Long-Term Adrenomedullin Infusion Improves Survival in Malignant Hypertensive Rats

Yosuke Mori, Toshio Nishikimi, Naohiko Kobayashi, Hidehiko Ono, Kenji Kangawa, Hiroaki Matsuoka

Abstract—Previous studies have demonstrated that adrenomedullin has inhibitory effects on the proliferation and DNA synthesis of mesangial cells and vascular smooth muscle cells in vitro and that plasma adrenomedullin levels are markedly elevated in malignant hypertension. This study was designed to examine whether chronic adrenomedullin infusion has renoprotective effects in malignant hypertensive rats. We studied the following 3 groups: control Wistar Kyoto rats, deoxycorticosterone acetate–salt spontaneously hypertensive rats, and adrenomedullin-treated deoxycorticosterone acetate–salt spontaneously hypertensive rats. Chronic adrenomedullin infusion using an osmotic minipump was started simultaneously with deoxycorticosterone acetate–salt treatment. After 3 weeks of the treatment, malignant hypertensive rats were characterized by higher blood pressure, kidney weight, urinary protein excretion, glomerular injury score, plasma renin concentration, aldosterone level, endogenous rat plasma adrenomedullin level, renal cortical tissue angiotensin II level, angiotensin-converting enzyme mRNA level, and transforming growth factor-β1 mRNA level in the renal cortex, and by lower creatinine clearance, compared with the control rats. Chronic adrenomedullin infusion significantly improved these parameters (kidney weight: 6.5%, urinary protein excretion: 63.8%, glomerular injury score: 38.3%, plasma renin concentration: 52.4%, aldosterone: 23.2%, rat adrenomedullin: 28.6%, renal angiotensin II: 28.1%, renal angiotensin-converting enzyme mRNA: 38.3%, renal transforming growth factor-β1 mRNA: 56.2%, and creatinine clearance: 20.5%) without significant reduction of mean arterial pressure (−4%). Kaplan-Meier survival analysis showed that adrenomedullin infusion significantly prolonged survival time. These results suggest that subdepressor dose of chronic adrenomedullin infusion has renoprotective effects in this malignant hypertension model, at least in part, via inhibition of the circulating and intrarenal renin-angiotensin system.

Key Words: adrenomedullin ■ hypertension, malignant ■ renal disease ■ renin-angiotensin system ■ renal circulation

Adrenomedullin (AM) is a potent vasodilator peptide that was discovered in human pheochromocytoma tissue. AM is a 52-amino acid peptide with a ring structure and C-terminal amidation. AM immunoreactivity and gene expression are widely distributed in mammalian organs, including the kidney. AM is present in human plasma and urine, and the urinary levels are higher than plasma levels, indicating that the kidney is one of the major organs for AM production.

Previous studies using immunohistochemistry showed that the level of AM immunoreactivity is high in the glomerulus, distal tubules, and collecting duct cells. AM mRNA was detected in the glomerulus, cortical collecting duct cells, outer collecting duct cells, and inner collecting duct cells using the reverse transcription-polymerase chain reaction (RT-PCR) technique. Considerable evidence also suggests a role for AM in regulating the renal function and in modulating the pathophysiology of renal disease. Intrarenal infusion of AM significantly increases the renal blood flow, glomerular filtration rate, and urinary sodium excretion. Direct effects of AM have been demonstrated in cultured mesangial cells, fibroblasts, and vascular smooth muscle cells, where AM induces an increase in the intracellular cyclic AMP (cAMP) level, followed by inhibition of proliferation of these cells. In normal healthy controls, AM circulates at picomolar concentrations in the plasma, but the levels are elevated 2- to 5-fold in patients with essential hypertension, malignant hypertension, and chronic renal failure compared with normal subjects.

More recently, we reported that plasma AM levels, urinary excretion of AM, and renal mRNA levels of AM are markedly higher in malignant hypertensive rats compared with normal rats. Considering the physiological actions of AM in the kidney, increased plasma and renal AM observed in these conditions may have renoprotective effects. Indeed, a recent study revealed that gene therapy involving AM deliv-
ery significantly attenuated the renal damage in a hypertensive rat model with marked elevation of plasma AM levels, suggesting that chronic elevation of plasma AM has renoprotective effects. However, because AM gene delivery decreased blood pressure considerably, it is still unclear whether AM has a blood pressure–lowering independent renoprotective effect in vivo. We therefore designed this study to determine whether a chronic subdepressor dose of AM infusion has renoprotective effects in a malignant hypertensive rat model.

