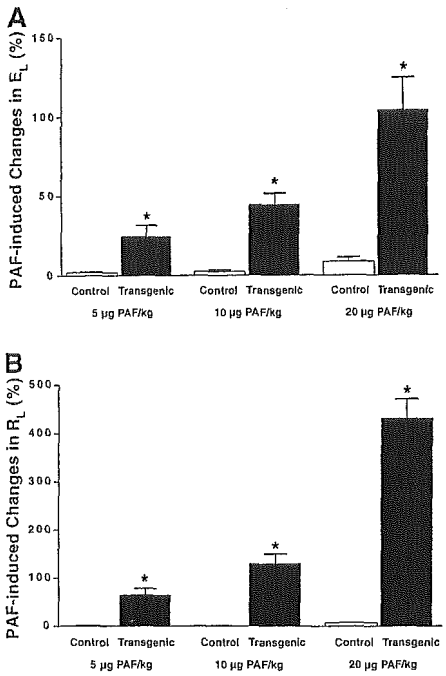

Figure 4. PAF-induced responses in EL (*A*) and RL (*B*) in control and PAFR-transgenic mice (n = 3 or 4 for each group). *p < 0.001 compared with control mice.

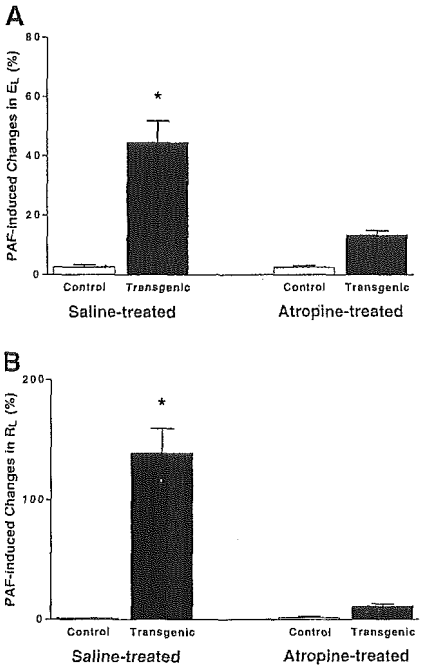

Figure 5. Effects of atropine on PAF-induced responses in EL (*A*) and RL (*B*) (n = 4 for each group). *p < 0.001 compared with other groups.

control and PAFR-transgenic mice (0.224 ± 0.018 and 0.215 ± 0.015, respectively), suggesting that the airway structure of PAFR-transgenic mice is not altered as compared with that of control mice.

DISCUSSION

The results of the experiments reported here show that airway responsiveness to MCh and PAF in PAFR-transgenic mice was significantly greater than in control mice. Blockade of the muscarinic pathway with atropine inhibited PAF-induced responses in the PAFR-transgenic mice, whereas the overexpression of PAFR did not change the binding activity of muscarinic receptor. Similarly, airway responsiveness to 5-HT was unaffected by overexpression of the PAFR gene, and morphometric analyses showed that airway structure, including airway smooth muscle, was not altered by overexpression of the PAFR gene. These findings suggest that the muscarinic pathway may have a key role in AHR associated with overexpression of the PAFR gene. Thus, expression of the PAFR gene is involved in airway responsiveness to MCh in mice through a functional but not a structural mechanism.

Before the results of the study are discussed, the issue of anesthesia warrants consideration. In the study, it was necessary to reduce the vagal function of the animals used, since effects of the experimental procedures on vagal activity might have affected the results and interpretation of data. We therefore chose to use anesthesia with pentobarbital sodium and ketamine hydrochloride in combination with one another. It has been demonstrated that both pentobarbiturate and ketamine have inhibitory effects on vagal pathways (27–29). Although the vagus nerve was not surgically sectioned, we believe that vagal function was significantly attenuated with the anesthesia applied in this study and that comparisons between the control and PAFR-transgenic mice are meaningful.

The airway responsiveness to MCh of the PAFR-transgenic mice was markedly greater than that of control mice, suggesting that the transgenic mice have an asthmalike phenotype. There was also a marked difference in MCh-induced changes in lung elasticity between the asthmalike and normal phenotypes (Figure 2). It has been reported that changes in EL reflect lung parenchymal alterations and stiffening of the lungs induced by various contractile stimuli (30), although the contraction of conducting airways can also cause changes in EL (31). By comparison, increases in RL represent decreases in airway luminal cross-sectional area (30). In the present study, enhanced responses in both EL and RL to MCh administration were observed in the PAFR-transgenic mice, suggesting that overexpression of the PAFR gene elicits increased responses to MCh in both the lung parenchyma and airways.

It has been postulated that PAF may be related to AHR in various species, including human (14, 15). The administration of exogenous PAF increases airway responsiveness in humans (20), whereas a specific PAFR antagonist (Y-24180) reduces AHR to MCh in asthmatic patients (32). In their recent study, Henderson and colleagues (19) found that increasing plasma levels of PAF acetylhydrolase through its administration was effective in blocking late-phase pulmonary inflammation in a murine model of asthma. However, the exact mechanism for the involvement of PAF in AHR remains to be clarified. In the current study, the molecular and pathophysiologic mechanisms underlying AHR were examined with PAFR-transgenic mice overexpressing the PAFR gene.

One possible mechanism for the involvement of PAF in AHR is that PAF and PAFR gene expression affect airway structure, and especially the lamina propria and airway smooth muscle. Airway remodeling, including thickening of airway smooth muscle, is a feature in asthmatic subjects and could be

TABLE 1. RESULTS OF BINDING ASSAYS FOR MUSCARINIC RECEPTOR

	Kd (*nM*)	Bmax (*fmol/mg protein*)
Control membrane + vehicle*	0.51 ± 0.04	109.2 ± 16.3
Control membrane + PAF*	0.55 ± 0.02	122.2 ± 21.2
PAFR-transgenic membrane + vehicle*	0.56 ± 0.06	130.3 ± 2.3
PAFR-transgenic membrane + PAF*	0.54 ± 0.06	127.4 ± 0.3

Definition of abbreviations: Bmax = binding maximum; Kd = dissociation constant for PAF from PAF receptor; PAF = platelet activating factor; PAFR = platelet activating factor receptor.

* n = 3 for each group.

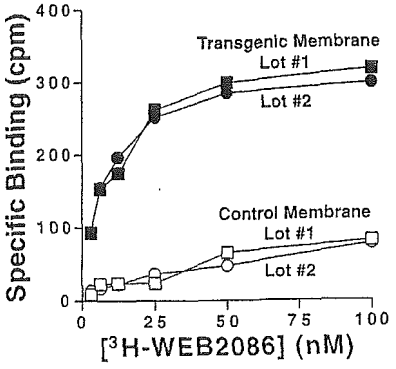

Figure 6. Binding assays for PAFR in control and PAFR-transgenic mouse lung membranes, showing isoforms for binding of [^{3}H]-labelled WEB2086 to membrane fractions. Because of low specific binding to control membranes, Scatchard analysis was not performed. Results shown are means from duplicate wells. Two independent membrane fractions (Lots #1 and #2), from whole lungs for each genotype, were used.

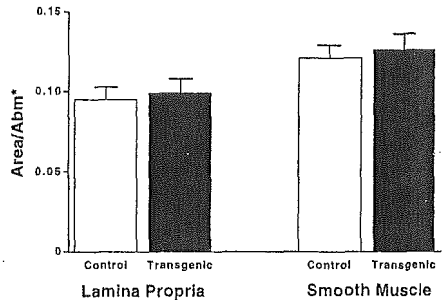

Figure 7. Thickness of lamina propria and airway smooth muscle in control and PAFR-transgenic mice. Area/Abm* = area normalized with ideally relaxed airway size.

involved in bronchial hyperresponsiveness (33, 34). Lambert and Paré have shown that a marked increase in airway responsiveness is theoretically induced by thickening of the airway wall, including its smooth muscle layer (33). In the current study, however, no significant difference in thickness of either the lamina propria or airway smooth muscle was observed in the control as opposed to the PAFR-transgenic mice. These results suggest that overexpression of the PAFR gene has little effect on airway remodeling in mice. Whereas PAF may have proliferative effects on various cells (35), it seems that in the mouse model used in our study, the effect of PAF on airway smooth muscle proliferation is not remarkable. In this model, AHR elicited by overexpression of the PAFR gene may be associated with airway dysfunction, but not with airway remodeling.

It has been shown that in PAFR-transgenic mice, a high level of transgenic mRNA exists in the trachea (12), and one could assume from this that airway smooth-muscle cells overexpressing PAFR may play a role in PAF-induced bronchopulmonary responses. Consistently, the binding activities for PAFR in the PAFR-transgenic mouse lungs in our study were remarkably augmented in comparison with those of the controls. After each dose of PAF given *in vivo*, marked pulmonary responses were observed in the PAFR-transgenic mice as compared with the controls. Notably, the effects of PAF were more remarkable at the higher doses in the PAFR-transgenic mice, indicating that PAF *per se* has a significant role in the AHR of PAFR-transgenic mice to PAF. On the other hand, blockade of the muscarinic pathway completely ablated PAF-induced responses in the PAFR-transgenic mice. This indicates that PAF-induced bronchoconstriction is mediated by an atropine-sensitive pathway. Stimler-Gerard (36) has postulated that PAF may stimulate neural elements proximal to the end plate to release constrictive neurotransmitters including acetylcholine, which might explain the mechanism for the findings in our study.

