Role of Endogenous Adrenomedullin in the Regulation of Vascular Tone and Ischemic Renal Injury

Studies on Transgenic/Knockout Mice of Adrenomedullin Gene


Abstract—Adrenomedullin (AM) is a potent depressor peptide whose vascular action is suggested to involve nitric oxide (NO) release. To explore the role of endogenous AM in vascular and renal function, we examined the effects of acetylcholine (ACh), AM, and AM receptor antagonists AM(22-52) and CGRP(8-37) on the renal perfusion pressure (RPP) of kidneys isolated from AM transgenic (TG)/heterozygote knockout (KO) mice and wild-type littermates (WT). Furthermore, we evaluated the renal function and histology 24 hours after bilateral renal artery clamp for 45 minutes in TG, KO, and WT mice. Baseline RPP was significantly lower in TG than in KO and WT mice (KO 93.4±4.6, WT 85.8±4.2, TG 72.4±2.4 mm Hg [mean±SE], P<0.01). ACh and AM caused a dose-related reduction in RPP, but the degree of vasodilatation was smaller in TG than that in KO and WT (%ΔRPP 10−7 mol/L: ACh: KO −48.1±3.9%, WT −57.5±5.6%, TG −22.8±4.8%, P<0.01), whereas Nω-nitro-L-arginine methyl ester (L-NAME) caused greater vasoconstriction in TG (%ΔRPP 10−7 mol/L: KO 35.1±3.3%, WT 53.5±7.2%, TG 152.6±21.2%, P<0.01). Both AM antagonists increased RPP in TG to a greater extent compared with KO and WT mice (%ΔRPP 10−6 mol/L: CGRP(8-37): KO 12.8±2.6%, WT 19.4±3.6%, TG 41.8±8.7%, P<0.01). In mice with ischemic kidneys, serum levels of urea nitrogen and renal damage scores showed smaller values in TG and greater values in KO mice (urea nitrogen: KO 104±5>WT 98±15>TG 38±7 mg/dL, P<0.05 each). Renal NO synthase activity was also greater in TG mice. However, the differences in serum urea nitrogen and renal damage scores among the 3 groups of mice were not observed in mice pretreated with L-NAME. In conclusion, AM antagonists increased renal vascular tone in WT as well as in TG, suggesting that endogenous AM plays a role in the physiological regulation of the vascular tone. AM is likely to protect renal tissues from ischemia/reperfusion injury through its NO releasing activity. (Circ Res. 2002;90:657-663.)

Key Words: adrenomedullin ■ nitric oxide ■ cGMP ■ endothelium ■ ischemia

Adrenomedullin (AM) is a potent vasodilating peptide that was originally isolated from human pheochromocytoma cells.1 AM has been considered a member of the calcitonin gene-related peptide (CGRP) superfamily based on their structural similarities. In fact, both peptides are reported to share common receptors. At present, it is known that AM-producing cells widely distribute in the whole body including the adrenal glands, heart, lungs, and kidneys. However, there is no step-up in AM levels of the venous plasma drained from various organs.2 It has also been reported that cultured vascular smooth muscle cells and endothelial cells synthesize and secrete AM.3,4 These findings suggest that circulating AM derives mainly from vascular walls and plays a role in the vascular system.

Because AM is isolated on the basis of the cAMP-increasing activity,1 it at first cAMP was considered to be the sole second messenger for AM-induced vasodilation.5,6 However, it was reported that AM dilates vessels in an endothelium-dependent manner and that cGMP is another second messenger for AM.7–11 We have already reported that denudation of rat aortic endothelial cells and inhibition of guanylate cyclase substantially inhibited AM-induced vasodilation.11 Furthermore, AM increased nitric oxide (NO) release from rat perfused kidneys and inhibition of NO synthase decreased both NO release and vasodilation caused by AM.8 These findings suggest that AM-induced vasodilation is partly dependent of activation of the NO-cGMP pathway.

