volunteers or uncomplicated essential hypertensive patients [38].

Receptors Mediating AM Actions

Many of the AM actions were shown to be mediated by intracellular cAMP, though the subtype of AM receptors has been controversial [2, 3]. McLatchie *et al.* identified three subtypes of receptor-activity-modifying protein (RAMP1 to 3), an accessory protein required for the transport of calcitonin-receptor-like receptor (CRLR) to the cell membrane [39]. CRLR can function as either an AM receptor or a calcitonin gene-related peptide (CGRP) receptor, depending on co-expression with the subtype of RAMP: CRLR serves as a CGRP receptor when coexpressed with RAMP1, while it functions as an AM receptor when coexpressed with either RAMP2 or 3 [39]. Meanwhile, not all the actions of AM can be fully explained by this receptor system linked to adenylate cyclase: some have been shown to be independent of cAMP [40, 41]. This suggests the presence of unknown receptor systems and further studies are required to clarify the intracellular signaling systems for AM.

PRODUCTION OF AM IN VARIOUS ORGANS OR TISSUES AND CIRCULATING AM IN THE BLOODSTREAM

AM was initially isolated from pheochromocytoma tissue, but subsequently, the AM gene was found to be expressed in various organs and tissues in humans as well as in rats: those are adrenal glands, cardiac atria and ventricles, kidneys, blood vessels and lungs [4, 42, 43]. Consistent with the gene expression, AM was found to be produced and secreted from cultured cells isolated or derived from these organs and tissues [43-50]. For example, cultured vascular endothelial and smooth muscle cells have been shown to express the AM gene, secreting AM peptide into culture media [43, 44]. We previously reported the production of AM in cultured cardiomyocytes and cardiac fibroblasts isolated from ventricles of neonatal rats [45, 46]. In addition, a number of types of cultured renal cells, such as mesangial cells and tubular cell lines derived from different species, were found to produce and secrete AM [47-49]. The secretion from cultured cells isolated from the organs and tissues where AM has been shown to exert biological actions, suggests a role as an autocrine or paracrine factor [45-47, 50]. Another important finding we should mention is that both AM gene expression and AM peptide secretion in these cultured cells increased in response to mechanical stress and humoral factors, such as angiotensin II and endothelin-1, which not only participate in regulating blood pressure but also are involved in the development of end organ damage associated with hypertension [45, 46, 50]. The factors affecting AM production will be discussed under the section "Production of AM in animal models of hypertension".

Radioimmunoassays for AM revealed that immunoreactive AM was circulating in the blood at mean plasma levels ranging from 2.4 to 10 fmol/ml in healthy human subjects [51-54]. Immunoreactive AM in plasma or tissues consists of two molecular forms: a mature form with an amidated C-terminal (mAM) and an intermediate form with an unamidated glycine-extended C-terminal (iAM) (Fig. (**2**)) [6]. In the process producing mAM, iAM is cleaved from the AM precursor peptide, and then converted to mAM by amidation enzyme [6]. The major molecular form of AM in plasma was found to be iAM, while that in tissues is mostly mAM [6, 51-55]. As detailed in the next section, plasma levels of immunoreactive AM have been shown to be elevated in patients with hypertension, while both mAM and iAM levels are elevated without a notable difference in the ratio of mAM to total AM (tAM), which is the sum of mAM and iAM, when compared with control subjects [53]. Similarly, there was found to be no significant difference in the ratio of mAM to tAM between Dahl salt-sensitive hypertensive and control rats [56]. iAM is thought to have no biological effects by itself, while our *ex vivo* study showed that iAM dilated rat aorta following its conversion to mAM probably in the aortic wall [57]. In any case, very little information is currently available as to the role of iAM, which should be clarified further with experiments *in vivo*.