TABLE 2. MORPHOMETRIC DATA FOR AIRWAYS

	Control Mice	PAFR-Transgenic Mice
Total number of airways	40	40
Number of airways obtained from individual animals	10.0 ± 0.7	10.0 ± 0.4
Pbm, mm	0.780 ± 0.039	0.772 ± 0.040
D_2/D_1	0.831 ± 0.015	0.826 ± 0.015

Definition of abbreviations: D_2/D_1 = index of airway roundness (airways with a D_1/D_2 ratio > 0.6 were analyzed); PAFR = platelet activating factor receptor; Pbm = length of basement membrane.

The results of the binding assays indicate that PAFR overexpression *per se* has little effect on the binding activity of muscarinic receptors in either the absence or presence of PAF. The interaction between PAF and muscarinic receptors may therefore instead occur through an indirect pathway. It has been reported that PAF may affect the biologic actions of other potent mediators, such as thromboxanes and leukotrienes, which are involved in AHR (37). Potentially, overexpression of the PAFR gene may modulate the production levels of these potent mediators.

Airway responsiveness to 5-HT was not affected by overexpression of the PAFR gene. This observation suggests that PAF and the PAFR gene may be specifically related to bronchial responsiveness to MCh, but not to 5-HT. Recently, it was demonstrated that AHR induced by 5-HT and acetylcholine is inherited independently in mice, and that murine nonspecific AHR is determined by multiple genes (38). The results of the present study indicate that mutation of the PAFR gene affects muscarinic receptor-specific responsiveness, but not general bronchial responsiveness.

Genetic features are potentially associated with the etiology of asthma. On the basis of the inheritance pattern of bronchial asthma a number of genes could have substantial roles in its pathogenesis (39). Murine models of asthma have recently been used to investigate individual genes associated with AHR (40). Since PAF may be one of the potent mediators involved in bronchial asthma (14, 15, 20), genes regulating the function and metabolism of PAF could be targets of study in the pathogenesis of asthma. These genes consist of the PAFR gene (11, 41) and genes encoding PAF-metabolic enzymes, including PAF acetylhydrolases (42, 43). The PAFR-transgenic mice used in the present study may contribute to study of the genetic roles of PAF in bronchial asthma.

PAF has pleiotropic and pathophysiologic effects on various cells and organs (1–4). It exerts its actions at concentrations as low as 10^{-12} M in some cells, and almost always at levels of at least 10^{-9} M as an intercellular messenger (2). PAF activates phospholipase A_2 (PLA_2), protein kinase C, protein tyrosine phosphorylation, and gene expression (1, 2, 44). It has been shown that PAFR knockout mice exhibit markedly reduced anaphylactic responses as compared with wild-type mice, suggesting that PAF plays an important role in the development of anaphylactic shock (13). On the other hand, PAFR-transgenic mice show an increased lethality to bacterial lipopolysaccharide endotoxin (12). Melanogenesis and melanocytic proliferation were observed in the skin of PAFR-transgenic mice, and melanoma was occasionally seen in aged PAFR-transgenic mice (12). Recent studies done with genetically-engineered mice have shown that cytosolic PLA_2 ($cPLA_2$) is essential in the production of PAF (24, 45), indicat-

ing that both $cPLA_2$ and PAF are key mediators in the development of inflammatory disorders. For example, both $cPLA_2$ and PAF play significant roles in the molecular mechanism underlying acute lung injury (46–48). The PAFR-transgenic mice used in our study may further provide novel insights for study of the pathophysiologic roles of PAF and PAFR *in vivo*.

In summary, the PAFR-transgenic mice in our study exhibited hyperresponsiveness to MCh and PAF, but not to 5-HT. The muscarinic pathway may have a key role in PAF-induced responses in these PAFR-transgenic mice. Meanwhile, binding activities for muscarinic receptor were not altered by the overexpression of PAFR. We observed no differences in airway structure between the control and PAFR-transgenic mice to suggest that PAFR gene overexpression would be involved in MCh airway responsiveness by acting as a functional mediator. The PAFR-transgenic mice may provide appropriate models for studying molecular and pathophysiologic mechanisms underlying diseases related to PAF metabolism.

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Resistance to Neointimal Hyperplasia and Fatty Streak Formation in Mice With Adrenomedullin Overexpression

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Objective—Several in vitro studies have implicated that adrenomedullin (AM) plays an important role in the pathogenesis of vascular injury and fatty streak formation. To test this possibility in vivo, we evaluated 2 experimental models using transgenic mice overexpressing AM in a vessel-selective manner (AMTg mice).

Methods and Results—Placement of a periarterial cuff on femoral arteries resulted in neointimal formation at 2 to 4 weeks to a lesser extent in AMTg mice than in their wild-type littermates (at 28 days, intima/media area ratio 0.45±0.14 versus 1.31±0.41, respectively; $P<0.001$). This vasculoprotective effect observed in AMTg mice was inhibited by N^{ω}-nitro-L-arginine methyl ester. We further examined the effect of AM on hypercholesterolemia-induced fatty streak formation by crossing AMTg mice with apolipoprotein E knockout mice (ApoEKO mice). The extent of the formation of fatty streak lesions was significantly less in ApoEKO/AMTg mice than in ApoEKO mice (percent lesion area 12.0±3.9% versus 15.8±2.8%, respectively; $P<0.05$). Moreover, endothelium-dependent vasodilatation as indicative of NO production was superior in AMTg/ApoEKO mice compared with ApoEKO mice.

Conclusions—Taken together, our data demonstrated that AM possesses a vasculoprotective effect in vivo, which is at least partially mediated by NO. (*Arterioscler Thromb Vasc Biol.* **2002;22:1310-1315.**)

Key Words: adrenomedullin ■ transgenic mice ■ neointimal hyperplasia ■ fatty streak ■ apolipoprotein E knockout mice

Many vasoactive factors derived from endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are known to regulate regional vascular tone as well as cellular proliferation and migration, thereby affecting vascular structure. Dysregulated production of these factors is assumed to make a contribution to pathological states, such as hypertension, atherosclerosis, and postangioplasty restenosis.

Adrenomedullin (AM), originally identified as a potent vasodilatory peptide produced by human pheochromocytoma,[1] has been shown to be released from ECs[2] and, to a lesser extent, VSMCs.[3] In addition to its direct[4] and NO-mediated[5] vasodilatory activities, in vitro studies have demonstrated its direct inhibitory effect on the migration[6] and proliferation[7] of VSMCs and its antiapoptotic effect on ECs[8,9] via NO. Therefore, it is suggested that AM has a compensatory effect on deteriorating physiological status and a protective effect against vascular injury as a possible counterpart against vasoconstrictive and mitogenic peptides, including angiotensin II and endothelin-1. However, direct evidence for such in vivo roles has still not been well documented.

Recently, we established transgenic mice overexpressing AM (AMTg mice) in a vessel-selective manner driven by the murine preproendothelin-1 promoter.[10] AMTg mice exhibited an increase in AM levels by 2- to 4-fold in the plasma and major organs and by 2- to 8-fold in the aorta by radioimmunoassay. Immunohistochemistry detected intense AM signals in both ECs and VSMCs in the aorta and major arteries as well as in arterioles in vascular-rich organs, including the heart, kidney, and lung. Concerning hemodynamics, chronic AM overexpression in the vessel wall resulted in decreased blood pressure through, at least in part, the stimulation of NO production. We postulated that these mice could also be useful for analyzing the role of AM in the pathophysiological process associated with vascular injury and atherosclerosis. In the present study, we compared the formation of injury-induced neointimal hyperplasia and hypercholesterolemia-

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induced fatty streak formation between mice with and without AM overproduction to clarify whether AM could be involved in the protective mechanisms in the vasculature.

Methods

The experiments were performed in accordance with the Declaration of Helsinki and the University of Tokyo Institutional Guidelines for Animal Experiments. The mice were housed in an animal room with a 12-hour light-dark cycle and a temperature of 22°C. The mice were given standard chow and water ad libitum, unless otherwise indicated.

Animals

The AMTg mouse line has already been established as previously described.[10] We crossed AMTg mice with apoE knockout (ApoEKO) mice.[11] To obtain AMTg/ApoEKO mice, homozygous AMTg mice were bred with homozygous ApoEKO mice. The resultant offspring, which overexpressed AM and were obligatorily heterozygous for the mutant apoE locus, were further bred with each other to obtain homozygous ApoEKO mice with or without the AM transgene. The parental ApoEKO mice were F2 hybrids between 129/Sv and C57BL/6, and the parental AM transgenic mice originated from hybrids from the B6C3F1 and C3H strains, which were backcrossed for at least 5 generations into the original background of the ApoEKO mice.

