Renoprotective Effect of Chronic Adrenomedullin Infusion in Dahl Salt-Sensitive Rats

Toshio Nishikimi, Yosuke Mori, Naohiko Kobayashi, Kazuyoshi Tadokoro, Xin Wang, Kazumi Akimoto, Fumiki Yoshihara, Kenji Kangawa, Hiroaki Matsuoka

Abstract—The present study was designed to examine whether chronic adrenomedullin infusion has renoprotective effects in hypertensive renal failure and the mechanism by which chronic adrenomedullin infusion exerts its effects. Dahl salt-sensitive rats and Dahl salt-resistant rats were fed a high salt diet starting at 6 weeks of age. Recombinant human adrenomedullin or vehicle was infused for 7 weeks in 11-week-old Dahl salt-sensitive rats. Dahl salt-resistant rat was used as a control. After 7 weeks, untreated Dahl salt-sensitive rats were characterized by decreased kidney function, abnormal morphological findings, increased hormone levels, increased renal tissue angiotensin II levels, and altered mRNA expressions of transforming growth factor β (TGF-β) and components of the renin-angiotensin system compared with Dahl salt-resistant rats. Chronic adrenomedullin treatment significantly improved renal function (serum creatinine −87%, creatinine clearance +114%, urinary protein excretion −59%) and histological findings (glomerular injury score −54%) without changing mean arterial pressure compared with untreated Dahl salt-sensitive rats. Interestingly, long-term human adrenomedullin infusion decreased the endogenous rat adrenomedullin level (−97%) with a slight increase of human adrenomedullin level. Chronic adrenomedullin treatment also significantly inhibited the increase of plasma renin concentration (−269%), aldosterone level (−82%), and renal tissue angiotensin II levels (−60%). Furthermore, adrenomedullin infusion significantly decreased the increases of mRNA expressions of TGF-β (−63%), angiotensin-converting enzyme (−137%), renin (−230%), and angiotensinogen (−38%) in renal cortex. These results suggest that increased endogenous adrenomedullin plays a compensatory role in chronic hypertensive renal failure and that long-term adrenomedullin infusion has renoprotective effects in this type of hypertension model, partly via inhibition of the circulating and renal renin-angiotensin system. (Hypertension. 2002;39:1077-1082.)

Key Words: adrenomedullin ■ hypertension, experimental ■ rats, Dahl ■ renin-angiotensin system ■ transforming growth factors

Adrenomedullin (AM) is a novel vasodilatory peptide originally discovered in human pheochromocytoma tissue.1 Subsequent studies demonstrated that AM is widely distributed in the cardiovascular system, including the kidney, heart, lungs, and blood vessels.1 AM gene transcripts and specific binding sites for this peptide are also present at high levels in the kidney, heart, lungs, and blood vessels.3 Immunohistochemistry of AM in the canine kidney has revealed AM immunoreactivity in glomeruli, cortical distal tubules, and medullary collecting duct cells.3 These results suggest that AM may play a role in the regulation of kidney function. Indeed, intrarenal infusion of AM increased the renal blood flow (RBF) and glomerular filtration rate (GFR) and low-dose intrarenal infusion of AM increased the urinary flow and sodium excretion without changes in GFR.3 These effects are mediated by AM binding to a G-protein–coupled receptor, which increases cytosolic cAMP.4 In fact, AM increases the intracellular cAMP levels in renal tubular cells, mesangial cells, endothelial cells, and vascular smooth muscle cells.5 More recently, investigators reported that AM inhibits proliferation and migration of rat mesangial cells, renal tubular cells, endothelial cells, and vascular smooth muscle cells, probably through a cAMP-dependent mechanism.5 In addition, plasma AM levels are significantly higher in patients with essential hypertension,6 malignant hypertension, and chronic renal failure than in normal subjects.5,7 Taken together, these findings suggest that prolonged elevation of plasma AM levels may have renoprotective effects against renal damage caused by severe hypertension. However, the effects of long-term infusion of AM on the development of renal impairment remain unknown. To address this question, we performed the present study, in which the effects of long-term infusion of AM on the development of renal impairment in Dahl salt-sensitive (DS) rats were investigated by examining urinary parameters, histological findings, plasma hormone levels, renal tissue angiotensin II levels, and...
mRNA expressions of transforming growth factor-beta (TGF-β), angiotensin II type-1 (AT1) receptor, angiotensin converting enzyme (ACE), renin, and angiotensinogen.