Methods
All procedures were in accordance with our institutional guidelines for animal research and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals and Experimental Design
Nine-week-old male Wistar Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) (Clea Japan, Tokyo, Japan) were studied. Deoxycorticosterone acetate (DOCA)-salt SHR were used as a malignant hypertensive rat model in this study because this model has extensive end-organ damage, including malignant nephrosclerosis. DOCA-salt SHR were produced as previously reported. DOCA-salt SHR were randomly divided into the following 2 groups: an AM treatment group (n=10) and an untreated group (n=9). Age-matched male WKY (n=9) drinking 1% NaCl served as a control group. After pentobarbital sodium anesthesia (30 mg/kg, IP), the rats were subcutaneously implanted with an osmotic minipump (Model 2 ML4, Alza Corporation), filled with recombinant human AM dissolved in 0.9% saline at the treatment group (500 ng/h) and 0.9% saline in the untreated group. This dose per body weight (BW) is a subdepressor dose with the biological effects previously reported. The AM treatment was started simultaneously with DOCA-salt treatment and continued for 3 weeks.

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 has extensive end-organ damage, including malignant nephrosclerosis. The AM treatment was started simultaneously with DOCA-salt treatment and continued for 3 weeks.

Hemodynamic Measurements and Blood Sampling
All rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg), and their BW was measured. A polyethylene catheter (PE-50) was inserted into the thoracic aorta via the right carotid artery to measure the heart rate (HR) and mean arterial pressure (MAP), as previously reported. Measurements of MAP and HR were carried out over a minute. After these hemodynamic measurements were finished, 3 mL of blood was obtained from the carotid artery and 2.5 mL of blood was transferred to a chilled glass tube containing aprotinin (500 U/mL) and disodium EDTA (1 mg/mL) for the measurement of the plasma hormone levels, and the remainder of the blood was transferred to another plastic tube for measurement of the serum level of creatinine. Immediately after the heart was arrested by the injection of 2 mmol of KCl, the right kidney was removed, weighed, and postfixed in 10% neutral buffered formalin.

Measurement of Angiotensin II Level in Renal Cortex
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, frozen in liquid nitrogen, and stored at −80°C. The radioimmunoassay for angiotensin II in renal cortical tissues was performed as reported previously.

Hormone Analysis
Human AM was measured using a recently developed specific immunoradiometric assay (IRMA) kit (AM RIA, Shionogi). 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 with IRMA as previously reported. These IRMA systems specifically recognize rat total or rat mature AM and do not cross-react with human AM. The plasma renin concentration (PRC) was measured as previously reported after adding an excess of angiotensinogen in the form of binephrectomized rat plasma. The plasma aldosterone level was measured by radioimmunoassay as previously reported.

Histological Examination
The right kidney was excised and immersed in neutralized formalin for histological examination. Grading of glomerular injury score (GIS) at 2 depths, the subcapsular and juxtamedullary cortex, was performed as described previously.

Quantification of Messenger RNA (mRNA) Using RT-PCR
All procedures used for the mRNA extraction and cDNA synthesis were described in detail in our previous report. PCR for transforming growth factor (TGF)-β1 and ACE and quantification of PCR products was performed as previously reported. The numbers of PCR cycles for the 3 genes examined were as follows: TGF-β1, 29; ACE, 29; GAPDH, 21.

Effect on Survival Rate
To examine the effect of long-term administration of AM on survival rate in DOCA-salt SHR, another 36 DOCA-salt SHR were randomly divided into 2 groups: an AM treatment group (n=18) and 0.9% saline in the untreated group (n=18) as described above in “Animals and Experimental Design.” A WKY group (n=10) was also produced. After 4 weeks of treatment with AM or saline, a new replacement osmotic minipump filled with recombinant human AM or saline was implanted. Animals were carefully monitored and deaths were recorded every day. Survival rates were compared among the groups at 11 weeks after the start of drug treatment.

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 After 3-Week Treatment of DOCA Salt, With and Without AM Infusion
The BW, kidney weight (KW), MAP, and HR in the 3 groups are presented in the Table. There were no differences in BW among the 3 groups. In contrast, DOCA-salt SHR had higher kidney weight/BW compared with control WKY. Chronic AM infusion therapy in DOCA-salt SHR significantly decreased kidney weight/BW. DOCA-salt SHR had markedly
BW, MAP, KW, and HR in WKY, DOCA-salt SHR, and AM-treated D-SHR

<table>
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<td>10</td>
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<td>BW, g</td>
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<td>HR, bpm</td>
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</table>

Data are expressed as mean±SD; *P<0.01 vs WKY; †P<0.05 vs D-SHR.