Hemodynamics

We evaluated blood pressure with a programmable sphygmomanometer (BP98 Softron) by use of the tail-cuff method.[12] All measurements were repeated 10 times for each animal, and the average was obtained. To evaluate the effect of blood pressure reduction, several mice were treated with hydralazine at a dose of 5 mg/kg per day in their drinking water.[13]

Vascular Injury by Cuff Placement

We used 10- to 12-week-old AMTg and wild-type mice from the same genetic background. The cuff placement surgery was performed according to a method described previously.[14,15] In brief, the mice were anesthetized with ketamine (70 mg/kg) and xylazine (4 mg/kg) by intraperitoneal injection. The left femoral artery was isolated from the surrounding tissues. A polyethylene tube (PE-50, 2 mm long, inner diameter 0.56 mm, outer diameter 0.965 mm; Becton-Dickinson) was cut longitudinally, loosely placed around the artery, and closed with a suture. After the experimental period, the mice were killed, and arterial tissues were fixed in 10% formalin and embedded in paraffin. The middle segment of the artery was cut into 5 subserial cross sections at intervals of 200 μm. The sections were stained by elastica van Gieson or hematoxylin and eosin staining. The areas of the neointima, media, and adventitia were measured by using image-analyzing software (NIH image). The average of 5 sections was taken as a representative value for each animal. To evaluate DNA synthesis, bromodeoxyuridine (BrdU, Sigma Chemical Co) was injected at doses of 100 mg/kg SC and 30 mg/kg IP 18 hours before euthanasia and then at a dose of 30 mg/kg IP 12 hours before euthanasia.[16] Immunohistochemistry using anti-BrdU antibody in serial sections was performed (BrdU Staining Kit, Zymed Laboratories), and the BrdU index (the ratio of BrdU-positive nuclei versus total nuclei) was calculated.

To evaluate the chronic inhibition of NO synthase, N^{ω}-nitro-L-arginine methyl ester (L-NAME) was dissolved in the drinking water at a concentration of 200 mg/L[17] for 28 days. Moreover, several mice were treated with the AM receptor antagonist CGRP(8-37) to evaluate the effect of AM antagonism. Alzet micro-osmotic pumps (model 1002, Alza) were implanted intraperitoneally at the time of cuff placement. The pumps delivered vehicle (saline) or CGRP(8-37) (20 mg/kg per day, Peptide Institute)[18] continuously for 28 days at a rate of 0.125 μL/h.

Evaluation of Fatty Streak Formation

Two diets were used: (1) a normal chow diet (MF diet from Oriental Yeast Co) that contained 5.6% (wt/wt) fat with 0.09% (wt/wt) cholesterol, and (2) an atherogenic diet, which consisted of the MF diet containing 0.15% (wt/wt) cholesterol and 15% (wt/wt) butter.[19] Eight-week-old ApoEKO/AMTg and ApoEKO mice were fed the atherogenic diet for 2 months and euthanized by an overdose of anesthetic, and the extent of fatty streak formation was evaluated by 2 methods: (1) the en face surface lesion area[20] and (2) the cross-sectional lesion area of aortic origin.[21] In brief, for evaluation of the entire aorta, the aorta from the aortic sinus to the iliac bifurcation was dissected, and lipid-rich atheroma were visualized by staining with Sudan IV. As cross-sectional lesions of the aortic sinuses, 4 serial sections at intervals of 60 μm were prepared, stained with oil red O, and counterstained with hematoxylin.

Vascular Reactivity to Endothelium-Dependent and -Independent Vasodilators

Vascular reactivity to endothelium-dependent and -independent vasodilators was tested by using the descending thoracic aorta to evaluate endothelial function as previously described.[22] The aortic rings were suspended under 1.0 g of tension and preconstricted with phenylephrine (5×10^{-8} mol/L). Acetylcholine (ACh) (10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (SNP, 10^{-8} to 10^{-5} mol/L) were added cumulatively to the organ bath. For ACh-induced vasodilatation after blockade of NO production, L-NAME (10^{-5} mol/L) was administered to evaluate endogenous NO production. Data are expressed as the percentage relaxation of phenylephrine-induced preconstriction.

Statistical Analysis

Quantitative values are expressed as the mean±SD. Comparisons of means were made by using the Student t test for unpaired values; when >2 means were compared, an ANOVA with repeated measurements was used. If a significant F value was found, the Scheffé post hoc test for multiple comparisons was used to identify any differences among groups. A value of $P<0.05$ was considered significant, unless otherwise indicated. In aortic ring experiments, the negative logarithm of the concentration of ACh or SNP that produced half the putative maximal response was referred to as ED_{50}. Maximum vasorelaxation was shown as E_{max} (a percentage).

Results

Neointimal Formation Induced by Cuff Placement

We compared neointimal formation induced by cuff placement between AMTg and wild-type mice. In contralateral sham-operated arteries, there was no neointima in either the wild-type or AMTg mice (Figure 1A). On the other hand, cuff placement resulted in neointimal hyperplasia, which grew for up to 28 days. Intimal hyperplasia was significantly smaller in AMTg mice than in wild-type mice at 14 days and also at 28 days after surgery (Figure 1A). Twenty-eight days after surgery, the ratio of the neointima area to the intimal/medial area was 16 390±1890 μm^2/1.31±0.41 in wild-type mice (n=10) and 5830±1620 μm^2/0.45±0.14 in AMTg mice (n=10, $P<0.001$; Figure 1B).

For DNA synthesis of VSMCs, we examined BrdU uptake 3 and 7 days after injury. BrdU uptake in subendothelial layers mainly composed of VSMCs was significantly higher in the wild-type mice (n=7) than in the AMTg mice (n=7). BrdU incorporation was relatively high within 1 week after injury and almost completely diminished ≥14 days after injury in AMTg and wild-type mice. These data indicate that AM overexpression inhibited the proliferation of VSMCs (Figure 2A).

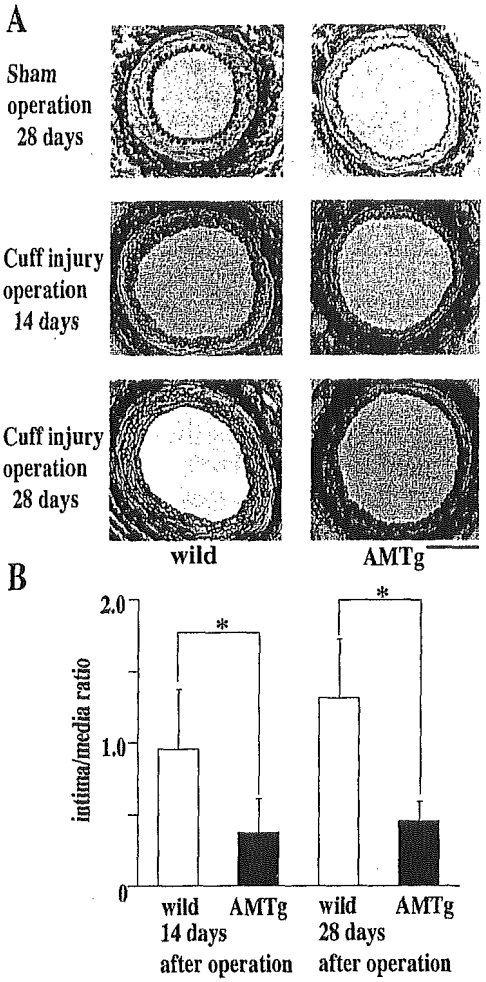

Figure 1. A, Photomicrographs showing representative cross sections of the femoral arteries 14 and 28 days after cuff placement. Sections of 5-μm thickness were stained by elastica van Gieson staining. B, Morphometric analysis of the cuffed femoral arteries. Cross-sectional areas of media and intima were evaluated at 14 days and 28 days after cuff placement, and the intima/media ratio was calculated from the area of intima divided by the area of media. *$P<0.001$.

Inflammatory cell infiltration around the vessels was prominently observed, especially within 1 week after injury. The number of inflammatory cells was not different between AMTg and wild-type mice at 3 and 7 days after injury, indicating that the inflammatory process at the adventitia may not be affected by AM overexpression (Figure 2B).

