed with an external probe (probe A; a). Wild-type (+) and mutant (−) loci generated 7.2-kb and 1.5-kb fragments, respectively. Single integration of targeting vector was confirmed by probe NEO. c, Southern blot analysis of genomic DNA from offspring of AM\textsuperscript{−/−} intercrosses. Genotyping was performed with probe A as described in b. d. Genotypes of embryos from intercrosses between AM\textsuperscript{−/−} were determined by PCR using primers P1 and P2 as indicated in a. A 380-bp PCR fragment predicted mutated AM locus, whereas a 300-bp PCR fragment was generated from wild-type AM locus.
Drug Treatment
Animals were maintained ad libitum. Ang II (Sigma) was administered by osmotic minipump (Alza) at a dose of 640 ng·kg⁻¹·min⁻¹ i.p. for 12 days. During the 12-day experimental period, a high-salt diet (8% NaCl) or regular-salt diet (0.66% NaCl) was given, and free access to water was allowed.

Measurement of Blood Pressure
Before and after the osmotic minipump had been implanted, mouse systolic blood pressure was measured by the tail-cuff method every other day in conscious and unrestrained status. Twelve days after surgery, mice were anesthetized with pentobarbital (1 g/kg body wt i.p.), the left carotid artery was isolated, and a PE-10 tubing catheter was inserted into the artery, secured with silk, and tunneled subcutaneously to exit at the back of the neck. Mice were allowed to recover from surgery for ≥6 hours. The arterial line was connected to a pressure transducer (model TP-200T, Nihon-Kohden), and systolic blood pressure and heart rate were measured with a computerized data acquisition system.

Histopathology and Immunofluorostaining
Heart, kidneys, and brain from mice were fixed in PBS containing 4% paraformaldehyde for 6 to 12 hours at 4 °C, dehydrated, embedded in paraffin, and cut into sections 4 µm in thickness. These were stained with hematoxylin and eosin. Hearts were stained by Azan staining to distinguish fibrotic changes from smooth muscle cells. For immunofluorostaining of p67phox and gp91phox, components of NAD(P)H oxidase, 20 we applied the following method. Hearts were cryoprotected in 20% sucrose in PBS and embedded in Tissue-Tek OCT compound (Sakura Fintek). Frozen sections were cut 4 µm thick, fixed in cold acetone, air-dried, washed, and blocked with 1% goat serum for 30 minutes at room temperature. The sections were stained with rabbit anti-mouse p67phox or gp91phox (Upstate Biotechnology), diluted 200 and 100 times, respectively, at 37 °C for 1 hour. After being washed with PBS, the sections were incubated in the dark for 45 minutes at room temperature with secondary antibodies, mixtures of FITC-conjugated anti-rabbit IgG (diluted 100 times) for either p67phox or gp91phox and rhodamine-conjugated phalloidin (Wako Chemical) for F-actin. Sections were washed with PBS and mounted. Sections were examined by fluorescence microscopy (MRC-1000, Bio-Rad), FITC- and rhodamine-labeled samples were excited by the 488 and 540 laser lines of the krypton/argon ion laser (model 5470K; Ion Laser Technology), respectively. Three different samples were examined for each mouse.

For immunohistochemistry of 3-nitrotyrosine, paraffin-embedded heart samples were used. After removal of paraffin and rehydration, slides were treated with 10 mmol/L citric acid (pH 6.0). Tissue sections were heated with microwaves, and nonspecific binding was blocked with 10% normal goat serum (pH 7.4) for 30 minutes before incubation with anti-nitrotyrosine antibody (Upstate Biotechnology) with 1% BAS for 1 hour at room temperature. Tissue sections were then incubated for 45 minutes at room temperature with a biotinylated anti-rabbit IgG secondary antibody with the Vectastain ABC kit (Vector). Diaminobenzidine substrate was used to visualize positive immunoreactivity.

Measurement of 8-Hydroxydeoxyguanosine and 8-Iso-Prostaglandin F₂α (Isoprostane)
Mice were placed in metabolic cages (KN-645, Natsume Co Ltd) to collect urine for a 24-hour sample for 3 consecutive days from 10 days after osmotic pump implantation, and urine samples were kept frozen until the assay. Urinary excretions of creatinine were comparable among groups (AM°+/°− with Ang II/salt 46.1±8.0 mg/d, AM°+/°− with Ang II/salt 43.3±4.5 mg/d, AM°+/°− with Ang II/salt 39.3±9.3 mg/d, AM°+/°− with Ang II/salt 49.3±8.9 mg/d). 8-Hydroxydeoxyguanosine (8-OHdG) was measured by ELISA according to the manufacturer’s instructions (Nikken Food). Isoprostane was measured by electroimmunoassay according to the manufacturer’s instructions (Assay Designs, Inc). For measurement of isoprostane, urine samples were stored separately with 10 µg/mL of indomethacin to avoid de novo synthesis of prostaglandins. The samples were diluted 100 times. Total excretion of 8-OHdG (ng/d) and isoprostane (pg/d) were calculated.