PLASMA AM IN ESSENTIAL HYPERTENSION

The biological action originally ascribed to AM was a blood pressure-lowering effect largely due to potent vasodilatation [1, 7], therefore initial interest focused on the role of this bioactive peptide in the regulation of blood pressure. Measurements with specific radioimmunoassays revealed plasma levels of AM to be higher in patients with essential hypertension than in normotensive controls [58, 59]. Cross-sectional, observational studies showed that the increase was related to blood pressure levels and to the severity of damage to target organs [58-60]. Kohno *et al.* demonstrated that plasma AM levels in untreated hypertensive patients remained unchanged after treatment with calcium channel blockers, despite a reduction in blood pressure [61]. Instead, they found an intimate relationship between the plasma AM and serum creatinine levels, which suggests that an increase in the plasma AM concentration is associated with impaired renal function [61]. In addition, a single administration of angiotensin converting enzyme (ACE) inhibitor lowered blood pressure in a short period of time, but the plasma AM level remained unchanged [62]. Sumimoto *et al.* reported that the plasma AM level of hypertensive patients complicated with left ventricular hypertrophy (LVH) was higher than that in those without LVH, despite the similar blood pressure levels in the two groups of patients [63]. Thus, the increased plasma AM levels in patients with essential hypertension seem to be correlated not directly with blood pressure levels but with organ damage associated with the elevation of blood pressure. This, however, is not the case in patients with a more severe form of hypertension: plasma AM levels in patients with malignant hypertension rose to a much higher level, and dropped, along with blood pressure levels, following antihypertensive treatment within one to three weeks [60].

Because AM peptide was abundantly detected in the adrenal medulla, we measured plasma AM concentrations of the adrenal vein, but found no step-up compared with the inferior vena cava [64]. Moreover, Nishikimi *et al.* were unable to find any site in the blood vessels showing a higher plasma AM concentration in patients with ischemic heart disease, though a step-down was observed in the aorta compared with pulmonary artery [65]. According to our previous

study with patients with ischemic heart disease, significant step-ups were observed in plasma mature AM (mAM) levels between the femoral artery and vein and between the aortic root and coronary sinus [66]. These data were not necessarily from patients with essential hypertension, though various organs or tissues including the heart and vasculature may contribute to plasma AM in hypertensive patients.

PLASMA AM IN PATIENTS WITH PHEOCHROMOCYTOMA AND OTHER FORMS OF SECONDARY HYPERTENSION

In addition to essential hypertension, there has been a number of reports on plasma AM levels in patients with secondary hypertension. Interest was aroused in plasma AM levels of patients with pheochromocytoma, in which AM was originally isolated and abundant AM expression is detected [1, 4]. Unexpectedly, plasma AM levels were not necessarily higher in patients with pheochromocytoma than those with essential hypertension [65]. Moreover, no elevation of plasma AM was observed during the paroxysms of this disease despite the elevation of blood pressure and plasma catecholamine levels [65]. This appears consistent with the notion that the adrenal medulla is not the major site secreting AM, but rather multiple organs or tissues contribute to the plasma AM level.

The plasma AM levels are higher in patients with primary aldosteronism, renovascular hypertension and Cushing's syndrome than controls, as they are in those with essential hypertension [62, 64, 67]. A significant positive correlation was found between the plasma AM level and mean blood pressure in patients with primary aldosteronism [64]. Meanwhile, no significant reduction in the plasma AM level was observed following surgical resection of an aldosterone-producing adrenal adenoma despite reduced blood pressure [62], a finding comparable with the effect of anti-hypertensive treatment on plasma AM levels in essential hypertensive patients [61].