Effect of L-NAME, Hydralazine, and CGRP(8-37) on Neointimal Hyperplasia

The systolic blood pressure measured by the tail-cuff method was significantly lower in the AMTg mice than in the wild-type mice (101.2±9.3 versus 115.3±13.2 mm Hg, respectively; $P<0.05$). When L-NAME was chronically administered, the systolic blood pressure was significantly elevated, and the difference between the 2 groups diminished (136.5±7.3 versus 141.2±11.4 mm Hg for AMTg versus wild-type mice, respectively; P=NS). For the vascular injury, the vasculoprotective effect in AMTg mice was diminished, and there was no difference in the neointimal response

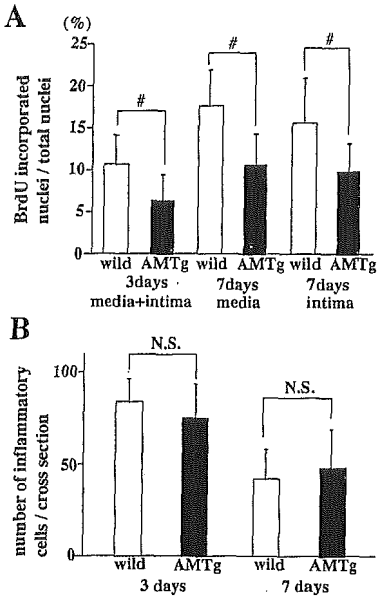

Figure 2. A, BrdU uptake in the media and neointima of the cuffed femoral arteries. The BrdU index (the number of BrdU positive nuclei/number of total nuclei) was analyzed 3 and 7 days after cuff placement. B, Number of inflammatory cells invading around injured arteries. The average of 5 sections was taken as the value for each animal. Error bars indicate SD. #$P<0.05$. NS indicates not significant.

between the AMTg and wild-type littermates (Figure 3A), suggesting that the vasculoprotective action of AM is mediated via an NO-dependent mechanism. Next, to confirm that the vasculoprotective effect of AM is independent of its hypotensive effect, we evaluated the neointimal responses between wild-type mice with and without hydralazine administration. The systolic blood pressure of wild-type mice treated with hydralazine was 98.3±8.5 mm Hg, which was almost equivalent to that of AMTg mice. We evaluated the neointimal formation 28 days after injury in the wild-type mice treated with hydralazine, finding that the intima/media ratio was 1.25±0.50, which was not significantly different from that of wild-type mice without hydralazine treatment (1.31±0.41, P=NS; Figure 3B). Therefore, the protective effect of AM was independent of the pressure reduction. Moreover, to test the effect of AM antagonism, we treated wild-type mice and AMTg mice with CGRP(8-37) or vehicle. This demonstrated that CGRP(8-37) infusion partially inhibited the beneficial effect of AM overexpression compared with vehicle administration and did not significantly affect neointimal formation in the wild-type mice (Figure 3C).

Fatty Streak Formation in ApoEKO and AMTg/ApoEKO Mice

Next, we examined the protective effect of AM on fatty streak formation by breeding AMTg with ApoEKO mice. In AMTg/ApoEKO mice, AM transgene expression was detected in the aorta, heart, lung, and kidney, as evaluated by reverse transcription–polymerase chain reaction and Northern blot analysis (data not shown), and plasma AM levels evaluated by radioimmunoassay[10] were significantly higher in AMTg/ApoEKO mice than in ApoEKO mice

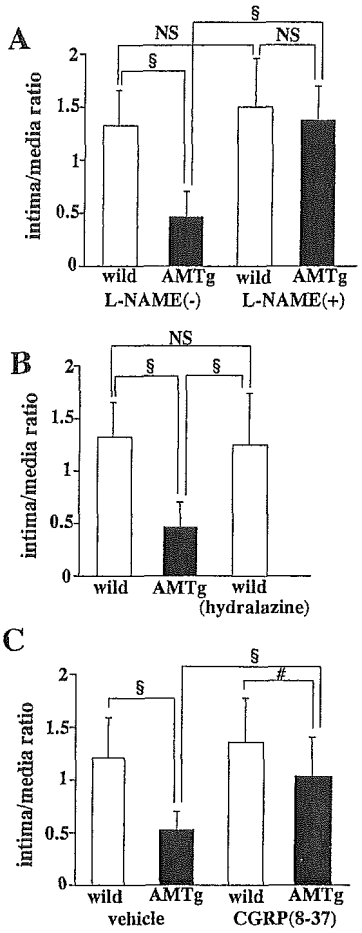

Figure 3. Effects of several drug interventions on neointimal formation. A, L-NAME. B, Hydralazine. C, CGRP(8-37). #$P<0.05$ and §$P<0.01$.

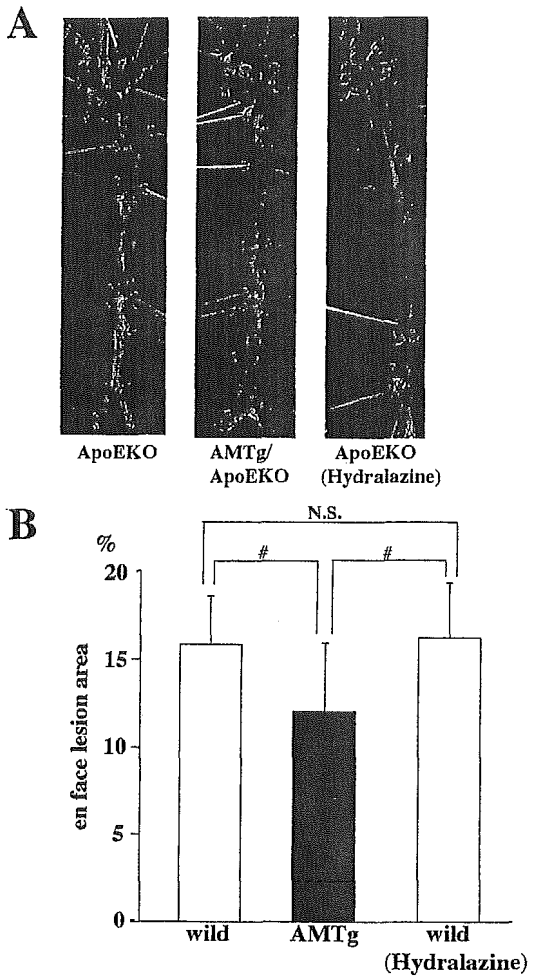

Figure 4. Hypercholesterolemia-induced atherosclerotic lesions. A, Representative photographs of en face atherosclerotic lesions in the entire aorta. B, Percentage of en face atherosclerotic lesions. #$P<0.05$.

(8.36±2.32 versus 4.93±1.07 fmol/mL, respectively; $P<0.01$; n=7 each).

ApoEKO mice and ApoEKO/AMTg mice were fed normal chow until they were 8 weeks old, and then they were fed the atherogenic diet for 2 months. Plasma lipid and lipoprotein concentrations were markedly elevated after an atherogenic diet. However, there were no significant differences in lipid profiles between the AMTg/ApoEKO mice and ApoEKO mice (data not shown). The AMTg/ApoEKO mice (n=16) were significantly more hypotensive than the ApoEKO mice (n=16); systolic blood pressure was 110±15 versus 125±14 mm Hg, respectively ($P<0.01$). Thus, to evaluate the direct effect of hypotension on fatty streak formation, we also evaluated the hydralazine-treated ApoEKO mice, whose systolic blood pressure (104±17 mm Hg) was almost equivalent to that of the AMTg/ApoEKO mice. The representative photographs of en face surface atherosclerotic lesions are shown in Figure 4A. The fatty streak formation of the AMTg/ApoEKO mice (n=12) was significantly smaller than that of the ApoEKO mice (n=12); the en face lesion area was 12.0±3.9% versus 15.8±2.8%, respectively ($P<0.05$, Figure 4B). The same result was observed in the cross-sectional lesion area (223 000±56 000 versus 290 000±45 200 μm^2 for AMTg/ApoEKO mice versus ApoEKO mice, respectively; $P<0.01$). However, the atheromatous lesion formation was not changed in ApoEKO mice when they were treated with hydralazine (en face lesion area 16.2±3.1%, cross-sectional lesion area 301 400±52 100 μm^2; P=NS), suggesting that AM inhibits fatty streak formation via a mechanism other than its hypotensive effect.

Endothelial Function and NO Production

To test endothelial function in ApoEKO/AMTg mice and ApoEKO mice, we examined endothelium-dependent and -independent vasodilatation with the use of aortic rings isolated from mice fed the atherogenic diet (n=8 for each group, Figure 5). As controls, wild-type mice and AMTg mice were used for the aortic ring experiment. There was no significant difference in the tension after preconstriction by phenylephrine among the 4 groups. The relaxation induced by ACh was significantly deteriorated in the apoE-deficient mice compared with the AMTg and wild-type mice. However, endothelium-dependent vasorelaxation was significantly better in ApoEKO/AMTg mice (E_{max} 94.6±2.5%, ED_{50} [−log molar] 7.31±0.14) than in ApoEKO (E_{max} 85.2±4.6%, ED_{50} 6.83±0.25; $P<0.05$). Concerning the 2 control groups, the

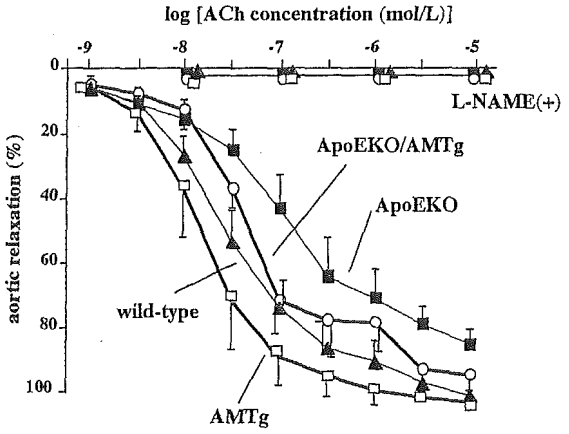

Figure 5. Endothelium-dependent vasorelaxation of aortas from the 4 groups: wild-type (closed triangle), AMTg (open square), ApoEKO (closed square), and AMTg/ApoEKO (open circle) mice (n=8 to 10). Values are mean±SEM. #$P<0.05$.