Renal Morphological Findings

The morphological appearance of the interstitium, glomeruli, and arterioles were considered normal in WKY (Figures 2A, 2D, and 2G). The renal histological appearance in the DOCA-salt SHR revealed severe interstitial fibrosis, nephrosclerosis, and arteriolar sclerosis (Figures 2B, 2E, and 2H). Chronic AM treatment reduced these changes, especially in the glomeruli (Figures 2C, 2F, and 2I). The GIS at 2 depths, the subcapsular and juxtamedullary cortex, are shown in Figures 2J and 2K. The subcapsular GIS and juxtamedullary cortex GIS were significantly higher in DOCA-salt SHR than in WKY. Chronic AM treatment caused significant amelioration of these changes, especially in the glomeruli in juxtamedullary cortex (Figure 2K). AM treatment also tended to attenuate the GIS at the subcapsular level; however, the attenuation did not reach statistical significance (Figure 2J).

Plasma Hormone Levels

The PRC and the aldosterone, rat AM, and human AM levels in the 3 groups are shown in Figures 3A to 3E. The plasma renin concentration and aldosterone, rat total, and mature AM levels were higher in DOCA-salt SHR than in WKY (Figures 3A to 3D). Long-term human AM infusion to DOCA-salt SHR significantly reduced the elevation of these hormone levels. Human AM immunoreactivity was only detected in AM-treated, DOCA-salt SHR (Figure 3E).

Tissue Angiotensin II Levels and Expression of TGF-β and ACE mRNAs in Renal Cortex

The renal cortical tissue levels of angiotensin II and of TGF-β and ACE mRNAs are shown in Figures 4A to 4C. The tissue angiotensin II level in the renal cortex was significantly higher in DOCA-salt SHR than in WKY (Figure 4A). TGF-β/GAPDH mRNA levels and ACE/GAPDH mRNA levels in the renal cortex were also higher in DOCA-salt SHR than in WKY (Figures 4B and 4C). Chronic AM infusion significantly attenuated the increase of the tissue angiotensin II level and also reduced the TGF-β/GAPDH mRNA level and ACE/GAPDH mRNA level in the renal cortex.

Survival Rate

Survival rate was analyzed at 3 weeks after the start of AM infusion treatment. All DOCA-salt SHR treated with saline died between 4 and 8 weeks after treatment. Kaplan-Meier survival analysis showed that, statistically, long-term AM infusion treatment significantly prolonged the survival time of DOCA-salt SHR (P<0.01) (Figure 5).

Discussion

In the present study we investigated the effect of infusion of a chronic subdepressor dose of AM in rats with malignant hypertension induced by DOCA-salt SHR. DOCA-salt SHR were characterized by histological findings of malignant nephrosclerosis, increased blood pressure, proteinuria, elevation of the PRC, plasma aldosterone level, plasma rat AM level, renal cortical tissue angiotensin II level, and renal cortical ACE and TGF-β mRNA levels, decreased Ccr, and poor survival. Chronic AM treatment significantly improved these findings with a reduction of the plasma rat endogenous

higher MAP than WKY. Long-term AM infusion therapy did not significantly affect the MAP. There were no significant differences in HR among the 3 groups.

Urinary Parameters

The urinary sodium excretion, urine flow, urinary protein excretion, and Ccr in the 3 groups are presented in Figures 1A to 1D. Urinary protein excretion was significantly increased in DOCA-salt SHR compared with WKY (Figure 1C). Ccr was significantly decreased in DOCA-salt SHR compared with WKY (Figure 1D). Chronic AM infusion therapy significantly improved urinary protein excretion and Ccr in DOCA-salt SHR. There were no significant differences in urinary sodium excretion or urine flow among the 3 groups (Figures 1A and 1B).

Figure 1. A to D, Effects of chronic adrenomedullin (AM) infusion on urinary sodium excretion (UNaV), urinary volume (UV), urinary protein excretion (U-Pro), and creatinine clearance (Ccr). These indices were measured over 24 hours at the end of 3 weeks DOCA, with/without AM infusion. WKY indicates Wistar Kyoto rats; D-SHR, DOCA-salt spontaneously hypertensive rats; and D-SHR/AM, AM treated D-SHR. Data are expressed as mean±SD; *P<0.01 versus WKY; †P<0.05 versus D-SHR; and ‡P<0.01 versus D-SHR.
AM level. These results suggest that chronic AM infusion has renoprotective effects and that increased endogenous plasma AM in renal failure acts in a compensatory mechanism.