Plasma levels of AM are elevated in patients with hypertension, heart failure, or renal failure.2,12,13 Because AM exerts vasodilatory and natriuretic effects, the increased levels of AM have been considered to play a compensatory role under such pathological conditions. However, plasma
levels of AM in the healthy populations are low and they show only several-fold increases even in patients with heart failure.21,12,13 Thus, it is unclear as to whether endogenous AM plays a significant role under physiological conditions. Recent progress of genetic technologies has enabled us to determine the in vivo activities of endogenous bioactive substances. We have recently established some mice strains in which AM genes are overexpressed or disrupted. Mice showing overexpression of AM genes, that is AM transgenic (TG) mice, had hypotension,14 whereas disruption of the AM gene was lethal. Heterozygote mice with a disrupted AM gene (AM+/− mice; KO) showed slight increases in blood pressure, compared with wild-type (WT) mice.15

Although NO has various cardiovascular effects, its role in ischemia/reperfusion renal injury is still controversial.16–19 We have reported that in ischemic acute renal failure (iARF) increases in endothelin-derived NO mitigated renal injury and vice versa.19 If AM releases substantial amounts of NO from the renal vasculature, increases in endogenous AM may be beneficial for ischemic renal injury. Thus, we analyzed the role of endogenous AM in the regulation of aortic and renal vascular tone and in renal injury caused by ischemia/reperfusion using AM TG mice and KO mice.

Materials and Methods

Animals
All mouse studies were performed in concordance with the university guidelines for animal experiments. AM TG mice and KO mice were established as previously reported. TG mice were established using fusion cDNA of the AM gene with the preproendothelin-1 gene, resulting in overexpression of AM in the vascular wall, particularly in the endothelium, and 2- to 5-fold increases in AM expression in the aorta and kidneys.14 AM KO mice were established by replacing exon 1 to 4 with the neomycin-resistant gene. Because AM homozygote KO mice were lethal, we used heterozygote mice as AM KO mice in which the AM content in the heart and lung decreased to 50% of that in WT mice.15

Radioimmunoassay for Adrenomedullin
Adrenomedullin concentration in the kidney of 12-week old AM TG mice, AM KO mice, and WT mice was measured by radioimmunoassay (RIA) as previously described.20 Under anesthesia induced with 40 mg/kg pentobarbital IP, the right kidney was isolated and homogenized (n=5). The homogenate was concentrated using Sep-Pak C18 cartridges and then dissolved in 100 μL of RIA buffer.

Isolated Perfused Kidney
Male AM TG (n=7), KO (n=6), and WT (n=6) mice were anesthetized with 40 mg/kg pentobarbital IP, then the right kidney was isolated and perfused as previously described.12,21 In brief, after an abdominal incision, we punctured the superior mesenteric artery with a needle and positioned the tip in the right renal artery. Perfusion was then started at 1.5 mL/min with Krebs-Henseleit buffer, and the kidney was isolated without ischemia. The buffer was saturated with 95% O2/5% CO2 at 37°C and contained 10−6 mol/L angiotensin II and 10−7 mol/L indomethacin to maintain perfusion pressure at about 100 mm Hg. Renal perfusion pressure (RPP) was monitored at the renal artery through the needle connected to a pressure transducer (Datex-Ohmeda K.K., Tokyo).

Measurement of NO Release
NO concentration in the perfusate was measured using a chemiluminescence assay.22 The venous effluent was introduced into a rotatory mixer with a chemiluminescence probe of 10 mmol/L H2O2, 18 μmol/L recrystallized luminol, 2 mmol/L potassium carbonate, and 150 mmol/L desferrioxamine. The mixture of the perfusate and probe then entered a chemiluminescence detector. The chemiluminescent signal was measured continuously and recorded on a standard pen recorder. The NO signal was calibrated using an NO solution.

