Hypotension and Resistance to Lipopolysaccharide-Induced Shock in Transgenic Mice Overexpressing Adrenomedullin in Their Vasculature

Takayuki Shindo, MD, PhD; Hiroki Kurihara, MD, PhD; Koji Maemura, MD, PhD; Yukiko Kurihara, MD, PhD; Tomoyuki Kuwaki, PhD; Taro Izumida, MD, PhD; Naoto Minamino, PhD; Ki-Hwan Ju, PhD; Hiroyuki Morita, MD, PhD; Yoshio Oh-hashi, MD; Mamoru Kumada, MD, PhD; Kenji Kangawa, PhD; Ryozo Nagai, MD, PhD; Yoshio Yazaki, MD, PhD

Background—Adrenomedullin (AM) is a vasodilating peptide involved in the regulation of circulatory homeostasis and in the pathophysiology of certain cardiovascular diseases. To determine the extent to which chronic AM overproduction affects circulatory physiology under normal and pathological conditions, we used a preproendothelin-1 promoter to establish transgenic mouse lines overexpressing AM in their vasculature.

Methods and Results—Transgenic mice overexpressing AM mainly in vascular endothelial and smooth muscle cells exhibited significantly lower blood pressure (BP) and higher plasma cGMP levels than their wild-type littermates. Blockade of NO synthase with \( N^G \)-monomethyl-L-arginine elevated BP to a greater degree in AM transgenic mice, offsetting the BP difference between the 2 groups. Despite their lower basal BP, administration of bacterial lipopolysaccharide elicited smaller declines in BP and less severe organ damage in AM transgenic mice than in wild-type mice. Furthermore, the 24-hour survival rate after induction of lipopolysaccharide shock was significantly higher in the transgenic mice.

Conclusions—A chronic increase in vascular AM production reduces BP at least in part via an NO-dependent pathway. In addition, smaller responses to LPS in transgenic mice suggest that AM is protective against the circulatory collapse, organ damage, and mortality characteristic of endotoxic shock. (Circulation. 2000;101:2309-2316.)

Key Words: adrenomedullin ■ genes ■ vasculature ■ blood pressure ■ shock

Adrenomedullin (AM), a polypeptide originally isolated from human pheochromocytoma,¹ has been characterized as a potent endothelium-derived vasodilator² whose structure resembles those of calcitonin gene–related peptide and amylin. As with calcitonin gene–related peptide, the vasodilatory effects of AM are mediated in part by an elevation in cytoplasmic cAMP leading to relaxation of vascular smooth muscle cells.³ In addition, AM acts on endothelial cells to stimulate nitric oxide (NO) production via \( Ca^{2+} \)-dependent activation of endothelial constitutive NO synthase (eNOS), which may also contribute to the vasodilatation.⁴,⁵

Normally, AM is found primarily in the plasma, adrenal medulla, heart, lung, and kidney.⁶ In addition, plasma AM levels are elevated in various pathological states, including hypertension, renal failure, heart failure, and endotoxic shock.⁷–⁹ Together with its potent biological activity, these findings lead us to speculate that AM may participate in the regulation of blood pressure (BP) and pathophysiology of various cardiovascular diseases. In particular, AM expression is dramatically upregulated in endotoxic shock, suggesting that AM may play a role in endotoxin-induced circulatory collapse together with other vasoactive factors. However, whether the increased AM production is beneficial or harmful to patients remains unknown, and from a clinical viewpoint, this remains a very important issue.¹⁰–¹²

To clarify the physiological and pathophysiological functions of AM, mouse models in which AM production has been genetically manipulated serve as excellent tools. In particular, vessel-specific overexpression of AM can focus on the role of AM in the regulation of vascular tone and its disorder as an autocrine/paracrine factor. For this purpose, we used the murine preproendothelin-1 (PPET-1) promoter to establish transgenic mouse lines overexpressing AM in their vasculature.
vasculature, after which the phenotype was analyzed with reference to BP regulation and the pathophysiology of endotoxic shock.