PLASMA LEVELS OF AM IN CARDIOVASCULAR AND RENAL DISEASES ASSOCIATED WITH HYPERTENSION

Heart Failure

It has been demonstrated that plasma AM levels are increased in patients with congestive heart failure as compared with controls [68-70]. Interestingly, in comparison with other humoral factors or hemodynamic parameters, the plasma AM level was significantly correlated with pulmonary capillary wedge pressure, pulmonary artery pressure, plasma renin activity (PRA) and plasma levels of atrial and brain natriuretic peptides (ANP and BNP) [69, 70]. The elevated plasma levels gradually decreased following successful treatment, together with the levels of ANP and BNP, in patients with heart failure [71]. Kobayashi *et al.* subgrouped patients with chronic heart failure based on the primary cause of heart disease, but failed to find any particular heart disease in which the plasma AM level rises higher than in the others [70]. Thus, the increased levels appear to be closely related to the degree of depressed cardiac function, not to the primary cause of heart failure.

Arteriosclerosis

There has also been a number of reports showing increased plasma AM levels in patients with arteriosclerotic vascular disorders [72-75]. Kuwasako *et al.* found a possible association between plasma AM levels and endothelial damage by comparing the plasma levels of AM with those of endothelin and thrombomodulin, markers of endothelial damage, in patients with cerebrovascular disease [72]. Similarly, in patients with chronic ischemic stroke, the increased plasma AM level was shown to be associated with the degree of carotid atherosclerosis [73]. Recently, Suzuki *et al.* reported that the plasma AM concentration was elevated in patients with peripheral arterial occlusive disease in proportion to its severity [74]. It has been recognized that arterial stiffness is an important cardiovascular risk factor. Kita *et al.* measured plasma AM levels in patients with various degrees of atherosclerosis and compared the plasma levels with indirectly measured pulse wave velocity, a parameter often used to assess arterial stiffness and sclerosis [75]. Their multiple regression analysis revealed a significant correlation between the plasma AM levels and pulse wave velocity, a relationship independent of blood pressure [75]. These findings are indirect, but indicative of a possible pathophysiological role of AM in arteriosclerotic vascular diseases.

Chronic Renal Failure and End-Stage Renal Disease

The plasma AM concentration progressively increased in proportion to the impairment of renal function in patients with chronic renal failure, irrespective of the basal renal disease [59, 76]. Since peptide hormones and small fragments of peptides are metabolized in the kidneys, the possibility of a decreased clearance of AM should be taken into account as an explanation for the increased AM concentration. Meanwhile, according to Nishikimi *et al.*, no significant step-down of the plasma AM levels was noted between the renal artery and vein of hypertensive patients [65]. This finding does not support the notion that the kidneys are the major clearance sites of AM circulating in the blood. The elevated plasma AM levels in patients with chronic renal failure may not be simply attributable to the impaired renal function.

In line with the findings in patients with chronic renal failure, plasma AM levels are markedly increased in those with end-stage renal disease. It should be of interest to compare the plasma AM levels before and after hemodialysis, where reductions of blood pressure or fluid volume occur in a short period of time, but there have been inconsistencies regarding changes in plasma levels following hemodialysis. Some investigators found reduced plasma AM concentrations in a hemodialysis session [77-79], while others reported that AM levels remained unchanged [80, 81]. The plasma AM levels were shown to be reduced by hemodialysis with a high-flux dialyzer, but not with a low-flux dialyzer [82]. Kanozawa *et al.* reported that AM levels were lowered in patients undergoing ultrafiltration, but remained unchanged in those without ultrafiltration [83]. The discrepancy in changes of plasma AM levels in the hemodialysis patients may be partly explained by differences in subtracted fluid volume or in the types of dialyzer used, though according to Tokura *et al.*, other factors also appear to be involved [79].