Methods

Experimental Animals and Protocols

After weaning, male inbred DS rats (Eisai Co, Ltd, Tokyo, Japan) were fed a 0.3% NaCl (low-salt) diet until the age of 6 weeks. Thereafter, they were fed a diet containing 8% NaCl (high-salt). At 11 weeks of age, the rats were randomly divided into 2 groups: the AM-treatment group and an untreated group. After pentobarbital sodium anesthesia (30 mg/kg, IP), the rats were subcutaneously implanted with an osmotic minipump (Model 2 ML4, Alza) filled with recombinant human AM dissolved in 0.9% saline in the AM-treatment group (500 ng/h per rat) and 0.9% saline in the untreated group. At about the age of 15 weeks, a new osmotic minipump filled with recombinant human AM or saline was implanted. Age-matched male Dahl salt-resistant (DR) rats fed the same diet served as a control group. All procedures were conducted in accordance with our institutional guidelines for animal research.

Urinary Collection

Twenty-four-hour urine samples were collected from rats in metabolic cages at 7 weeks after infusion of AM for measuring electrolytes, protein, and creatinine levels. Urine electrolytes, urinary protein, creatinine in serum and urine, and serum blood urea nitrogen (BUN) were analyzed by standard methods. Creatinine clearance (Ccr) was calculated using standard formulas.

Hemodynamic Measurements and Blood Sampling

After approximately 7 weeks (50±3 days, mean±SD) of infusion of human recombinant AM or saline, hemodynamic studies were performed as previously reported.8 Measurement of blood pressure was carried over a minute. After hemodynamic measurement, 3 mL of blood was obtained from the carotid artery for the measurement of plasma hormone levels as previously reported.8 Immediately after the heart was arrested by the injection of 2 mmol KCl, the right kidney was removed, weighed, and postfixed in 10% neutral buffered formalin.

Renal Morphology and Glomerular Injury Score

After the right kidney sections were stained with hematoxylin and eosin, periodic acid-Schiff, and periodic acid-methenamine silver for semiquantitative evaluation, glomerular injury scores (GIS) were determined as previously reported.9

Recombinant Human AM

Human recombinant AM was kindly provided by Shionogi & Co, Ltd, Osaka, Japan. The method of production of human recombinant AM is briefly described below. AM with a glycine extension residue in the C-terminus (AM-gly) was expressed in Escherichia coli in a fused form with thioredoxin at the N-terminus. The product was recovered as inclusion bodies and digested with site-specific protease after denaturation. The resulting AM-gly was amidated by peptidyl-amidating enzyme for conversion to mature AM with an amidated C-terminus. Then, AM was purified by column chromatography, lyophilized, and stored at −80°C.

Hormonal Analysis

Human AM was measured using a recently developed specific immunoradiometric assay (IRMA) kit (AM RIA, Shionogi).10 This IRMA kit is specific for human AM and does not cross-react with rat AM. Rat total AM and rat mature AM were also measured by IRMA systems using 2 monoclonal antibodies against rat AM, one specifically recognizing a ring structure of rat AM in both assay kits and the other specifically recognizing the carboxy-terminal sequence in the rat mature AM kit or AM(25–36) in the rat total AM kit. The assay measures rat mature AM or rat total AM by sandwiching it between the 2 antibodies without the extraction of plasma. These IRMA systems specifically recognize rat total or mature AM and do not cross-react with human AM. The plasma renin concentration was measured by a gamma-coat plasma renin activity kit (Dade Behring Co) and determined by the RIA of angiotensin I generated by the incubation of plasma after adding an excess of angiotensinogen provided by binephrectomized rat plasma.11 The plasma aldosterone concentration was measured by RIA using a SPAC-S aldosterone kit (Daiichi Radioisotope Labs).12 This aldosterone antibody shows very low cross-reactivity with dexamethasone (<0.000003), corticosterone (0.03), cortisol (0.0002), cortisone (<0.0003), and deoxycorticosterone (0.05). Plasma and urinary cAMP and cGMP were measured using RIA kits (cAMP and GMP assay kit, Yamasa Shoyu Co).

Measurement of Angiotensin II Levels in Renal Cortex

Immediately after the heart was arrested by the injection of 2 mmol KCl, the left kidney was perfused with 30 mL of cold phosphate-buffered saline (pH 7.4) as previously reported and separated into the cortex and medulla,12 frozen in liquid nitrogen, and stored at −80°C. The radioimmunoassay (RIA) for angiotensin II in renal cortical tissues was performed as reported previously.13

Quantification of Messenger RNA (mRNA) Using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

All procedures used for the mRNA extraction, cDNA synthesis, PCR, and quantification of PCR product were described in detail in our previous reports.14 PCR was done using synthetic oligonucleotide primers as previously reported.15,16 The numbers of PCR cycles for the 5 genes examined were as follows: TGF-β, 29; AT1-receptor, 28; ACE, 29; renin, 30; angiotensinogen, 28.