ED_{50} was also significantly better in AMTg mice (7.39±0.30) than in wild-type mice (7.77±0.16). However, there was no significant difference in E_{max} between AMTg mice (97.7±2.4%) and wild-type mice (99.7±0.4%, P=NS). On the other hand, there was no significant difference in aortic relaxation in response to SNP among the 4 groups. ACh-induced relaxation was almost completely attenuated by L-NAME, suggesting that the vasorelaxation induced by ACh is almost exclusively mediated by endothelium-derived NO. These data suggest that endothelial function was preserved by AM overexpression when damaged by remarkable hypercholesterolemia. Moreover, plasma cGMP levels were also significantly higher in AMTg/ApoEKO mice (7.9±2.0 pmol/mL) than in ApoEKO mice (6.1±1.8 pmol/mL, $P<0.05$), implicating that the steady-state NO production was also upregulated in mice with AM overexpression. On the other hand, the endothelial NO synthase (eNOS) expression levels in aorta detected by reverse transcription–polymerase chain reaction were not different between mice with and without AM overexpression (data not shown), suggesting that the function of eNOS may be regulated by AM at the posttranslational or protein levels.

Discussion

In the present study, we examined the involvement of AM in the formation of vascular lesions by analyzing 2 different experimental models. We evaluated neointimal formation induced by cuff placement and hypercholesterolemia-induced fatty streak formation and found that mice carrying AM transgenes were significantly resistant to vascular injury and fatty streak formation. This is the novel important evidence that supports the vasculoprotective effect of AM in vivo.

Rodents are naturally resistant to neointimal hyperplasia and fatty streak formation. Therefore, several experimental models of neointimal hyperplasia or atherosclerosis have been proposed. In the present study, we adopted the cuff-injury model, in which the endothelial layer remains intact and thrombus formation is very rare because no intravascular manipulation is performed. Using this model, we demonstrated that AM overexpression suppressed neointimal hyperplasia and that BrdU-positive nuclei in the subendothelial area were reduced in the cuff-injured femoral arteries, indicating that the proliferation of VSMCs was downregulated by AM. This evidence is consistent with previous in vitro experiments.[7] On the other hand, there was no significant difference in inflammatory cells around the injured arteries between AMTg and wild-type mice, suggesting that inflammation may not be affected by AM overproduction.

AM can act on endothelial cells to stimulate NO production via calcium-dependent activation of eNOS.[5,23] We tested whether inhibition of NO production diminished the protective effect of AM. Chronic administration of L-NAME abolished the beneficial effect of AM overexpression on vascular injury. Therefore, the vasculoprotective effect of AM was thought to be at least partially mediated by NO. The beneficial effect of NO produced by endothelium has been previously demonstrated in many reports. eNOS knockout mice have been reported to exhibit abnormal intimal hyperplasia after vascular injuries in the cuff placement.[14] eNOS overexpression has also been shown to inhibit neointimal formation significantly in the rat carotid balloon injury model.[24] These previous studies and the present result lead us to postulate that the activation of eNOS by AM may be effective in preventing the formation of vascular proliferative lesions.

Shimizu et al[18] reported that the AM antagonist CGRP(8-37) inhibits neointimal hyperplasia after balloon injury in the rat carotid artery, indicating that endogenous AM in the injured tissue may promote the proliferation of VSMCs. This observation seems to contradict our data. In the present study, AM overexpression inhibited neointimal formation, and CGRP(8-37) infusion partially inhibited the beneficial effect of AM overexpression. This discrepancy may be due to differences in the experimental animals and systems. In our 2 models, the endothelial layers remained intact, and AM was overexpressed mainly in the endothelium. On the other hand, in a rat carotid artery balloon-injury model, the endothelial layers were denuded, and the beneficial effect of AM on endothelial layers was abolished. The injured VSMCs were exposed directly to the blood stream; thus, the phenotypic changes in VSMCs might occur more easily. Concerning other type of cells, AM has an antiproliferative effect of glomerular mesangial cells[25] but stimulates cell proliferation in Swiss 3T3 cells.[26] Therefore, it is likely that the effect of AM on cell proliferation depends on the phenotypes or experimental situations.

To date, apoE knockout mice have been useful in the analysis of cholesterol-induced fatty streak formation. Several experiments on crossbreeding with other genetically altered mice or on pharmacological intervention in ApoEKO mice have been reported.[19,27] Thus, we crossbred AMTg mice with ApoEKO mice to clarify the effect of AM on hypercholesterolemia-induced fatty streak formation. We demonstrated that the presence of AM transgenes suppresses the formation of atheromatous lesions and that this is independent of the blood pressure reduction and lipoprotein profiles. It is worth noting that the vasculoprotective effect of AM was also confirmed in this model.

Endothelial dysfunction is characterized by impaired endothelium-derived NO-mediated vasorelaxation in atherosclerosis in humans and experimental animals. A previous report[28] demonstrated that endothelial dysfunction was impaired in isolated aortic rings of ApoEKO mice compared

with wild-type mice, which was replicated in the present study. In the present study, we found that endothelium-dependent vasodilatation was superior in AMTg/ApoEKO mice compared with ApoEKO mice, although severe dyslipidemia damaged endothelium-dependent vasorelaxation significantly. Moreover, the plasma cGMP level (taken to be indicative of steady-state NO production) was significantly higher in ApoEKO/AMTg mice than in ApoEKO mice. Therefore, our data suggest that the antiatherogenic effect of AM in ApoEKO is at least partially caused by endothelium-derived NO production by chronic AM overexpression.

In summary, we demonstrated the beneficial effects of AM in vivo against vascular injury at least partially via endothelial NO production with the use of genetically altered mice. However, further studies are needed to clarify the precise mechanism of the vasculoprotective effect of AM so that AM can be applied clinically for atherosclerosis and for postangioplasty neointimal hyperplasia.

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Evaluation of the atrial natriuretic peptide gene in stroke

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Abstract

The atrial natriuretic peptide (ANP) gene was, though inconclusive, implied to be etiologically related to stroke in rats and recently in humans. The present study tested the candidacy of ANP for stroke susceptibility by a combination of molecular genetic approaches. First, we undertook an association study using a reported ANP variant, G664A, in two case-control panels independently collected, which involved 970 Japanese subjects. Second, we compared the rat ANP gene sequences and neighboring marker alleles among stroke-prone SHR (SHRSP), normal SHR and WKY of an original inbred colony and we also compared brain ANP expression between SHRSP and normal SHR. In humans, we found no significant association between the 664A variant and stroke in the studied population. In rats, 21 polymorphic sites were identified by direct sequencing of 2170-bp ANP fragments, from which two distinct alleles, SHRSP- and WKY-types, were inferred. From a genealogical point of view, our data indicated that an SHRSP-type allele could not play a determinant role in stroke-proneness. Overall results did not support the disease relevance of ANP, disagreeing with previous reports. Thus, considerable caution should be taken when one attempts to transfer findings in the animal model to humans.

Keywords: Atrial natriuretic factor; Stroke; Genetics; Single nucleotide polymorphism; Association

1. Introduction

Several lines of evidence have supported the substantial involvement of genetic factors in the pathogenesis of stroke [1]. However, studies of the molecular genetics of stroke are not necessarily facile, especially in humans, due to a number of complex features of the disease, e.g. coexistent multifactorial disorders, such as hypertension, diabetes and dyslipidemia. An alternative strategy would be to first explore the predisposition to stroke in the animal model and thereafter, test the corresponding findings in humans, instead of investigating certain candidate genes without any clue. Among animal models thus developed to date, the stroke-prone spontaneously hypertensive rat (SHRSP) is considered of particular interest, since it promotes severe hypertension from the early age of life and also manifests the propensity for stroke on a high salt and low potassium diet [2–4]. Accordingly, this animal has been widely used as a model organism to investigate the etiological relationship between hypertension and stroke.

Recently, two study groups have undertaken genome-wide searches of quantitative trait loci (QTLs) for 'stroke-associated' phenotypes in F2 progeny derived from SHRSP [5,6] and have suggested that predisposition to stroke is at least, in part, under genetic controls independent of hypertension. One group explored genetic susceptibility for latency until the manifestation

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of stroke in F2 progeny between SHRSP and normal (or stroke-resistant) SHR [5], while the other did so for infarct volume after the middle cerebral artery (MCA) occlusion in F2 progeny between SHRSP and Wistar Kyoto (WKY) rats [6]. These genome screens successfully identified four QTLs on three separate rat chromosomes, among which a region around the atrial natriuretic peptide (ANP) gene has drawn substantial attention because markers in the vicinity of ANP on rat chromosome 5 were significantly linked to both phenotypic traits.

Then, the following questions may be asked: (1) whether the ANP gene itself can underlie either (or both) of the 'stroke-associated' phenotypes? (2) whether findings in rats are directly extendable to human stroke? Remarkably, one of the study groups above-mentioned has further proposed ANP as a candidate susceptibility gene based on two lines of evidence. First, SHRSP of a Heidelberg colony (SHRSP/Heidelberg) showed alterations in structure, expression and in vitro function of ANP compared to normal SHR [7]. Second, a case-control study involving 696 white subjects indicated that a single nucleotide polymorphism, G664A, located in exon 1 of the human ANP gene was associated with the risk for stroke [8]. On the contrary, SHRSP of a Glasgow colony (SHRSP/Glasgow) showed no significant differences in either structure or expression of ANP compared to WKY [9]. Rat ANP data from two study groups thus appear discordant and need further confirmation.