DOCA-salt SHR have been employed extensively as a model of malignant hypertension accompanied by extensive end-organ damage, including malignant nephrosclerosis.\textsuperscript{10,12} Malignant nephrosclerosis causes renal failure, leading to poor survival. In the present study, the histological examination of the kidneys in DOCA-salt SHR revealed glomerulosclerosis, obstruction of the small arteries with fibrinoid necrosis, and interstitial fibrosis, results consistent with those of previous studies.\textsuperscript{10,12} This model also shows renal functional alterations such as increased proteinuria and decreased creatinine clearance. More recently, we reported that plasma AM levels, urinary AM levels, renal cortical tissue AM levels, and renal cortical AM mRNA and its receptor mRNAs are significantly increased in this model compared with control rats.\textsuperscript{10} In the present study we also showed that the plasma levels of mature AM and total AM are increased in this model compared with control rats. Thus, this model is useful for investigating the pathophysiological role of AM.

Many studies have investigated the acute effects of AM on blood pressure and other cardiovascular parameters.\textsuperscript{5} However, few studies have investigated the effects of chronic AM infusion on the cardiovascular system in vivo. Khan et al\textsuperscript{24} reported that chronic administration of AM at a rate of 1000 ng/h significantly reduced blood pressure in rats with renovascular hypertension. More recently, Dobrzynski et al\textsuperscript{11} reported that adenovirus-mediated AM gene delivery in DOCA-salt hypertensive rats induced chronic elevation of the plasma AM level, reduced the blood pressure, and ameliorated the renal damage, suggesting that chronic plasma AM elevation has renoprotective effects. However, because AM gene therapy reduced blood pressure considerably, it is still unclear whether the beneficial effect of AM is due to a direct effect of AM or to reduction of the blood pressure per se.

Yoshihara et al\textsuperscript{13} previously reported that chronic infusion of AM at 200 ng/h to relatively small rats with 120 to 140 g BW did not reduce the blood pressure but attenuated pulmonary vascular remodeling in pulmonary hypertensive rats. In the present study, we therefore used a subdepressor dose of AM to determine whether AM has a blood pressure-lowering independent effect in malignant hypertensive rats.

The locally activated renin-angiotensin system plays an important role in the process of renal damage. After this activation, there is stimulation of the downstream cascade involving multiple growth factors.\textsuperscript{25} Although high salt intake suppresses the renin-angiotensin-aldosterone system, the locally activated system plays an important role in the process of renal damage in DOCA-salt hypertensive rats. Kim et al\textsuperscript{26} reported that administration of TCV-116, a specific angiotensin II antagonist, protected against renal functional and morphological deterioration without changing the blood pressure, suggesting an important role of angiotensin II for renal impairment in this rat model. They also demonstrated that the renal level of TGF-\(\beta\)1 mRNA is increased in DOCA-salt hypertensive rats and that a subdepressor dose of angiotensin II antagonist decreased the level of TGF-\(\beta\)1 mRNA. In the present study, we demonstrated that the plasma renin concentration, plasma aldosterone level, renal cortical tissue angiotensin II level, and levels of ACE and TGF-\(\beta\)1 mRNAs in the renal cortex were significantly increased in DOCA-salt SHR.
compared with salt-loaded WKY. Chronic AM treatment significantly decreased the plasma renin concentration, plasma aldosterone level, renal cortical tissue angiotensin II level, and levels of ACE and TGF-β1 mRNAs in the renal cortex with a concomitant improvement of renal functional and morphological changes. Such inhibition of the renin-angiotensin system by AM is in good agreement with prior in vivo and in vitro studies showing that chronic AM infusion inhibited the plasma renin activity in rats with renovascular hypertension and that AM inhibits the production of aldo-

**Figure 3.** A to E, Effects of long-term AM infusion on plasma renin concentration (PRC) (A), aldosterone (B), rat total (C), and mature AM (D) and human AM levels (E). Data are expressed as mean±SD. †P<0.05 versus WKY, *P<0.01 versus WKY, ‡P<0.05 versus D-SHR, and ¶P<0.01 versus D-SHR.

**Figure 4.** A to C, Effects of long-term AM infusion on renal cortical tissue angiotensin II level (Ang II) (A) and level of expression of TGF-β (B) and ACE mRNAs (C) in the renal cortex. Data are expressed as mean±SD. †P<0.05 versus WKY, *P<0.01 versus WKY, ‡P<0.05 versus D-SHR, and ¶P<0.01 versus D-SHR.
sterone in dispersed rat adrenal zona glomerulosa cells. 