PLASMA AM LEVEL AND PRODUCTION OF AM IN ANIMAL MODELS OF HYPERTENSION

Substantial efforts have so far been made to clarify the pathophysiological role of AM in hypertension by measuring AM concentrations of plasma or tissues and AM mRNA expression in various rat models of hypertension [56, 84-87]. Shimokubo *et al.* reported that both AM peptide and gene expression levels in the cardiac atrium and ventricle were higher in spontaneously hypertensive rats (SHR) than the control Wistar-Kyoto rats (WKY) [84]. The Dahl salt-sensitive rat, a model of volume-dependent hypertension, was examined for AM by at least two groups including ours. Similar to SHR, left ventricular AM content and mRNA expression of Dahl salt-sensitive rats fed on a high salt diet were found to be progressively elevated in association with left ventricular hypertrophy (LVH) and with transition from LVH to congestive heart failure [56, 85]. The plasma AM levels also increased in Dahl-salt sensitive rats on a high salt diet as compared with those on a low salt diet or Dahl salt-resistant rats [56, 85]. In addition to the increased AM expression, mRNA levels for the AM receptor components of calcitonin-receptor-like receptor (CRLR) and receptor-activity-modifying protein (RAMP) type 2 and 3 were found to increase in the left ventricle of Dahl salt-sensitive rats, suggesting up-regulation of the AM effector systems [56]. To examine AM expression in hypertension dependent on the renin-angiotensin system, we measured plasma and tissue AM levels in 2-kidney, 1-clip (2K-1C) renovascular hypertensive rats [86]. Although the plasma level was found to be similar between the 2K-1C and control rats, the AM concentration in the left ventricle of the hypertensive rats increased, showing a significant correlation with the degree of left ventricular hypertrophy [86] (Fig. (**3**)).

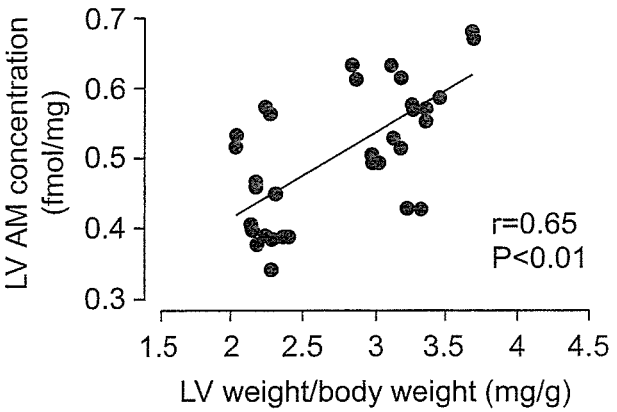

Fig. (3). Relationship between degree of left ventricular (LV) hypertrophy and LV AM concentration in 2K-1C renovascular hypertensive rats. Reprinted from reference 86 with permission from the Japanese Society of Hypertension.

In kidneys of SHR and Dahl salt-sensitive rats, neither the peptide nor mRNA expression level of AM was increased in comparison with the respective controls [84, 85]. In the meantime, Nishikimi *et al.* reported that both AM content and mRNA expression were augmented in renal medulla of SHR given deoxycorticosterone acetate and salt (DOCA salt SHR), a model of malignant hypertension, concomitantly with elevated plasma AM levels and with increased urinary excretion of AM [87]. Although the mRNA level for CRLR in the renal medulla remained unchanged, levels for RAMP1 and 3 were augmented in this model of hypertension [87]. Taking the blood pressure-lowering properties of AM into account, we hypothesize that AM functions as a factor acting systemically or locally against elevations of blood pressure in the animal models of hypertension.

The mechanisms for augmented AM production in the heart or kidneys of hypertensive rats are not totally understood at present, but we may be able to discuss this important issue based on the findings from cell culture studies, as well as from animal experiments. According to our previous studies, AM production in cultured cardiomyocytes and cardiac fibroblasts was increased by the pressor peptides angiotensin II and endothelin-1 or simply by mechanical stretching of the cells [45, 46, 88]. Yoshihara *et al.* showed that production of AM in cultured cardiomyocytes was increased by hydrogen peroxide (H_2O_2), suggesting the involvement of oxidative stress [89]. The production of AM by cultured mesangial cells and vascular smooth muscle cells was found to increase in response to such humoral factors or cytokines as angiotensin II, aldosterone, interleukin-1β and tumor necrosis factor-α [44, 47, 90]. In vascular endothelial cells, AM production has been shown to be modulated by glucocorticoid or the alteration of shear stress [91, 92]. Hence, various factors seem to be involved in augmented AM production in the animal models of hypertension and possibly in human hypertension.