Statistical Analysis

All values are expressed as mean±SD. Statistical comparisons among the 3 groups were carried out by analysis of variance (ANOVA) and the Bonferroni’s post-hoc test for multiple comparisons. A probability value <0.05 was considered statistically significant.

Results

Physiological Profiles

The physiological profiles of the 3 experimental groups are summarized in the Table. Body weight (BW) was significantly lower in DS rats than in DR rats. In contrast, DS rats had higher kidney weight/BW compared with DR rats. Long-term AM infusion therapy in DS rats significantly increased BW and significantly decreased kidney weight/BW; however, there were still differences between DR and AM-treated DS rats. Regarding hemodynamics, DS rats had markedly higher systolic, diastolic, and mean arterial pressure than DR rats. Long-term AM infusion therapy did not affect the systolic, diastolic, or mean arterial pressure. There were no significant differences in HR among the three groups.

Urinary and Serum Parameters

Urinary parameters are shown in Figure 1. Urine volume, urinary sodium excretion, and urinary protein excretion were significantly increased in DS rats compared with DR rats (Figures 1A, 1B, and 1D). Ccr was significantly decreased in DS rats compared with DR rats (Figure 1E). Chronic AM infusion significantly increased urinary sodium excretion in DS rats (Figure 1B). In addition, chronic AM infusion significantly reduced urinary protein excretion (Figure 1D)
Physiological Profiles and Serum and Urinary Parameters of the 3 Experimental Groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DR</th>
<th>DS</th>
<th>DS-AM</th>
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<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Body weight, g</td>
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<td>344±35*‡</td>
<td>397±16*¶</td>
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<td>192±20*</td>
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<tr>
<td>Mean arterial pressure, mm Hg</td>
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<td>212±30*</td>
<td>215±23*</td>
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<td>Heart rate, bpm</td>
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<td>418±45</td>
<td>442±18</td>
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<td>0.29±0.10‡</td>
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<tr>
<td>Serum BUN, mg/L</td>
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<td>31.2±12.7†</td>
<td>17.7±4.6†</td>
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<td>74.5±44.9*‡</td>
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<tr>
<td>Plasma cGMP, pmol/mL</td>
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<td>Urinary cAMP, nmol/day</td>
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<td>Urinary cGMP, nmol/day</td>
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<td>2.8±1.8†</td>
<td>3.7±2.2†</td>
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</tbody>
</table>

DS-AM indicates DS rats treated with AM. Data are mean±SD. †P<0.05 vs DR; *P<0.01 vs DR; ‡P<0.05 vs DS; ¶P<0.01 vs DS.

and increased Ccr (Figure 1E). There were no significant differences in urinary potassium excretion among the 3 groups (Figure 1C). Serum creatinine and BUN levels were also increased in DS rats compared with DR rats (Table). Long-term AM infusion therapy decreased these increased creatinine and BUN levels.

Renal Morphology and Glomerular Injury Score

The morphological appearance of arteries, glomeruli, and the interstitium were considered normal in the DR rats. Renal injury of the untreated DS rats was revealed by segmental and global glomerular sclerosis and arterioarteriolar sclerosis associated with inflammatory cell infiltration, interstitial fibrosis, atrophic and dilated tubules, and tubular casts. In addition, these DS rats demonstrated marked medial and intimal thickening, with proliferation of vascular smooth muscle cells in the interlobular arteries. Chronic AM treatment reduced these changes, especially in the glomeruli. Figure 2 shows typical light micrographs in 3 groups. DR rats showed few pathological findings. Untreated DS rats demonstrated severe nephrosclerosis, and the damage was reduced in AM-treated DS rats. As a result, the total GIS was significantly greater in untreated DS than in DR rats. AM treatment significantly reduced this score (Figure 2).

Hormonal Responses to Long-Term AM Infusion

Plasma levels of hormones are shown in Figure 3. Plasma renin concentration, aldosterone, rat endogenous total AM, and rat mature AM levels were all significantly elevated in DS rats compared with DR rats (Figures 3A through 3D). Long-term human AM infusion significantly reduced these elevated hormone levels (Figures 3A through 3D). Interestingly, rat mature AM levels were about 20% of rat total AM. Human AM immunoreactivity was detected only in AM-treated DS rats (Figure 3E). DS had higher plasma cAMP and cGMP levels than DR. Long-term AM treatment further increased these levels. In contrast to plasma, urinary excretion of cAMP and cGMP was significantly lower in DS rats than DR rats. Long-term AM treatment tended to increase urinary excretion of cAMP and cGMP; however, they did not reach statistical significance (Table).