Quantification of Reactive Oxygen Species
We measured ·O₂⁻ by electron spin resonance (ESR) spectroscopy with the nitroxide radical hydroxy-TEMPO as a spin probe. 21 The ESR was set as follows: a microwave power of 10 mW, a range of external magnetic field of 10 mT, and a scan rate of 1 mT/s, and measurements were performed at room temperature with an ESR spectrometer (JES-FA-100; JEOL). Samples were homogenized in PBS containing protease inhibitors. The homogenate was immediately reacted with hydroxy-TEMPO (0.1 mmol/L), and its ESR spectra were recorded every 2 minutes. The peak heights of the ESR spectra of hydroxy-TEMPO were measured, and there was a linear relation in the semilogarithmic plot of peak signal intensity versus time. The rate of signal decay was calculated from the slope of this line.

Measurement of AM
As in the previous report, 22 with polyclonal antibodies, AM and PAMP concentrations were measured in the lung, adrenal gland, heart, and plasma. The concentrations in the lung, heart, and adrenal gland were standardized with their weight (grams).

Statistical Analysis
All values were expressed as mean±SEM. Comparisons among groups were made by ANOVA followed by Scheffé’s method, and probability values of P<0.05 were considered to indicate statistical significance.

Results
Generation and Characterization of AM-Knockout Mice
To investigate AM function, we developed AM peptide–disrupted mice with normal PAMP production. To disrupt only the AM gene without affecting PAMP, we inserted a stop mutation at the starting point of the AM coding sequence of exon 4 following the strategy outlined in Figure 1a. The disruption of AM gene alleles was confirmed by the absence of immunoreactive AM in the fetus. The AM°− was a lethal mutation in the mice; however, AM°+/− mice were both viable and fertile. Their AM levels in organs and plasma were almost as half those of AM°+/° mice (Table 1). Their PAMP levels in plasma and other organs were comparable to those of AM°+/° mice (Table 1).

| Table 1. Baseline Contents of AM and PAMP in the Lung, Adrenal Gland, and Plasma |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | AM (fmol/g)     | PAMP (fmol/g)   | AM (fmol/g)     | PAMP (fmol/g)   | AM (fmol/g)     | PAMP (fmol/g)   |
| Lung            | 22.61±0.37      | 36.30±7.01      | 35.30±8.55      | 29.60±3.43      | 75.23±8.97      | 10.42±2.55      |
| Adrenal Gland   | 10.33±0.21      | 15.32±1.94      | 17.27±9.61      | 22.02±6.62      | 130.34±33.08    | 12.51±3.61      |

AM and PAMP were measured as described in the Methods section. Data are expressed as mean±SEM. Comparisons among groups were made by ANOVA followed by Scheffé’s method. *P<0.01.
Ang II and Salt Loading

We assessed blood pressure, tissue AM contents, oxidative stress formation, and histological changes with Ang II/salt loading in AM−/− and AM+/+ mice. Eight-week-old male mice were treated with Ang II loading on a high-salt diet for 12 days. As shown in Figure 2, baseline blood pressure did not differ between AM−/− and AM+/+ mice. Blood pressure was gradually elevated by treatment with Ang II and high-salt diet in both AM−/− and AM+/+ mice and similarly reached significantly greater levels on day 6 than in sham-operated mice. By direct method, systolic blood pressure was also higher in Ang II/salt–loaded mice than sham-operated mice; systolic blood pressure did not differ significantly between AM−/− and AM+/+ mice with Ang II/salt loading (Table 2). Inflammatory changes were time-dependently deteriorated at the perivascular area of the coronary artery in Ang II/salt–loaded AM−/− mice and AM+/+ mice (Figure 3, a, c, and e). In severe cases, coronary arteries showed fibrinoid necrosis. No such changes were observed in kidney or brain (data not shown).

Recent studies showed that Ang II–induced vascular damage was mediated by oxidative stress,15,23–26 and thus, we studied whether oxidative stress plays a role in coronary damage in AM+/+ mice. Urinary excretions of 8-OHdG and isoprostane, which are markers for oxidative stress,27,28 were significantly higher in AM+/+ mice with Ang II/salt loading than in AM+/− mice without Ang II/salt loading, but Ang II/salt loading did not significantly elevate urinary 8-OHdG and isoprostane in AM+/− mice.

Table 2. Systolic Blood Pressure, Urinary Excretion of Oxidative Markers, ROS Production, and AM Concentration in the Heart of AM+/− and AM+/+ Mice With and Without Ang II/Salt Loading

<table>
<thead>
<tr>
<th>Ang II/Salt Loading</th>
<th>− (n=14)</th>
<th>+ (n=16)</th>
<th>− (n=15)</th>
<th>+ (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>103±7</td>
<td>171±5*</td>
<td>108±8</td>
<td>159±10*</td>
</tr>
<tr>
<td>8-OHdG, ng/d</td>
<td>44±6</td>
<td>58±2</td>
<td>45±5</td>
<td>80±6†</td>
</tr>
<tr>
<td>8-iso-prostaglandin F2α, pg/d</td>
<td>166±72</td>
<td>237±74</td>
<td>205±69</td>
<td>512±83‡</td>
</tr>
<tr>
<td>AM concentration in heart, fmol/g</td>
<td>1.26±0.52§</td>
<td>2.97±0.46‡§</td>
<td>0.69±0.35</td>
<td>0.67±0.34</td>
</tr>
</tbody>
</table>

Systolic blood pressures were directly measured 12 days after drug treatments. 8-OHdG, 8-iso-prostaglandin F2α, and AM were measured as described in the Methods section. Data are expressed as mean±SEM. Comparisons among groups were made by ANOVA followed by Scheffé’s method.