Methods

Transgene

A rat AM cDNA fragment containing the entire open reading frame was obtained from rat lung cDNA by reverse transcription–polymerase chain reaction with primers deduced from the previously reported sequence. The amplified 0.72-kb fragment was then inserted into a plasmid containing a 9.2-kb fragment of the murine PPET1 gene 5′-flanking region, a 131-bp sequence of exon 1, and a 0.7-kb SV40-derived sequence with an additional intron and poly-A signal (Figure 1A).

Generation of Transgenic Mice

The transgenic construct was liberated from the vector by XhoI digestion and purified by agarose gel electrophoresis and a GeneClean kit (BIO101). Donor eggs were then prepared from B6C3F1 mice. After microinjection with the construct, eggs were transferred into the oviducts of pseudopregnant ICR foster mothers. Founder mice were identified by Southern blot analysis of tail DNA carried out with a 2-kb fragment of the PPET1 promoter sequence as a probe. When the genomic DNA was digested by HindIII, a 6.0-kb transgene band was distinguishable from the 8.0-kb authentic gene band. All experiments were performed in accordance with the Declaration of Helsinki and were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Northern Analysis

Total RNA samples were extracted from tissues with RNAzol (BIOTEX). A 370-bp fragment of the 3′-noncoding region of the AM cDNA was used for 32P-labeled antisense riboprobe, and the RNA samples were subjected to Northern blot analysis. The blots were rehybridized with a radiolabeled mouse GAPDH riboprobe for an internal standard of each sample.

Radioimmunoassay

Tissue and plasma AM levels were measured by radioimmunoassay with an anti-AM antibody recognizing the C-terminal amide structure common to both human and rat AM as reported previously. Plasma cGMP levels were measured with a radioimmunoassay kit (Yamasa).

Immunohistochemistry

Tissue samples were embedded in OCT compound and cut into 8-μm frozen sections on a cryostat. After preincubation with goat
nonimmune serum, tissue sections were serially treated with a rabbit anti-rat polyclonal AM antibody, biotinylated goat anti-rabbit IgG, and avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories) and then developed with 0.004% H2O2 and 0.02% diaminobenzidine tetrahydrochloride. As a negative control, some samples were incubated with preimmune serum instead of the primary anti-rat AM antibody.

BP Measurement
Male transgenic mice (8 to 10 weeks old) heterozygous for the transgene and their wild-type littermates were anesthetized with halothane and ventilated with room air (Harvard rodent ventilator model 683), and a femoral artery in each mouse was cannulated with polyethylene tubing (ID 0.28 mm, OD 0.61 mm). After the mice were allowed to recover, pulsatile BP was recorded with the mice conscious and unrestrained and was analyzed as described previously. To examine the effect of the NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA; Sigma), a second catheter was placed in the femoral vein and BP was recorded for 30 minutes under halothane anesthesia, after which L-NMMA (250 μmol/kg in 0.5 mL/kg 0.9% saline) was injected intravenously.

In those experiments in which bacterial lipopolysaccharide (LPS) was used to induce shock (see below), BP was measured indirectly with a tail cuff and a 98A Softron system.

LPS-Induced Shock Model
Eight- to 10-week-old male transgenic heterozygotes and wild-type mice, chosen from their littermates of the same age and sex, were injected intraperitoneally with 100 μg/kg body wt LPS (Escherichia coli, serotype 055: BE, BACTO 3923–25–9) and 8 mg D-galactosamine (D-GalN) (Wako) in 0.2 mL pyrogen-free 0.9% saline. At the same time, 100 μmol/kg body wt of L-NMMA in 0.05 mL pyrogen-free 0.9% saline was administered into the tail veins of some mice. Survival rate was calculated at 24 hours after LPS and D-GalN administration. Blood samples were drawn from the inferior vena cava 5 hours after LPS administration, and measurements were made for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH), 3 markers of liver injury. For histological examination, organs were collected 6 hours after LPS treatment. Formalin-fixed and paraffin-embedded specimens were cut into 6-μm sections and stained with hematoxylin-eosin.

Statistical Analysis
Data are expressed as mean±SEM. Student’s t test and χ2 test were used to determine significant differences. Values of P<0.05 were considered significant.