Renal Tissue Angiotensin II Levels and mRNA Expressions of TGF-β, AT1-Receptor, ACE, Renin, and Angiotensinogen

Tissue angiotensin II levels in renal cortex were significantly increased in DS rats compared with DR rats at approximately 18 weeks of age (Figure 4A). TGF-β/GAPDH mRNA levels, ACE/GAPDH mRNA levels, renin/GAPDH mRNA levels, and angiotensinogen/GAPDH mRNA levels in the renal cortex were also increased in DS rats compared with DR rats (Figures 4B and 4D through 4F). In contrast, AT1-receptor/GAPDH mRNA levels were significantly decreased in the renal cortex in DS rats compared with DR rats (Figure 4C). Long-term AM infusion therapy significantly decreased these increased tissue angiotensin II levels and also reduced TGF-β/GAPDH mRNA levels, ACE/GAPDH mRNA levels, renin/GAPDH mRNA levels, and angiotensinogen/GAPDH mRNA levels in the renal cortex (Figures 4A and 4B and 4D through 4F). Chronic AM infusion significantly increased the decreased AT1-receptor mRNA level in the renal cortex (Figure 4C).

Discussion

In the present study, we demonstrated that chronic AM infusion prevented renal functional and morphological changes without reducing blood pressure. There are many studies that have investigated the effects of AM on blood pressure and other cardiovascular parameters. However, few studies have investigated the chronic effects of AM on blood pressure. Khan et al. reported that chronic administration of AM at a rate of
1000ng/h significantly reduced blood pressure in rats with hypertension. Yoshihara et al. reported that chronic infusion of AM of 200ng/h to relatively small rats with 120 to 140g body weight did not reduce blood pressure but attenuated pulmonary vascular remodeling. In Yoshihara’s study, the AM dose/body weight was almost the same as that in the present study. More recently, Zhang et al. reported the effect of AM gene delivery on blood pressure in hypertensive rats. AM gene delivery caused plasma AM levels to become elevated to close to 100 times higher than the normal rat AM level, with a concomitant reduction of blood pressure. Thus, the effect of chronic AM administration on the blood pressure level may depend on the dose of AM administered.

In this study, DS rats had higher plasma renin and aldosterone concentrations than DR rats, indicating activation of the circulating renin-angiotensin-aldosterone system in DS rats. Interestingly, long-term AM infusion significantly decreased the plasma renin concentration in DS rats. In previous studies, the effects of acute administration of AM on plasma renin activity in vivo were controversial, because excessive reduction of blood pressure induced by AM may induce renin secretion. However, Khan et al. reported that chronic administration of AM significantly reduced the plasma renin activity in renovascular hypertensive rats, suggesting direct inhibitory effects of chronic AM infusion on renin secretion. Because the effect of AM on the vasculature is partly mediated via the nitric oxide–cGMP pathway in the kidney and cGMP is an intracellular mediator of inhibition of renin release, it is possible that AM may inhibit renin release via a nitric oxide–cGMP pathway. Furthermore, long-term AM infusion significantly reduced aldosterone secretion in DS rats with renal failure. In vitro, AM has been shown to inhibit production of angiotensin II–induced aldosterone by dispersed rat adrenal zona glomerulosa cells. Thus, the beneficial effects of long-term administration of AM may be attributable, in part, to direct inhibition of the circulating renin-angiotensin-aldosterone system.
In addition to the circulating renin-angiotensin system, the locally activated renin-angiotensin system also plays an important role in the process of renal damage.\textsuperscript{24} After this activation, there is stimulation of the downstream cascade involving multiple growth factors.\textsuperscript{25} The activation of the intrarenal renin-angiotensin-aldosterone system in DS rats was suggested by recent studies. Otsuka et al\textsuperscript{26} reported that administration of TCV-116, a specific angiotensin II antagonist, markedly protected DS rats against renal functional and morphological deterioration without changing blood pressure. In the present study, we found that DS rats with renal failure exhibited activation of the renal tissue renin-angiotensin-TGF-\(\beta\) system compared with DR rats. Renal angiotensin II concentrations were significantly increased in the renal cortex in DS rats compared with DR rats. The gene expression levels of renin, ACE, angiotensinogen, and TGF-\(\beta\) were also significantly increased in the renal cortex in DS rats compared with DR rats. Long-term AM administration significantly inhibited these increases of tissue angiotensin II levels and the levels of expression of ACE, renin, angiotensinogen, and TGF-\(\beta\) mRNAs. The precise mechanisms by which the chronic infusion of AM inhibits the renal tissue renin-angiotensin system and TGF-\(\beta\) remain to be elucidated, but direct effects of AM on the intrarenal renin-angiotensin-TGF-\(\beta\) axis are possible. Previous studies showed that AM inhibited the DNA synthesis and migration of mesangial cells induced by angiotensin II in a cAMP-dependent manner,\textsuperscript{27} suggesting that AM antagonizes the renin-angiotensin system. Another study also reported that AM inhibited the proliferation and migration of vascular smooth muscle cells induced by angiotensin II.\textsuperscript{5} In addition, AM suppressed the mitogen-activated protein kinase activity in mesangial cells,\textsuperscript{28} the activation of which is a critical mediator of TGF-\(\beta\) induction. These results support the notion that the reduction of the intrarenal renin-angiotensin-TGF-\(\beta\) axis may be involved in the protective effects of AM against the development of renal failure in DS rats.