Another approach to clarifying the mechanisms behind the augmented AM expression is to examine the changes in levels of AM peptide or mRNA expression following antihypertensive treatment. Pressure overload to the heart by aortic banding elevated left ventricular AM levels in rats, but this increase was inhibited by reducing blood pressure with quinapril, an angiotensin-converting enzyme inhibitor [93]. Using Dahl-salt sensitive rats, Kobayashi *et al.* demonstrated that the increased AM level in the left ventricle was reduced by a sub-depressor dose of the angiotensin II type 1 receptor blocker TCV-116 [94]. Consistent with the cell culture experiments, either mechanical stress applied to the myocardium or the renin-angiotensin system appears to be involved in the augmented AM expression in the left ventricle in these rat models of hypertension.

LONG-TERM MODULATION OF BLOOD PRESSURE BY AM

Soon after the discovery of AM, the hypotensive effects of bolus AM injection or infusion in a relatively short period of time were well characterized in animal experiments and in human studies [7, 8, 16]. However, the plasma AM concentration was shown to rise to a level 100-fold higher than the physiological concentration following such a bolus injection [8], therefore we are unable to discuss the roles of endogenous AM in the long-term regulation of blood pressure using these reports. To examine the effects of chronically administered AM, we continuously infused synthetic human AM for two weeks, into spontaneously hypertensive rats (SHR) and 2-kidney, 1-clip (2K-1C) renovascular hypertensive rats [95, 96]. As shown in Fig. (**4**), the systolic blood pressure of the

2K-1C rats was reduced by the chronic AM infusion, which raised the plasma AM level to within the physiological range [96]. Blood pressure reduction by prolonged AM infusion at a physiological plasma AM level was also observed by Rademaker *et al.*, who infused AM over four days into an ovine model of heart failure [97]. Meanwhile, in other rat models of hypertension, such as the Dahl salt-sensitive rat or SHR given deoxycoricosterone acetate and salt (DOCA salt SHR), chronically infused AM failed to reduce blood pressure despite the infusion of AM at doses similar to those of the above-mentioned studies [98, 99].

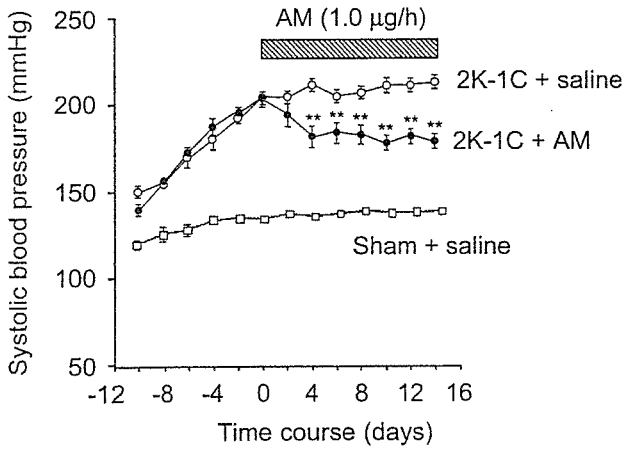

Fig. (4). Blood pressure-lowering effect of long-term infusion of AM in 2K-1C renovascular hypertensive rats. The 2K-1C rats were infused with 1.0 μg/h of synthetic human AM for 14 days. **P<0.01, *vs.* 2K-1C rats infused with saline. Reprinted from reference 96 with permission from Elsevier.