Under these circumstances, the present study was designed to evaluate the candidacy of ANP in human and rat stroke. Initially the ANP association was tested in two case-control panels involving 970 Japanese subjects; 270 brain infarction patients and 359 sex- and age-matched controls were included in one panel and 178 patients and 163 controls were included in another panel. This association was primarily tested because ANP could constitute a potential, though not strong, candidate gene for cerebrovascular disorders, whether rat ANP is the susceptibility gene in question or not. Moreover, from a genealogical point of view, disease susceptibility was investigated among SHRSP, normal (or stroke-resistant) SHR and WKY of an original inbred colony, all of which originated from a Wistar rat colony in Japan [4].

2. Methods

2.1. Human subjects

This study was approved by an institutional review committee. Informed consent for participation was obtained from all subjects. Two study panels were independently collected according to classification criteria previously described [10,11]. Briefly, participants in the first panel comprised 270 cases, who were enrolled at the Kitamura Neurosurgery Clinic, Tokyo from September 1996 to May 1997, more than 2 months after the incident of stroke and 359 controls frequency matched by age and sex, who were selected from outpatients at the cardiovascular clinic of the Institute for Adult Diseases, Asahi Life Foundation, Tokyo [10]. Both institutes are in the same area of the megalopolis. The second panel involved three participant groups; (1) 104 subjects with silent brain infarction (SBI) were consecutively enrolled from people undergoing a health screening examination between January 1995 and December 1997 at the Shimane Institute of Health Science; (2) 163 subjects without evidence of SBI on MRI were selected from the same population as controls; and also (3) 74 subjects with symptomatic subcortical infarction were enrolled from outpatients at Shimane Medical University in the corresponding period [11]. Clinical characteristics were defined as a dichotomous phenotype except for age, as depicted in Table 1.

2.2. Genotyping of the G664A polymorphism and statistical analysis

The G664A polymorphism of human ANP was genotyped by the mutagenically separated PCR (MS-PCR) method (Fig. 1) as described in our previous report [12], in which this polymorphism was designated as G191A according to the nucleotide position relative to the transcription start site. Two other single nucleotide polymorphisms (SNPs), C-664G and T1766C, were also genotyped as described previously [12]. The three SNPs had been shown to represent principal ANP polymorphisms in Japanese subjects.

The χ^2-test statistic was calculated between the genotype distribution (or allele frequencies) and stroke status. Because pathological findings of cerebral lesions in SHRSP are similar to those of subcortical (or lacunar) infarction in humans [13], patients with subcortical infarction were analyzed separately in the first panel. Also, because the rat ANP locus is assumed to confer stroke susceptibility independently of hypertension [5,6], each group of cases and controls was stratified by hypertension status to remove its effects on association analysis. Furthermore, confounding influences of six variables listed in Table 1 were assessed in a multiple logistic regression model. The power of a case-control study of the available sample size was determined by calculating the smallest detectable relative risk with 80% power at a 5% Type I error probability [14].

2.3. Experimental animals

We use the name SHR for rats with a low incidence of spontaneous stroke (stroke-resistant SHR or 'SHRSR')

Table 1
Characteristics of the study populations and association analysis of the G664A polymorphism in relation to cerebral infarction

	First panel			Second panel		
	Cases		Controls	Symptomatic subcortical infarction	Silent brain infarction	Controls
No. of subjects (M/F)	270 (127/143)		359 (170/189)	74 (51/23)	104 (62/42)	163 (97/66)
Age (years)	69.5±7.8		68.6±6.4	65.8±10.7*	69.4±9.8*	57.0±7.9
Hypertension (%)	60		57	82*	69*	18
Diabetes (%)	22*		13	28*	12	11
Hypercholesterolemia (%)	57		59	34	52*	37
Smoking (%)	38		34	41	39	34
Genotype distribution for the G664A polymorphism	Total cases	(Subjects with subcortical infarction)				
G/G	230 (85%)	(104 (85%))	298 (83%)	64 (86%)	88 (85%)	127 (78%)
G/A	37 (14%)	(16 (13%))	58 (16%)	10 (14%)	14 (13%)	35 (21%)
A/A	3 (1%)	(3 (2%))	3 (1%)	0 (0%)	2 (2%)	1 (1%)
Frequency of 664A allele	8.0%	(8.9%)	8.9%	6.8%	8.7%	11.3%

In the original report by Rubattu et al. [8], the 664A allele was assumed to confer an increased risk for stroke. First and second panels were collected independently, according to classification criteria previously described in detail [10,11]. Cases in the first panel comprised patients with focal neurological symptoms due to cerebral infarction and excluded those with intracerebral or subarachnoid hemorrhage or those with cardiovascular complications, such as atrial fibrillation, vasculitis and a history of cardiac surgery. Controls in the first panel were ascertained from the same area with the frequencies of age and sex being matched. In the second panel, all subjects were evaluated by MRI; 104 subjects with silent brain infarction and 163 with cerebral infarctions (control group) were selected from people undergoing a health screening examination, while 74 patients with symptomatic subcortical infarction were also included in the study. Classification criteria for confounding factors were as follows: Hypertension was defined by either (1) BP measurements exceeded systolic BP ≧ 160 mmHg and/or diastolic BP ≧ 95 mmHg on two consecutive visits for untreated subjects or (2) chronic antihypertensive treatment of patients. Diabetes mellitus was defined by either (1) fasting plasma glucose concentration ≧ 7.7 mmol/l and/or a diabetic pattern was observed after an oral glucose challenge or (2) those under chronic treatment with oral hypoglycemic agents or insulin. Hypercholesterolemia was defined by either (1) serum cholesterol levels ≧ 5.70mmol/l for those untreated or (2) patients receiving cholesterol-lowering drugs.

* $P < 0.05$ versus controls by t-test of χ^2-test.

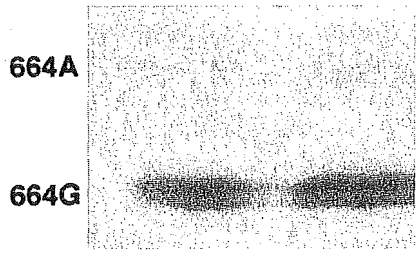

Fig. 1. A G664A polymorphism used for genotyping in the case-control analysis. After MS-PCR, the products were electrophoresed in 6% polyacrylamide/7 M urea gels and transferred to nylon membranes. The membranes were then hybridized with a ^{32}P-labeled primer and subjected to autoradiography.

and SHRSP as a whole hereafter. Ten SHR substrains derived from three colonies were used for DNA analysis in the present study. These included: three SHRSP ($A_{1\text{-}sb}$, A_3 and A_4) and four SHRSR (B_1, B_2, CH and CL) substrains from a colony kept at Kyoto University, Kyoto, Japan (SHRSP/Izm and SHRSR/Izm), SHRSP and SHRSR from the Max Delbruck Center for Molecular Medicine in Berlin-Buch, Germany (SHRSP/Heidelberg and SHRSR/Heidelberg) and SHRSR from the Genetic Resource Section, the National Institute of Health, Bethesda (SHRSR/NIH). The WKY rat from a colony kept at our institution (WKY/Izm) was used as a normotensive control strain. The genealogy of SHR substrains was described elsewhere [2,3,15,16]. Briefly, selective breeding was originally made for stroke-proneness to separate SHRSP from the *A* subline of SHR during $F_{24\text{-}25}$ generations (in 1971), by which time three distinct sublines of SHR (the *A*–*C* sublines) had been maintained in Kyoto. Stroke-resistant SHR, SHRSR, was thereafter derived from the *B* and *C* sublines and seven substrains of SHR (three SHRSP and four SHRSR substrains) have been kept at our institution, all direct descendants of the original colony. SHRSR/NIH was separated from a Japanese colony as early as F_{13} generation and established as an inbred strain at the NIH. SHRSP/Heidelberg was separated from the A_3 substrain of SHRSP/Izm at F_{36} generation (in 1975), but we do not know the details of the origin of SHRSR/Heidelberg. Two SHR substrains of a Japanese colony, descendants of SHRSP (A_3) and SHRSR (B_1), were further investigated in the gene expression study. For this purpose, animals were fed on a regular rat chow diet and sacrificed under pentobarbital anesthesia at 13 weeks of age. All procedures were in accordance with institutional guidelines.

2.4. Sequence comparison of rat ANP among substrains of SHRSP and SHRSR and WKY/Izm

Genomic ANP fragments, 2170-bp in size, were sequenced and compared among seven SHR substrains of a Japanese colony and WKY/Izm. Additionally, a 408-bp fragment spanning from intron 1 to intron 2 was sequenced for SHRSP/Heidelberg, SHRSR/Heidelberg, and SHRSR/NIH. Five overlapping sets of PCR primers were designed to cover a 625-bp 5′-untranslated region (UTR), three exons and two introns and a 591-bp 3′UTR in the rat ANP gene (Fig. 2). After PCR amplification, the products were gel-purified and subjected to cycle-sequencing according to the manufacturer's protocol (Dye-Terminator Cycle sequencing kit) on an ABI 377 DNA sequencer (Applied Biosystems). Information of the PCR primers can be obtained from the authors upon request.