Results
Establishment and Characterization of AM Transgenic Mice
Microinjection of the transgenic construct into fertilized mouse eggs gave rise to 350 live-born offspring whose overall appearance, body weight, behavior, and fertility were not distinguishing. Among them, however, 28 were founders carrying the transgene, which was identified by Southern blot. Furthermore, among those carrying the transgene, Northern blot analysis confirmed that 8 lines also transcribed it (Figure 2A). Two lines, designated AMC35 and AMC15, were found to be high expressers of the transgene and were selected for further experimentation. The former carried 30 copies of the transgene, and the latter carried 5 copies. Immunoreactive (ir)-AM levels in plasma and tissues were elevated in these lines (Figure 2B). In AMC35 mice, for example, plasma AM levels were 2.3-fold higher than in their wild-type littermates, and tissue AM levels were elevated 2.1-fold in the kidney and 8.5-fold in the aorta.

Localization of AM transgene expression was analyzed immunohistochemically. ir-AM was heavily labeled in endothelial cells and medial smooth muscle cells in the aortas of transgenic mice (Figure 3A and 3B). In wild-type mice, by contrast, AM expression was only faintly detected in the aortic wall (Figure 3C). Similarly, in the kidneys of transgenic mice, ir-AM was detected in the glomeruli and arterioles, whereas little ir-AM was detected in the kidneys of wild-type mice (Figure 3E through 3G). These patterns of AM expression were identical to those of PPET1-luciferase, confirming that the same vasculature-selective expression of the transgene occurred when its expression was driven by the PPET1 promoter.
BP in AM Transgenic Mice

The effect of AM overexpression on arterial BP was assessed mainly in the AMC35 line. BP, which was measured in 8 transgenic and 14 wild-type mice while they were conscious and unrestrained, was significantly lower in the transgenic mice (109.3 ± 4.7 versus 124.4 ± 2.7 mm Hg; *P* < 0.01) (Figure 4A). No significant change in the heart rate accompanied the reduction in BP (738.5 ± 19 versus 738.4 ± 12 bpm) (Figure 4B). In the AMC15 line, BPs fell between those of the AMC35 and the wild-type mice (data not shown).

Figure 3. Immunohistochemical labeling of aortas and kidneys of representative transgenic mice and their wild-type littermates. A and B, Aortic sections from a transgenic mouse. Note that endothelial and medial smooth muscle cells were intensely labeled with AM. C, Aortic section from a wild-type mouse showing faint labeling in aortic wall. D, Control section from a transgenic mouse labeled with preimmune serum. E through G, Kidney sections from transgenic and wild-type mice. E, Section from a transgenic mouse. Specific AM labeling was detected in glomeruli and arterioles. F, Section from a wild-type mouse. No specific signals were detected. G, Control section from a transgenic mouse labeled with preimmune serum. Magnification ×100 (A, C through G) and ×400 (B).
AM causes vasodilatation through 2 mechanisms: a direct effect on smooth muscle cells and an indirect effect through NO release from endothelial cells. To determine the extent to which the reduced BP seen in transgenic mice was due to increased NO release, we studied the effect of L-NMMA, an NOS inhibitor, on BP under halothane anesthesia. BP was also significantly lower in anesthetized transgenic mice than in their wild-type littermates (79.0 ± 2.4 versus 86.8 ± 2.2 mm Hg; P < 0.05) (Figure 5A). Interestingly, the pressor response elicited by intravenous injection of L-NMMA was significantly higher in transgenic mice (21.1 ± 3.3% versus 10.7 ± 1.3%; P < 0.01) (Figure 5A). Interestingly, the pressor response elicited by intravenous injection of L-NMMA was significantly higher in transgenic mice (21.1 ± 3.3% versus 10.7 ± 1.3%; P < 0.01) (Figure 5A). Moreover, plasma cGMP concentrations were significantly higher in transgenic than in wild-type mice (Figure 5B), all of which are indicative of steady-state activation of the NO-cGMP pathway.

Responses to LPS Administration in AM Transgenic Mice

The bacterial LPS model was used to evaluate the effects of AM overexpression on organ damage and survival in septic shock. At the dosages used, LPS plus D-GalN lowered BP in wild-type mice by ~25 mm Hg within 3 hours, whereas the BP decrease in transgenic mice (AMC35) was only ~10 mm Hg (Figure 6A and 6B). Thus, despite relatively low baseline pressure, transgenic mice were less sensitive to LPS-induced hemodynamic changes than wild-type mice.