*P<0.01 and †P<0.05 vs without Ang II/salt loading; ‡P<0.01 vs AM+/− with Ang II/salt loading; §P<0.01 vs equally Ang II/salt-loaded AM+/−.
In the present study, we showed that endogenous AM could protect coronary artery injury by Ang II/salt loading. The cardiovascular protective action of AM may be partly based on the inhibition of Ang II effects of inducing oxidative stress and cell proliferation.

AM−/− mice showed almost half the level of AM in plasma, kidney, and other organs compared with AM+/+ mice, but their baseline blood pressures were comparable. No significant differences in baseline blood pressure were found between 10-month-old AM−/− and AM+/+ mice (data not shown). The present finding of comparable baseline blood pressures in AM+/+ and AM−/− mice are consistent with the results of the previous studies using the technique of gene disruption, indicating that the disruption of vasoactive factors, such as angiotensinogen, atrial natriuretic peptides, and nitric oxide synthase genes, could change blood pressure significantly in homozygotes but did not affect blood pressure in heterozygotes.29 Unfortunately, AM−/− mice were embryonic lethal. Therefore, we could not evaluate the role of AM per se in the regulation of blood pressure.

A recent study showed that when both PAMP and AM were disrupted, the mutation was lethal to the embryo.10 They reported that hydrops fetalis and cardiovascular abnormalities cause lethality by E14.5. The present study revealed that AM disruption caused embryonic lethality at the same embryonic age, E14.5 to E15.5, but only mild subcutaneous edema appeared without hydrops or cardiovascular abnormalities (data not shown). This discrepancy could be attributed to the different methods used to disrupt genes; the former study disrupted both PAMP and AM, and we disrupted only AM, with normal PAMP production.

We and others have demonstrated that Ang II and salt loading is a model of hypertension associated with severe organ damage in rats.13,14 Then we used this model in AM−/− mice to study organ-protective effects of AM. Despite the comparable elevation of blood pressure, AM−/− mice showed marked coronary artery injuries with Ang II/salt loading compared with AM+/+ mice. In severe cases, moreover, marked arterial intimal hyperplasia associated with luminal occlusions was observed; these changes are similar to atherosclerosis seen in coronary artery disease.30 In contrast to rats, Ang II/salt loading caused little organ damage, including the heart, in the AM−/− mice.23,31 Accordingly, it should be noted that organ injury induced by Ang II/salt might be species-specific, because several investigators reported that Ang II/salt caused organ injury in mice to a lesser degree than in rats, probably through the overproduction of superoxide dismutase.33,34 Nevertheless, Ang II/salt loading could induce severe coronary injury in AM−/− mice that did not have an Ang II–induced increase in cardiac AM production (Table 2). Taken together, these findings suggest that endogenous AM deficiency might cause vascular damage.

Although the precise mechanism for Ang II/salt–induced arterial injury remains to be elucidated, a growing body of evidence suggests that Ang II–induced vascular injury is mediated by oxidative stress, the principal source of which is an NAD(P)H oxidase.15,25,26 Moreover, treatment with superoxide dismutase could inhibit Ang II–induced vascular in-
The present study consistently showed increased oxidative stress in AM–/– mice: (1) increased urinary excretion of 8-OHdG and isoprostane excretion; (2) increased immunostaining of 3-nitrotyrosine at the coronary endothelium as well as p67phox and gp91phox, subunits of NAD(P)H oxidase; and (3) increased cardiac reactive species production as measured by ESR. This suggests that oxidative stress might be involved in Ang II/salt–induced vascular injury. In contrast to Ang II, AM was reported to inhibit the generation of oxygen radical metabolites in cultured mesangial cells and macrophages. In the present study, Ang II/salt loading upregulated AM production in heart in AM–/– mice but not in AM+/+ mice (Table 2). Thus, AM deficiency could aggravate these vascular changes induced by Ang II/salt loading, possibly through the failure to reduce oxidative stress. Moreover, we and others have demonstrated that oxidative stress could increase AM production in vascular endothelial and smooth muscle cells. In Ang II–treated AM–/– mice, Ang II–induced proliferation and migration of vascular smooth muscle cells, possibly by inhibiting oxidative stress production. Taken together, this suggests that AM might be an endogenous organ-protective substance, possibly through the inhibition of free radical formation.

In summary, AM–/– mice showed marked vascular injury concomitant with increased oxidative stress by Ang II/salt loading. Endogenous AM may antagonize Ang II with respect to the generation of oxidative stress and thus result in the protection of the vasculature from atherosclerotic changes.

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