Also, we may be able to discuss the role of endogenous AM in long-term BP modulation based on the findings from AM gene-manipulated mice. The homozygote of AM gene-knockout mice was found to be embryo-lethal, but mean blood pressure of the heterozygote was significantly higher by about 10 mmHg than that of the wild-type littermates [100]. Comparable with this, transgenic mice over-expressing the AM gene showed a lower blood pressure with higher plasma AM levels [101]. Although the blood pressure-lowering effects of continuous, long-term infusion of AM appear dependent on animal models of hypertension, the results obtained by the AM gene manipulation suggest the participation of AM in the mechanisms acting against the elevation of blood pressure.

When injected in a relatively short period of time, AM exerts a potent blood pressure-lowering effect largely secondary to vasodilatation [7, 9, 16]. In such experiments, plasma renin activity was elevated probably by the activation of the sympathetic nerve system resulting from an acute reduction of blood pressure [16]. However, chronically infused AM reduced the plasma renin activity and aldosterone concentration of 2K-1C renovascular hypertensive rats [96]. Consistent with the direct inhibitory action of AM on the secretion of aldosterone [27], AM suppression of plasma aldosterone levels was observed not only in chronic experiments but also in relatively short-term infusion studies [102, 103].

As mentioned in the section "Biological actions of AM", vasodilatation is partly mediated *via* the NO-cGMP pathway [10, 12], which may also be involved in the chronic modulation of blood pressure by AM. Nishikimi *et al.* found an increased plasma level and urinary excretion of cGMP following prolonged AM infusion in Dahl salt-sensitive rats [104]. An increased plasma level of cGMP was also observed in the AM gene-overexpressing mice, whose blood pressure was lower compared with the wild type, but rose to a greater degree following the inhibition of NO production with N-monomethyl-L-arginine (L-NMMA), offsetting the blood pressure difference [101]. Conversely in mice deficient in a single copy of the AM gene, showing reduced production of NO in the kidneys, the blood pressure elevation induced by L-NMMA was smaller compared with their wild-type littermates [101]. The notion of reduced production of NO in AM knockout mice was further supported by the higher renal perfusion pressure *ex vivo*, which rose to less in response to N-nitro-L-arginine methyl ester (L-NAME), compared with the controls [105]. Collectively, though bolus injection of AM reduces blood pressure largely *via* vasodilatation, the blood pressure-lowering effects of chronically infused or overly expressed AM appear to be mediated by multiple mechanisms: inhibition of the renin-angiotensin system, direct inhibition of aldosterone secretion, stimulation of the NO-cGMP system, etc.

CARDIOVASCULAR AND RENAL PROTECTIVE EFFECTS OF AM *IN VITRO*

A number of cell culture experiments have been done to see whether or not AM has the potential to protect end organs from the damage associated with an elevation of blood pressure. AM production was found to progressively increase in cardiac ventricles of Dahl salt-sensitive or 2K-1C renovascular hypertensive rats in relation to the degree of ventricular hypertrophy [85, 86] (Fig. (**3**)). To examine the effect of AM on cardiac hypertrophy, we carried out a series of experiments *in vitro* with cultured cardiomyocytes isolated from cardiac ventricles of neonatal rats [45, 50, 88, 106]. Incubation with synthetic AM reduced *de novo* protein synthesis, cell size and atrial natriuretic peptide (ANP) production induced by angiotensin II or by mechanical stretching in the cultured cardiac myocytes, a finding indicative of an inhibitory effect of AM on cardiac ventricular hypertrophy [45, 50, 88, 106].