On macroscopic inspection after treatment with LPS, the most prominent finding in wild-type mice was a dark-colored and swollen liver (Figure 7A). Histological examination revealed hemorrhagic inflammation with neutrophil infiltration and severe hepatocyte damage (Figure 7C). In contrast, such changes were far less severe in transgenic mice (Figure 7B and 7D). The number of infiltrated neutrophils counted from 5 cross sections of liver was...
significantly lower in transgenic mice (wild-type, 993.2 ± 32.0/mm²; transgenic, 455.5 ± 36.7/mm²; P, 0.0001). Consistent with the comparatively minimal liver damage, serum ALT, AST, and LDH levels were all significantly lower in transgenic mice than wild-type mice (Figure 7E).

Survival was monitored after injection of LPS, and it was found that 44% of LPS-treated, wild-type mice (11 of 25), 78% of AMC35 mice (11 of 14), and 75% of AMC15 mice (12 of 16) survived for 24 hours (P, 0.05 in both AMC35 and AMC15) (Figure 7F). There were no deaths among mice treated with either saline or D-GalN alone. The survival rate among wild-type mice was unaffected by administration of L-NMMA, although it tended to decrease the survival rate among transgenic mice, thereby offsetting the difference in the survival rates (Figure 7F). Thus, consistent with the elevated plasma cGMP concentrations, the protective effect of AM overproduction against LPS-induced shock is probably mediated by an NO-dependent mechanism.

Discussion
In the present study, we established several lines of transgenic mice overexpressing AM primarily in the systemic vasculature and in the glomeruli and arterioles of the kidney. The selective overexpression of the AM gene in the vascular wall was achieved by use of the murine PPET1 promoter, which was previously shown to direct in vivo expression of a luciferase reporter gene within the intima and media of the aorta and other arteries of the systemic vasculature. Consistent with the elevation of plasma cGMP concentrations, the protective effect of AM overproduction against LPS-induced shock is probably mediated by an NO-dependent mechanism.

Hypotension in AM Transgenic Mice
One notable phenotypic characteristic of AM transgenic mice was the comparatively low BP in both conscious and anesthetized animals. It has been known that AM can elicit potent and long-lasting depressor effects in rats, sheep, and humans. For instance, an intravenous bolus administration of 3 mmol/kg AM can decrease BP by as much as 50 mm Hg. It was not clear, however, whether steady-state and physiologically relevant changes in AM production would also affect BP. Khan et al found that in rats, chronic infusion of AM with an osmotic minipump caused significant reductions in BP at plasma AM concentrations that were within the physiological range. In that context, the present results confirm that steady-state elevation of vascular AM production and the resultant elevation of plasma AM can induce a stable hypotensive response even when plasma AM levels remain within the physiological range.

Our experiments with L-NMMA and measurement of plasma cGMP suggest that AM-induced increases in NO synthesis are responsible for the reduced BP seen in AM transgenic mice. This is consistent with accumulating evidence that AM causes vasodilation via NO-cGMP-dependent as well as cAMP-dependent pathways. Hirata et al reported that AM decreased renal vascular resistance and increased NO release in the perfused rat kidney, effects that were reversed by L-NMMA. In cultured aortic endothelial cells, AM was found to mobilize intracellular free Ca²⁺, which can stimulate eNOS activity and increase levels of cGMP. Furthermore, chronic overexpression of eNOS in transgenic mice bred with the same promoter was recently shown to cause hypotension. It follows, then, that overexpression of AM, which would be expected to lead to increased eNOS activation, should reduce BP in transgenic mice.

Resistant to LPS-Induced Shock in AM Transgenic Mice
Septic shock, which has a high mortality rate due to circulatory collapse and fatal organ damage, is a systemic inflammatory process induced by LPS or other microbial products. During septic shock, plasma AM concentrations are known to be dramatically increased, more so than for any other pathological state. It is currently unknown, however, whether the elevated AM is beneficial or harmful to patients suffering from septic shock. To address that question using AM transgenic mice, we mimicked septic shock through intraperitoneal administration of LPS plus D-GalN and observed...
that LPS-induced depressor responses and liver damage were significantly less severe in transgenic mice than in wild-type mice. Furthermore, survival was greatly improved by the overexpression of AM, clearly indicating that steady-state increases in basal AM production are protective against LPS-induced shock.