Following a 60-minute equilibrium period, graded doses of ACh or AM were added to the buffer at 10-minute intervals through a 3-way cock. The responses to L-arginine methyl ester (L-NAME), an NO synthase (NOS) inhibitor, AM (22-52) and CGRP (8-37), AM receptor antagonists, or E-4021 (Eisai Co., Ltd.), a phosphodiesterase (PDE) inhibitor, were studied in the same manner. E-4021 is a type-V PDE inhibitor that has been reported to selectively inhibit cGMP-specific PDE.23 To evaluate the effects of NO or PDE inhibition, the effects of N5-monomethyl-L-arginine (L-NMMA) or E-4021 infusion on RPP were examined. NO release was normalized by kidney weight and expressed as fmol per minute per gram kidney weight.

Ischemic Acute Renal Failure
Twelve-week-old AM TG (n=10), KO (n=10), and WT (n=10) male mice were used in the present study. The in vivo model of iARF was prepared as described elsewhere.24 In brief, after anesthesia with pentobarbital sodium (40 mg/kg IP), a midline abdominal incision was made and bilateral renal arteries were clamped with plastic clips for 45 minutes after the injection of heparin (10 U/kg, IM); thereafter, the clamps were removed and the incision was closed. Twenty-four hours after the start of reperfusion, 0.3 mL arterial blood was drawn to determine the serum levels of urea nitrogen and creatinine. Thereafter, the kidneys were perfused with saline for histological studies. The NOS inhibitor, L-NAME (30 mg/kg), was administered by gavage to 5 of each mice group before induction of renal ischemia.

Histological Studies
Samples of renal tissue from the sham-operated and ischemic animals were fixed in 10% formaldehyde, stained with periodic acid-Schiff’s (PAS) reagent, and examined under an optical microscope in a blinded manner. The kidneys were histologically examined for the presence of dilatation of Bowman’s space, tubular dilatation and necrosis, loss of tubular epithelium, and tubular casts.

The degree of renal injury was evaluated using the criteria reported by Solez et al.24

Measurement of NOS Activity
The activity of NOS in vitro was determined by the conversion of L-[14C] arginine to L-[14C] citrulline, according to the method described previously.25 The renal medulla was dissected and homogenized in lysis buffer. Forty μL of the sample was incubated in 100 μL of assay buffer containing 0.5 μCi/mL L-[14C] arginine and incubated for 20 minutes at 37°C. To separate L-[14C] arginine from L-[14C] citrulline, the samples were loaded onto 1-mL columns of Dowex resin (AG50WX-8 Na+ form) and eluted with 500 μL distilled water. Aliquots were used for liquid scintillation counting. Calcium-dependent activity was determined as the difference between the L-[14C] citrulline produced from control samples and samples containing 3 mmol/L EGTA to bind calcium.

Drugs and Chemicals
Laboratory reagents and chemicals used to prepare Krebs-Henseleit solution and H2O2 were purchased from Wako Pure Chemicals (Osaka). AM, AM(22-52), and CGRP(8-37) were from the Peptide Institute (Osaka). All other chemicals were from Sigma-Aldrich Japan (Tokyo).

Statistical Analysis
Data are expressed as the mean±SEM. Statistical comparisons were made by analysis of variance followed by the Student-Neumann-Kuels test. To compare renal injury scores, the nonparametric
Kruskal-Wallis test was used. Differences with a value of \( P < 0.05 \) were considered statistically significant.

**Results**

**Renal Contents of AM**

We measured the renal contents of AM in the 3 groups of mice by RIA. As shown in Figure 1A, AM contents in the kidney of AM TG mice were significantly greater than WT mice, whereas those of AM KO mice were significantly less. These results were compatible with the findings of our previous reports.\(^{14,15}\)

**Isolated Perfused Kidney**

Kidneys from KO, TG, and WT mice were macroscopically normal and their weight did not greatly differ among the 3 groups. Baseline RPP in KO mice was significantly higher, whereas that in TG mice was lower, compared with RPP in WT mice kidneys (Figure 1B).