2.5. Substrain comparison of marker alleles on rat chromosome 5

In the region which was assumed to encompass QTLs for 'stroke-associated' phenotypes on rat chromosome 5 [5,6], we examined allele distribution patterns of microsatellite markers among ten substrains of SHR and WKY/Izm. Substrain comparison was made by scoring 42 markers, where the allele size of PCR products was determined in base pair on an ABI 377 DNA Sequencer (Applied Biosystems).

A genetic linkage map of the relevant chromosomal region was constructed by genotyping all informative markers on 110 male F_2 rats involving the A_3 substrain of SHRSP/Izm and WKY/Izm to complete our consensus map. Genotyping and linkage mapping were performed as previously described [17].

As for the ANP locus, to differentiate base substitutions identified in intron 2 of the gene, a set of MS-PCR primers were newly designed as follows:

FP, 5′-AGGATCTGAGCCACGAGCAC-3′
RP-WKY, 5′-TCCCACCAGCCACAGTCTG-3′
RP-SP, 5′-CCAGTGACCAAGTCTTAGCCACCAGC CACAGTCCA-3′
where deliberate differences and base substitutions are underlined [18].

2.6. Gene expression studies

Total RNA was extracted from the whole brain of SHRSP/Izm and SHRSR/Izm for Northern blotting. A semi-quantitative PCR assay was also performed using the full-length cDNAs synthesized from reverse-transcribed mRNA of the brain. The PCR primers were: 5′TCTGATGGATTTCAAGAACCTG3′ (forward) and 5′TCAATCCTACCCC-CGAAGCAG3′ (reverse), where a forward primer was designed to be located on separate exons (exons 1 and 2) in order to eliminate PCR amplification from contaminating genomic DNA. PCR was terminated during the exponential phase and the products were electrophoresed on a 2% agarose gel. Quantitative analysis was performed by densitometric scanning on an AlphaImager 2000 (Alpha Innotech) and normalized by GAPDH levels.

3. Results

3.1. Association of G664A with human stroke

Table 1 shows baseline characteristics in our study panels. Apart from the status of diabetes mellitus, five variables—sex, age, and the status of hypertension, hypercholesterolemia and smoking—were comparable between case and control groups in the first panel. On the other hand, some variables were not comparable among three groups in the second panel, which was inevitable due to the consecutive enrollment scheme. Genotype characterization of the G664A polymorphism was performed by MS-PCR (Fig. 1) and no significant association was seen between case and control groups in either of panels. The results were almost unchanged when each study group was stratified by the presence or absence of hypertension and when confounding factors

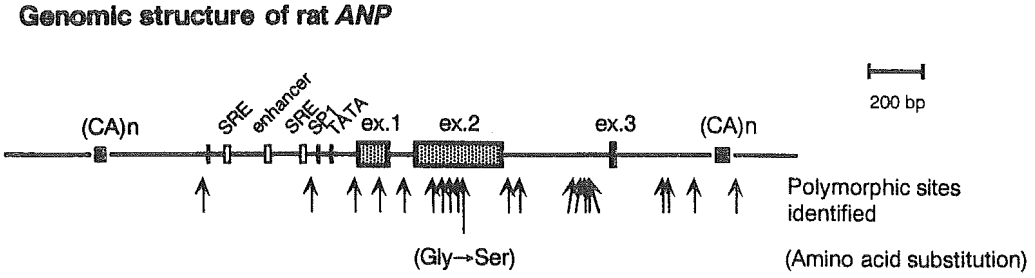

Fig. 2. Sequence differences identified between SP/Izm-type and WKY/Izm-type alleles of the rat ANP gene. Two distinct alleles were inferred from the 21 polymorphic sites identified in the sequenced region. Only a G-to-A substitution at position 485 from the CAP site resulted in an amino acid change, Gly-to-Ser, at residue 100 of prepro-ANP. SRE, serum response element; SP-1, promoter-specific transcription factor.

were adjusted by a multivariate analysis (data not shown). Restricting to patients with subcortical infarction did not influence the results for association. While no significant association was observed between C-664G and T1766C and stroke status, rarer allele frequencies did not exceed 3% in the Japanese population for either of two polymorphisms. This precluded haplotype analysis (data not shown). The prevalence of tested polymorphisms in each study group was consistent with Hardy–Weinberg equilibrium.

3.2. Sequence analysis of rat ANP

A total of 21 base-substitution polymorphisms were identified by direct sequencing of the rat ANP gene (Fig. 2). While six polymorphisms were located in exons, only a G-to-A substitution at position 485 from the CAP site (which was previously numbered as 1125 according to the deposited sequences, K02062 and K02063) [7] resulted in an amino acid change, Gly-to-Ser, at residue 100 of prepro-ANP. In a region of 625-bp upstream of the CAP site, we found three polymorphisms: G-to-T at position −463, C-to-T at position −88, and C-to-T at position 86. Here, a G-to-T substitution at position −463 was localized on the putative PEA-2 polyoma enhancer binding site [19] and a C-to-T substitution at position 86 was 1-bp upstream of the ATG starting codon [20]; these are considered functionally important. Two of the polymorphisms (a G-to-A substitution at position 485 and a G-to-T substitution at position −463) had been shown in a Heidelberg colony [7].

Two distinct types of ANP alleles were inferred from 21 polymorphic sites in the sequenced region. In a Japanese colony, all three SHRSP substrains possessed an identical allele (SP/Izm-type), while four SHRSR substrains and WKY/Izm shared the other allele (WKY/Izm-type). In addition, sequencing of a 408-bp genomic fragment, where six polymorphisms had been detected, revealed that SHRSP/Heidelberg possessed a SP/Izm-type allele, while SHRSR/Heidelberg and SHRSR/NIH possessed a WKY/Izm-type allele (Fig. 3). Judging from four exon 2 polymorphisms reported by Brosnan et al. [9], SHRSP/Glasgow and WKY of a Glasgow colony (WKY/Glasgow) were thought to possess a SP/Izm-type allele.

3.3. Allele distribution patterns on rat chromosome 5

To explore the relevance of substrain differences to 'stroke-associated' QTLs on rat chromosome 5, we determined allele distribution patterns of 42 microsatellite markers among ten substrains of SHR and WKY/Izm (Fig. 3). A small interval (< 10 cM in size) flanked by D5Mit11 and D5Rat24 differed between three SHRSP and four SHRSR substrains of a Japanese colony, whereas allele distribution patterns in the corresponding region were identical among two substrains of a Heidelberg colony and SHRSR/NIH. By contrast, the type of alleles at two adjacent loci, D5Mgh15 and ANP, differed between SHRSP and SHRSR substrains regardless of the origin of colonies.

3.4. Rat ANP gene expression

We found no detectable ANP mRNA expression of the rat brain by Northern blot analysis (data not shown), which appeared in agreement with previous observations in Heidelberg and Glasgow colonies [5,6]. A semi-quantitative assay by reverse-transcription PCR resulted in no significant differences in brain ANP expression between SHRSP/Izm and SHRSR/Izm. In two independent experiments, densitometric units for SHRSP were 91 and 112% of those for SHRSR, respectively, after normalization by GAPDH levels. An example of the reverse-transcription PCR assay is shown in Fig. 4.

4. Discussion

In the present study, we evaluated the ANP gene in relation to stroke susceptibility through a combination of genetic approaches. Our data failed to support the candidacy at large, but brought up several important issues that require careful interpretation. The issues include: (1) how much statistical value can be set on our association analysis in the Japanese subjects; (2) whether the type of rat ANP alleles accounts for part of phenotypic differences between SHRSP and SHRSR (or WKY) at all; and (3) what are the plausible explanations for the observed discrepancies between two previous studies concerning the rat ANP gene [5,6].