Figure 4. Effects of long-term AM infusion on renal cortical tissue angiotensin II levels and mRNA expressions of TGF-\(\beta\), AT1-receptor (AT1-R), ACE, renin, and angiotensinogen (ANG) in renal cortex. Left, Representative ethidium bromide stained agarose gels of RT-PCR products for TGF-\(\beta\), AT1-R, ACE, renin, ANG, and GAPDH at each PCR cycle. Right, Renal cortical tissue angiotensin II levels (A) and quantitative analysis of TGF-\(\beta\) (B), AT1-R (C), ACE (D), renin (E), and ANG (F) mRNA levels normalized relative to GAPDH mRNA levels. Data are expressed as mean±SD. *\(P<0.05\) versus DR, †\(P<0.01\) versus DR, ‡\(P<0.05\) versus DS, ¶\(P<0.01\) versus DS.

The biological effects of AM have been reported to be mediated by both cAMP and cGMP pathways.\textsuperscript{5} However, whether these beneficial effects of AM have been mediated by cAMP and/or cGMP signaling pathway in vivo remains unknown. Recent studies reported that gene delivery of AM improved cardiorenal histological findings in DS rats with a considerable reduction of blood pressure.\textsuperscript{19,29} In these studies, AM gene delivery markedly increased plasma AM and also increased urinary and tissue cAMP levels, but not cGMP levels, findings that differ from our study. In the present study, chronic AM infusion improved renal morphological and functional parameters with a small increase of active forms of AM levels without changing blood pressure. Chronic AM infusion did not change plasma or urinary cAMP or cGMP levels between AM-treated DS or untreated DS groups, although these levels tended to increase in the AM-treated group. Thus, the discrepancy between the results obtained by us and those of Zhang et al\textsuperscript{19} may be partly explained by the difference in the increased AM levels. Another possibility is that AM stimulated intracellular cAMP or cGMP at the tissue levels sufficiently to induce a biological response but insufficiently to induce a measurable rise in urinary level. Further studies are required to elucidate the exact mechanism of the beneficial effect of AM.

Interestingly, chronic human recombinant AM infusion therapy significantly decreased the elevated plasma levels of endogenous rat total AM and mature AM. To the best of our knowledge, there is no evidence for a direct negative feedback of AM on its own production. Therefore, this effect of exogenous AM administration might be due to an indirect effect. Plasma AM levels are known to be increased in proportion to the severity of renal failure.\textsuperscript{5} Although the plasma level of human AM was low, the beneficial effects of chronic AM infusion therapy on renal function, histological findings, hormone levels, and renal molecular markers were significant. Previous studies have shown that chronic AM infusion significantly reduced plasma renin activity in renovascular hypertensive rats\textsuperscript{17}
and attenuated the development of pulmonary hypertension, induced in rats by monocrotaline, with an increase of plasma AM levels to within the pathophysiologic range. These findings were consistent with the present results. The fact that such a slight increase of plasma AM was effective may be attributable to an increase of mature AM, an active molecular form of AM. Recent studies have shown that the major molecular form of human plasma AM is AM-glycine, an inactive molecular form of AM, and that mature AM only constitutes about 10% of plasma AM. In the present study, we first showed that mature AM is about 20% of rat plasma total AM, indicating that the major molecular form of rat plasma AM is also AM-glycine. Thus, the finding that exogenous AM administration reduces endogenous AM levels may support the hypothesis that increased plasma AM plays a compensatory role in the pathophysiology of renal failure.

In conclusion, chronic AM infusion has beneficial effects on renal function, histological findings, hormone levels, and the renal tissue renin-angiotensin-TGF-β axis in DS rats with renal impairment. Our findings may open up the possibility of a new therapeutic strategy for hypertension with renal impairment.

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