Figure 2 shows the effects of ACh on RPP and NO release in the 3 groups of mice kidneys. ACh lowered RPP of kidneys in either group in a dose-related manner. The degree of renal vasodilation caused by ACh was smaller in TG mice than in WT mice. The ACh-induced vasodilation was associated with an increase in NO release from the kidney. NO release caused by ACh was greater in TG mice and smaller in KO mice, compared with WT mice. Thus, TG mice kidneys showed hyporesponsiveness to NO. The responses to AM in the 3 groups of mice were similar to those to ACh (data not shown). Figures 3A and 3B show the effects of AM receptor antagonists AM(22-52) and CGRP(8-37) on RPP, respectively. Both antagonists alone significantly elevated RPP in a dose-related fashion. The increase in RPP was significant even in KO and WT mice. The response decreased in the following order: TG > WT > KO.

In order to explore the involvement of the NO-cGMP pathway in AM-induced vasodilation, we examined the effects of L-NMMA and E-4021 on RPP. As shown in Figure 3C, L-NMMA alone increased RPP in all 3 groups of kidneys. In contrast, E-4021 alone reduced RPP (Figure 3D). These changes in RPP caused by L-NMMA and E-4021 were dose-related and the responses decreased in the following order: TG > WT > KO.

**Ischemic Acute Renal Failure**

No mice died after renal ischemia/reperfusion. Serum concentrations of urea nitrogen markedly increased 24 hours after reperfusion in WT mice (Figure 4A). Decreases in renal excretory function were significantly greater in KO mice than in WT mice. However, the increases in urea nitrogen levels were significantly smaller in TG mice. Serum creatinine levels changed almost in parallel with urea nitrogen levels in the 3 groups of mice (Figure 4B). Pretreatment with L-NAME did not change the increases in serum urea nitrogen levels in WT and KO mice, whereas it significantly increased them in TG mice, resulting in no differences in serum urea nitrogen or creatinine levels among the 3 groups of mice.

Changes in renal excretory function induced by ischemia were confirmed by histological analysis. Figure 5 shows the renal histology of the 3 groups of mice. In WT mice, marked damage of renal tissues, particularly in the tubuli, were observed; these included detachment of epithelial cells of the proximal tubuli, interstitial edema, and tubular casts. Expansion of Bowman’s space was also observed. AM KO mice also had marked tubular damage. However, in AM TG mice...
renal injury was apparently mild. In fact, all 4 types of damage scores were significantly less marked in TG mice compared with the other 2 groups of mice. Figure 6 shows the means of 4 types of injury scores in each group of mice. AM KO mice kidneys subjected to ischemia showed significantly greater scores than WT mice and AM TG mice. Pretreatments with L-NAME also diminished differences in renal injury scores among the 3 groups of mice.

Renal NOS Activity
As shown in Figure 7, calcium-dependent NOS activity in the renal medulla was greater in TG mice, whereas it tended to be less in KO mice. Ischemia/reperfusion decreased the NOS activity in all 3 groups of mice. However, the NOS activity was still greater in TG mice kidneys. On the other hand, calcium-independent NOS activity was not detected in the kidney of any group of animals.

Discussion
Endothelial cells secrete many circulating and local vasoactive hormones. Among them, endothelin-1 and NO are potent vasoconstrictive and vasodilative substances, respectively, and they play important roles because their gene disruption results in death or in a hypertensive reaction, respectively. However, it has not been clarified whether endothelium-derived AM is a significant factor in the regulation of circulation.

The plasma concentration of AM in healthy subjects is at a picomolar level. However, the vasodilatory effects of AM appear at concentrations higher than 10^{-10} mol/L, according to previous reports. In the present study, significant vasodilation in the renal vessels and aorta was observed also at about 10^{-9} mol/L. That is, the physiological circulating level of AM is about a hundredth of the effective concentration on arteries, although it is possible that the vascular wall may be exposed to locally high concentrations of AM. Receptor antagonists are useful tools to explore the role of endogenous ligands. We examined 2 kinds of AM receptor antagonists, CGRP(8-37) and AM(22-52). Both antagonists increased the vascular tone in renal vessels precontracted by angiotensin II in a dose-related manner. This suggests that endogenous AM in the vasculature shows tonic inhibition on vasoconstrictive stimuli. This phenomenon may be explained by some agonistic action of the antagonists, particularly at higher concentrations. In fact, AM(1-25), a truncated peptide of AM, exerts vasoconstrictive activity. However, CGRP derivatives have not been reported to cause vasoconstriction.