Extending findings in rats to humans is certainly tempting, but results should be critically evaluated as far as the rat data remain undefined. While there was no standard method to etiologically stratify stroke patients according to clinical manifestations, we chose to enroll patients with radiographical evidence of cerebral infarction (i.e. abnormal areas on brain CT or MRI) in order to decrease a genetic diversity in overall stroke. Diagnostic criteria for case subjects were no less stringent than those in the original report [8], where cases were sampled in a population-based setting according to individual's medical records or autopsy results. Since our study was performed in a hospital-based setting, one may argue that the results of association could be affected by insufficient matching of controls or other uncontrolled factors. Several findings can refute this criticism. First, despite the use of different enrollment scheme, comparable results were provided for lack of disease association with the ANP locus in two independent populations. Second, the 664A allele frequencies

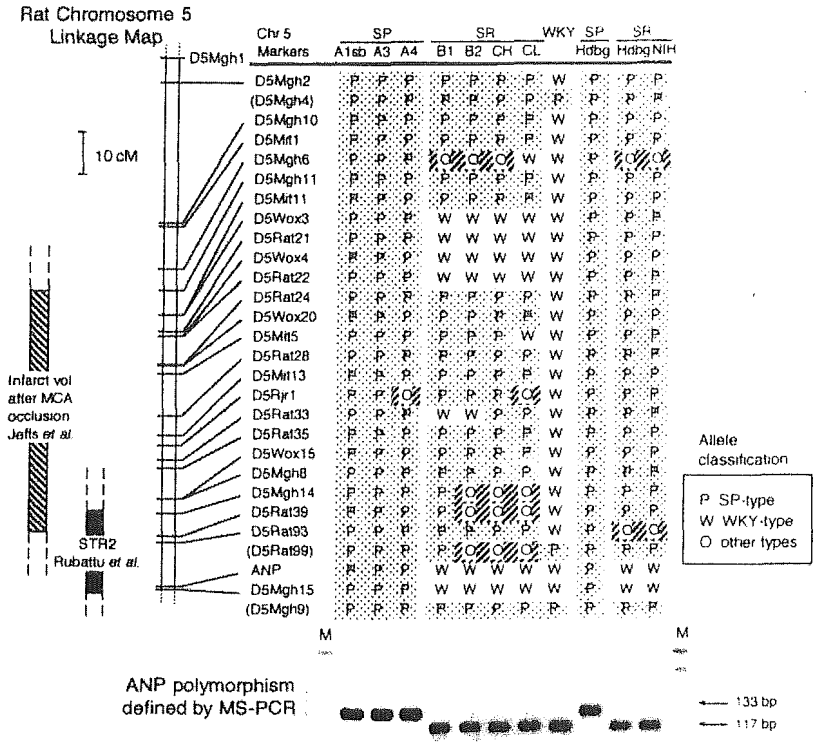

Fig. 3. Substrain comparison in the selected region on rat chromosome 5 (top) and results for an ANP polymorphism defined by MS-PCR (bottom). Marker alleles were categorized into three groups: SP/Izm-type allele (P), WKY/Izm-type allele (W) and alleles different from both types (O). Results for 28 selected markers are depicted in the figure: they were 25 markers informative between A_3 and WKY/Izm and three other markers shown in parentheses. For reference, positions of QTLs for 'stroke-associated' phenotypes are arbitrarily placed to the left of the linkage map, where a likely interval of the QTL for infarct volume after MCA occlusion cannot be precisely defined because Jeffs et al. [6] assigned the ANP locus to the middle of chromosome 5. M, ϕX174 DNA/*Hae*III marker.

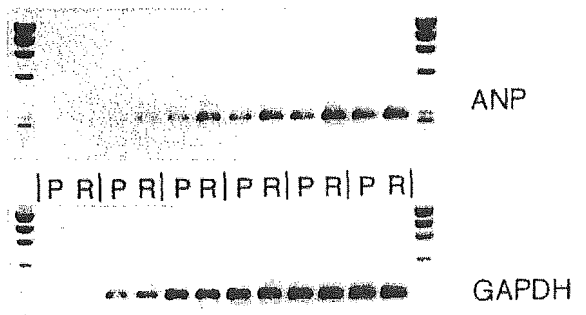

Fig. 4. A quantitative assay by reverse transcription-PCR of brain ANP expression. PCR products were shown with incremental cycles (three additional cycles) from left to right by two consecutive lanes, where products of SHRSP/Izm and SHRSR/Izm were placed in the left and right lanes, respectively. P, SHRSP/Izm; R, SHRSR/Izm.

observed in the present study proved to be concordant with the population frequency (8.9–9.2%) that we previously reported in the Japanese [12]. Furthermore, according to our calculation, a relative risk of >1.9 could have been detected in the available sample of 270 cases and 359 controls in the first panel alone with 80% power at a 5% type I error probability. This allowed us to test the odds ratio of 2.0, which was shown to be indicative of the risk increase in a white population [8]. Also, it has to be noted that the three tested SNPs could represent principal ANP polymorphisms in the Japanese, as demonstrated by our extensive screening [12].

Then we looked into the candidacy of ANP for rat stroke by comparing substrains of SHR. This strategy is based upon the hypothesis that a gene predisposing rats to stroke should have some functional differences between stroke-prone and stroke-resistant strains. Our results showed that two types of ANP alleles differentiated SHRSP from SHRSR substrains at a structural level. To our surprise, however, WKY/Glasgow appeared to possess a SP/Izm-type allele and, more importantly, apart from four substrains tested in the present study, there was a substrain of SHRSR with a SP/Izm-type allele, which had been extinct and only DNA was available from the frozen liver tissue kept at our institution (data not shown). The presence of 21 polymorphisms between two ANP alleles and the presence of a marker (D15Mgh15) showing the same allele distribution pattern as ANP implied that a certain chromosomal fragment rather than a single de novo mutation of the gene had been inherited during the inbreeding process. Taken together, the following situation is probable. There had been at least two distinct types of ANP alleles among ancestral laboratory rats in Kyoto, Japan, from which SHR and WKY were

developed. A few substrains of SHRSR and WKY should have inherited a SP/Izm-type allele, whereas four SHRSR substrains currently kept in our institution happen to have inherited a WKY/Izm-type allele of ANP.

Before we discuss a functional significance of a SP/Izm-type allele, it seems helpful to review 'stroke-associated' phenotypes in the literature. As for latency until the manifestation of stroke, SHRSP/Heidelberg is assumed to have a 'protective' allele of a QTL postulated on rat chromosome 5 compared with SHRSR/Heidelberg [5,21]. In theory, relating a 'protective' allele to SHRSP ironically counterbalances the propensity for stroke. Because such a QTL is unlikely to constitute a principal determinant for selective breeding of stroke-proneness, a 'protective' allele might as well be found in some substrains of SHRSR and WKY. As for infarct volume after the MCA occlusion (or ischemic vulnerability), on the other hand, significant linkage was identified in F2 progeny between SHRSP/Glasgow and WKY/Glasgow [6]. Even so, it remains unclear whether this trait by itself represents a phenotypic difference between stroke-prone and stroke-resistant SHR strains. Some studies have already shown that ischemic vulnerability is observable in SHRSR and may not account for substantial part of strain differences [22,23]. Thus, considerable care should be exercised when we extend the results for 'stroke-associated' phenotypes to stroke-proneness in SHRSP. Nevertheless, it must be stressed that these arguments do not undermine the original findings of linkage to the individual phenotypes because the existence of QTLs is not questionable under given circumstances, i.e. study design and statistical significance levels presented.

Results of the gene expression study fueled further confusion. We found no significant differences in the brain expression of ANP between SHRSP/Izm and SHRSR/Izm, whereas Rubattu et al. reported 3-fold lower expression in the brain of SHRSP/Heidelberg compared with SHRSR/Heidelberg [7]. This discrepancy may be partly attributed to technical problems, as different quantification methods were used to measure relatively low levels of ANP expression. We should also pay attention to differences in blood pressure (BP) profile between Japanese and Heidelberg colonies: SHRSP promotes a severer degree of hypertension from earlier age of life than SHRSR in an original colony [4], whereas both SHRSP and SHRSR are reported to develop a similar degree of hypertension in a Heidelberg colony [5]. It is therefore possible that BP differences in a Japanese colony (a maximal BP difference exceeds 30–40 mmHg) modify the brain ANP expression towards the reduction of strain differences shown in a Heidelberg colony [7]. In fact, elevated BP was supposed to induce higher mRNA expression of brain ANP in SHR when compared to WKY [24]. Alternatively, despite compelling evidence in experiments in vitro [7], functional alterations of a SP/Izm-type allele may not manifest themselves in vivo dependent on the genetic background, i.e. in SHRSP of a Japanese colony. Further investigations are currently in progress to resolve the uncertainties using a more sensitive quantification technique.

Although human and rat parts of our study do not appear complementary to each other, our data collectively lead to the argument that the ANP gene is unlikely to play a major role in the propensity for stroke. In view of a presumably complex interplay between causative genes and confounding phenotypes, such as hypertension, integration of various genetic strategies is required to clarify the pathophysiological role of the ANP gene in stroke. Findings in the current study are indispensable in this regard.

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

Studies on Transgenic/Knockout Mice of Adrenomedullin Gene

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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 N^G-nitro-L-arginine methyl ester (L-NAME) caused greater vasoconstriction in TG (%ΔRPP 10^{-4} mol/L: KO 33.1±3.3%, WT 55.5±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] 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

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levels of AM in the healthy populations are low and they show only several-fold increases even in patients with heart failure.[2,12,13] 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 endothelium-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.[21,22] 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% O_2/5% CO_2 at 37°C and contained 10^{-6} mol/L angiotensin II and 10^{-5} 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 H_2O_2, 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 N^G-nitro-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 NOS or PDE inhibition, the effects of N^G-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.[19] 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-[^{14}C] arginine to L-[^{14}C] citrulline, according to the method described previously.[19] 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 mCi/mL L-[^{14}C] arginine and incubated for 20 minutes at 37°C. To separate L-[^{14}C] arginine from L-[^{14}C] 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-[^{14}C] 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 H_2O_2 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-Keuls test. To compare renal injury scores, the nonparametric