Evidence now suggests that vasoactive peptides play key roles in the pathophysiology of endotoxin shock. For example, endothelin-1

Figure 7. A and B, Macroscopic examination of abdominal organs 6 hours after LPS administration. A, Representative LPS-treated wild-type mouse. Note prominent swelling and dark color of liver. B, Representative LPS-treated transgenic mouse. Liver appears intact, without swelling or color change. C and D, Histological examination of liver 6 hours after LPS administration. C, Liver of an LPS-treated, wild-type mouse. Note prominent hemorrhagic changes, neutrophil infiltration, and hepatocyte vacuoles. D, Liver of an LPS-treated transgenic mouse. Histological changes were minimal except for neutrophil infiltration of sinusoids. Bars represent 50 μm in ×200 magnification. E, Levels of liver enzymes in serum 5 hours after administration of LPS/D-GalN to transgenic mice (AMC35 and AMC15) and their wild-type littersmates. #P<0.05, *P<0.01 vs wild-type mice. F, Survival rates among LPS/D-GalN–treated transgenic and wild-type mice, with (solid columns) or without (open columns) L-NMMA administration. Survival was monitored for 24 hours. #P<0.05 vs wild-type mice.
(ET-1) expression in the liver is markedly induced after LPS treatment. The elevated ET-1 production is postulated to cause perisinusoidal cell constriction and to disrupt the microcirculation of the liver, exacerbating liver injury. Sinusoidal cells are composed of Ito cells (of smooth muscle lineage) and fat-sorting cells (hepatic stellate cells), on which ET-1 receptors are abundantly expressed. In primary culture, addition of AM causes relaxation of stellate cells, whereas ET-1 causes their constriction. Thus, AM may counteract the harmful effect of ET-1 on the hepatic microcirculation during endotoxic shock.

We found that L-NMMA administration tended to cancel the survival advantage afforded by overexpression of AM, suggesting that AM-activated NO production contributes to the protective effect of AM against shock. NO is currently regarded as a key factor in the pathophysiology of septic shock, and expression of the NO synthase-1 gene is generally repressed in an in vivo shock model. LPS and cytokines, including IL-1 and tumor necrosis factor-α, are strong stimulators of inducible NO synthase expression in cultured smooth muscle cells and macrophages. In addition, the inhibition of NO synthesis during septic shock promotes hepatic damage by compromising organ blood flow, which suggests that NO serves a protective function against circulatory disruption, most likely by virtue of its vasodilatory properties. It follows, then, that increased basal release of NO in AM transgenic mice, as is indicated by the effects of L-NMMA on BP and cGMP overproduction, would also contribute to the resistance to LPS-induced liver injury seen in AM transgenic mice.

Still other mechanisms may contribute to the beneficial effect of AM overproduction. For example, recent observations suggest that AM may regulate secretion of inflammatory factors, such as cytokine-induced neutrophil chemotactant, from alveolar macrophages. Although further studies are needed to completely define the mechanism underlying the involvement of AM in septic shock, the present study clearly shows that overexpression of AM is protective against LPS-induced shock and provides a clue to a novel therapeutic strategy for the treatment of endotoxic shock.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan; a Research Grant for Cardiovascular Diseases (11C-1) from the Ministry of Health and Welfare; the Ryoichi Naito Foundation for Medical Research; the Tokyo Biochemical Research Foundation; and the Mochida Memorial Foundation for Medical and Pharmaceutical Research. Dr Y. Kurihara is a Research Fellow of the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan.

References

Hypotension and Resistance to Lipopolysaccharide-Induced Shock in Transgenic Mice Overexpressing Adrenomedullin in Their Vasculature

Takayuki Shindo, Hiroki Kurihara, Koji Maemura, Yukiko Kurihara, Tomoyuki Kuwaki, Taro Izumida, Naoto Minamino, Ki-Hwan Ju, Hiroyuki Morita, Yoshio Oh-hash, Mamoru Kumada, Kenji Kangawa, Ryozo Nagai and Yoshio Yazaki

_Circulation_. 2000;101:2309-2316
doi: 10.1161/01.CIR.101.19.2309

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/101/19/2309

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/