In contrast, previous studies reported that acute AM infusion stimulated the renin release with a concomitant reduction of blood pressure. Moreover, Jensen et al. reported the direct stimulating effect of AM on renin release in mouse juxtaglomerular granular cells. Thus, interaction of AM and the renin-angiotensin system seems to be complex; however, the chronic effect of AM may be different from the acute effect of AM. The precise mechanism by which chronic AM treatment inhibits the renal tissue renin-angiotensin system remains to be elucidated. In addition, previous studies showed that AM inhibited the DNA synthesis and migration of mesangial cells and vascular smooth muscle cells induced by angiotensin II in a cAMP-dependent manner, suggesting that AM antagonizes the actions of angiotensin II. In addition, AM suppressed the mitogen-activated protein kinase activity in mesangial cells, the activation of which is a critical mediator of TGF-β induction. Thus, not only inhibition of the renin-angiotensin system, but also antagonism of the action of the renin-angiotensin system, may be, in part, involved in the renoprotective effects of AM.

In the present study, long-term human recombinant AM administration significantly decreased the elevation of the endogenous rat AM level with a concomitant improvement of renal functional, morphological, and biochemical changes; however, the plasma level of human AM was low. Previous studies showed that chronic AM infusion significantly reduced plasma renin activity in renovascular hypertensive rats and attenuated the development of pulmonary hypertension in rats induced by monocrotalin, with an increase of plasma AM levels within the pathophysiological range.

These previous findings were consistent with the results of the present study. The fact that a slight increase of human AM was effective may be attributed to 2 causes. One cause may be an increase of mature AM. Recent studies revealed that the major molecular form of human plasma AM is AM-gly, an inactive molecular form of AM, and that mature AM only constitutes about 10% of plasma AM. In this study we showed that the rat mature AM constitutes only about 15% to 20% of total AM, suggesting that the major molecular form of rat AM is also AM-gly. Thus, the reason that a small dose of AM infusion is effective may be the fact that recombinant human AM constitutes of mature AM. The other cause may be the timing of the administration of AM. In the present study, because we started to infuse AM simultaneously with the DOCA-salt treatment, a small dose of AM administration might effectively attenuate the glomerular injury and attenuate the following increase of plasma AM levels. On the other hand, the effect of AM might be blunted in severe renal damage of untreated DOCA-salt. These phenomena are observed in other vasoacting peptides, such as atrial natriuretic peptide (ANP). A small dose of ANP is very effective in mild heart failure; however, its effect is blunted in severe heart failure, despite a marked increase of plasma ANP levels. Thus, the finding that the chronic administration of AM has renoprotective effects suggests that increased AM in malignant hypertension may be a compensatory mechanism.

A limitation of the present study is that we assessed blood pressure only under anesthesia. However, we used the direct method and measured it over a minute. Therefore, it is possible that our method might be more accurate than the indirect method. In addition, we showed that chronic AM infusion treatment significantly decreased the renal renin-angiotensin–TGF-β axis with a concomitant improvement of renal functional and morphological changes, suggesting that inhibition of the renin-angiotensin system may be, in part, involved in the renoprotective effects of AM. However, we could not show the direct effect of AM on the renin-angiotensin system. Previous studies showed that AM has many physiological actions through many cascades, such as classical cAMP cascade, nitric oxide (NO) pathway, inhibition of oxygen stress, or the phosphatidylinositol 3-kinase/Akt pathway. Therefore, we could not deny the possibility that other cascades may be involved in the present observed beneficial effect of AM and that the decreased renin-angiotensin system may reflect the results of improved renal function.

**Perspectives**

Previous studies revealed that the plasma AM levels are increased in hypertension and renal failure. However, the role of increased AM in these conditions has not been fully understood. We recently reported that endogenous AM plays a role in the regulation of blood pressure and renal tubular function in malignant hypertensive rats. These findings, together with the direct inhibitory effect of AM on the proliferation and DNA synthesis of mesangial cells and vascular smooth muscle cells, suggest that long-term AM infusion may be effective for the treatment of hypertension. In the present study, we demonstrated that chronic AM infusion is, in fact, effective for the treatment of progressive renal injury in malignant hypertension. Furthermore, recent studies indicate that acute AM administration significantly improved hemodynamics and renal tubular function in patients with hypertension and renal disease. These results indicate that chronic AM administration may be a new therapeutic approach for treatment of certain forms of hypertension and renal disease. The development of an orally active AM agonist or specific inhibitor of AM degradation would be desired.
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References

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