Figure 3. Effects of adrenomedullin (AM) receptor antagonists, AM(22-52) (A) and CGRP(8-37) (B), on renal perfusion pressure (RPP) in TG, WT, and KO mice. Effects of E-4021 (C) and N^G-monomethyl-L-arginine (L-NMMA) (D) on renal perfusion pressure (RPP) in TG, WT, and KO mice. *P<0.05, †P<0.01 vs WT.

Figure 4. Serum urea nitrogen (A) and creatinine levels (B) in mice with ischemic acute renal failure. (I/R) indicates ischemia/reperfusion; L-NAME, N^G-nitro-L-arginine methyl ester - I/R.
Furthermore, because endothelial denudation of the aorta diminished the vasoconstriction caused by receptor antagonists (unpublished observation, 2001), it is unlikely that the two antagonists per se increased vascular tone. Thus, it appears that the antagonists-induced vasoconstriction is attributed to a direct blockade of the action of endogenous AM.

It is striking that the receptor antagonists significantly increased RPP even in WT mice, although vasoconstriction was greater in TG mice and less in KO mice. Baseline perfusion pressure in the kidney isolated from KO mice was significantly higher than that from WT mice. These findings suggest that endogenous AM actually regulates renal vascular tone under physiological conditions, at least in mice. Furthermore, the decrease in endogenous AM production by 50% may contribute to increases in renal vascular resistance. It is not exceptional that a 50% reduction of gene expression influences its function. For example, heterozygote eNOS KO mice showed slightly higher blood pressure and greater susceptibility to pulmonary hypertension due to hypoxia than WT mice. Although it is unknown whether there is a pathological state associated with a reduced production of AM, various cardiovascular diseases are associated with several-fold increases in circulating AM. In TG mice, expression of AM in the aorta and kidney increased by 2- to 5-fold. Therefore, increased AM in patients with hypertension and chronic renal failure may play a compensatory role with regard to blood pressure regulation. On the other hand, in septic shock in which the highest level of plasma AM was reported, AM may be one of the factors contributing to hypotension. In the present study, compared with WT mice, TG mice showed 2-fold increase and KO mice about 50% decrease in AM. Thus, the AM levels in TG and KO mice were pathological rather than physiological. In iARF, AM KO mice subjected to ischemia/reperfusion had significantly higher serum urea nitrogen and creatinine levels than WT mice. Furthermore, histological analysis revealed that renal damage was significantly more severe in KO mice than in WT mice. These results suggest that high levels of AM may mitigate renal damage and decreases of AM below the physiological level may abrogate it.

Not only AM-induced vasodilation but also ACh-induced vasodilation was attenuated in AM TG mice. This mechanism is unclear from the present study; however, eNOS TG mice

**Figure 5.** Photographs of renal histology showing tubular casts, interstitial edema, epithelial detachment, and expansion of Bowman’s capsule from AM transgenic (TG) mice, AM knockout (KO) mice, and wild-type (WT) mice.

**Figure 6.** Effects of ischemia and preischemic treatment with L-NAME on renal injury scores in TG, WT, and KO mice. Values are means of 4 types of injury scores. (I/R) indicates ischemia/reperfusion; L-NAME, L-NAME/7/H11001/I/R.

**Figure 7.** NO synthase activity in the kidneys of TG, WT, and KO mice. NO synthase activity was measured based on the conversion of L-arginine to L-citrulline. (I/R) indicates ischemia/reperfusion. *P<0.05 vs WT; †P<0.05 vs WT(I/R).
also showed hyporesponsiveness to endothelium-dependent vasodilators, but not to endothelium-independent vasodilators. In these mice, the activity of soluble guanylate cyclase and the expression of cGMP-dependent protein kinase decreased, probably due to continuous activation of the NO-cGMP pathway. A similar kind of downregulation of the signal transduction of the NO system may occur in AM TG mice. However, this mechanism may not totally explain the differences in vasodilatory responses of the 3 groups of mice because NO synthase inhibition increased RPP in AM TG mice to a greater extent. The responses to L-NMMA or E-4021 reflected the baseline NO releasing rates. The response of TG mice to NO may be attenuated. However, blood pressure and RPP in TG mice were still significantly lower, suggesting that the total vascular effects of endogenous NO in TG mice may overwhelm the attenuated response and be greater than those in WT mice at the baseline level. Thus, the differences in the responses to L-NMMA and E-4021 suggest the great differences in baseline NO release among the mice groups. On the other hand, when NO release was stimulated, nearly maximal renovasorelaxation in AM TG mice may at least in part explain the mechanism for attenuated vasodilatory responses to ACh. This is compatible with the findings of previous reports that L-NMMA caused greater vasoconstriction under increased NO release, including eNOS transgenic mice.

In the present study, 24-hour reperfusion following 45-minute ischemia caused iARF in mice. Although many factors may involve renal injury caused by ischemia/reperfusion, the role of NO has been considered to be important; however, it is still controversial as to whether NO increases or decreases and as to which NO synthase is responsible for the change in NO in iARF. We have recently reported that tetrahydrobiopterin (BH4), a cofactor of NO synthase, of renal tissue is deficient in iARF and that replacement of BH4 restored renal endothelium-derived NO and improved renal injury in rats. On the other hand, inhibition of NO synthesis by L-NAME aggravated renal injury. These findings suggest that endothelium-derived NO exerts cytoprotective effects in ischemia/reperfusion and its decrease enhances tissue injury. From this point of view, in TG mice the degree of renal injury and renal excretory dysfunction was less than in WT mice, suggesting that AM-induced NO release may contribute to mitigation of ischemic renal injury. In fact, renal NOS activity increased in AM TG mice. Pretreatment with L-NAME abolished the differences in renal function between TG mice and WT mice, supporting this possibility. It remains undetermined as to how NO mitigates ischemia/reperfusion injury. However, it is possible that NO is a potent antioxidant and traps superoxide during the reperfusion period. Furthermore, it has been reported that NO suppresses excessive increases in intracellular calcium by increasing cGMP. NO-induced vasodilation also contributes to it. AM is reported to protect cultured endothelial cells from apoptosis and this effect is mediated by the stimulation of NO production by endothelial cells.

From the results of L-NMMA administration and citrulline assay, we supposed that AM-induced renal protection might be exerted via NO release. The citrulline assay showed that the NOS activity was much lower in the renal cortex of all groups of mice than in the medulla. This finding was consistent with that of most previous studies, which found that NOS activity in the renal cortex was less than 10% of that in the medulla. Because it is difficult to differentiate a calcium-dependent activity of NOS from a calcium-independent one, we did not analyze the small activity detected in the renal cortex. Although it is possible that NO released in the cortex may also work, it is suggested that NO derived from medullary vessels and tubuli would play a major role in protecting renal function.

In conclusion, using genetically manipulated animals, we found that endogenous AM actually regulates renal vascular tone even by relatively small changes in its production. Furthermore, AM may exert a cytoprotective role in ischemic injury through its NO-releasing activity.

Acknowledgments

This study was supported in part by Grants-in-Aid Nos. 10218202, 13470141, and 13557061 from the Japanese Ministry of Education, Culture, Sports and Science. We thank Marie Morita, Reiko Sato, and Etsuko Taira for technical assistance.

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Circ Res. 2002;90:657-663; originally published online February 21, 2002;
doi: 10.1161/01.RES.000013697.55301.E7
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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