Making up two-thirds of the total number of cells in the heart, cardiac fibroblasts also play an important role in the myocardium: they contribute to the morphological integrity of the myocardium, while excessive proliferation and collagen production by the fibroblasts result in impairments of systolic and diastolic function. In addition to its effect on cardiomyocytes, AM inhibited the proliferation and protein synthesis of cultured cardiac fibroblasts [46]. Similar results were obtained by Horio *et al.*, who raised the possibility that AM inhibits the synthesis of collagen in fibroblasts [107]. Interestingly, a blockade of the actions of endogenous AM with a monoclonal antibody increased the protein synthesis or cell proliferation of cultured cardiac myocytes and fibroblasts, suggesting an autocrine or paracrine inhibition of cardiomyocyte hypertrophy and the proliferation of cardiac fibroblasts by AM [45, 46, 106]. These results led us to the

hypothesis that AM functions to inhibit cardiac hypertrophy or fibrosis induced by inappropriate mechanical or humoral stimuli associated with elevations of blood pressure.

The proliferation of vascular smooth muscle cells in the media and intima of arteries is involved in the progression of vascular remodeling or atherosclerotic lesions. Because AM is produced by the vascular wall [43, 108], its effects on the proliferation of vascular smooth muscle cells were tested *in vitro*; however, there has been an inconsistency regarding the actions of AM. Kano *et al.* reported that AM inhibited the proliferation of cultured vascular smooth muscle cells [109]. The same group also showed an inhibitory effect of AM on the migration of these cultured cells, and according to their experiments, the effects were mediated by intracellular cAMP [110]. Conversely, Iwasaki *et al.* found that AM stimulated the proliferation of cultured vascular smooth muscle cells in a mitogen-activated protein kinase-dependent manner [111]. These discrepancies may result from differences in the experimental conditions or types of cultured cells used. Meanwhile, as discussed in the next section, recent studies with AM gene knockout or transgenic mice have shown that AM inhibits fatty streak formation and periarterial cuff-induced intimal thickening [112, 113].

Secreting various vasoactive substances, vascular endothelial cells have an important role not only in regulating vascular tone but also in modulating the progression of vascular remodeling and atherosclerotic lesions. In line with this, cultured vascular endothelial cells were found to actively produce and secrete AM [43]. Kato *et al.* reported that AM inhibited serum deprivation-induced apoptosis of cultured rat vascular endothelial cells *via* a cAMP-independent manner [114]. Sata *et al.* also observed an anti-apoptotic effect of vascular endothelial cells, which was presumably mediated by a non-cAMP and non-cGMP dependent mechanism [115]. In addition to inhibition of apoptosis, an *in vitro* wound healing assay by Miyashita *et al.* showed that AM promoted endothelial regeneration in a protein kinase A- or phosphatidylinositol-3-kinase-dependent pathway [116]. Though the intracellular mechanisms of the action remain to be clarified, these results suggest that AM has a protective effect against the progression of vascular remodeling or atherosclerotic lesions by inhibiting apoptosis and promoting the regeneration of vascular endothelial cells.

A substantial level of AM mRNA was detected in kidneys [4, 42], and in accord with this, AM has been reported to be produced by cultured renal cells including mesangial cells [47-49]. Although mesangial proliferation is not a main feature of benign nephrosclerosis, hypertension is a factor exacerbating glomerular diseases, in which the inhibition of mesangial proliferation should be beneficial. There have been a significant number of reports showing an inhibitory effect of AM on the proliferation of cultured mesangial cells *in vitro* [47, 117-119]. This effect seems to be partly mediated by intracellular cAMP, because forskolin, a direct adenylate cyclase activator, mimicked the effect and H-89, a protein kinase A inhibitor, attenuated it [119]. In addition to the inhibition of mesangial cell proliferation, Parameswaran *et al.* reported AM-induced apoptosis of mesangial cells, showing the possibility that p38 mitogen-activated protein kinase was involved in the mechanism [119, 120]. These findings suggest that AM exerts a protective effect on the glomerulus by inhibiting the proliferation of mesangial cells in renal diseases.

CARDIOVASCULAR AND RENAL PROTECTIVE EFFECTS OF AM *IN VIVO*

As discussed earlier, the increased plasma AM concentration in patients with hypertension appears to be largely related to target organ damage such as left ventricular hypertrophy, reduced cardiac function, impaired renal function and arteriosclerosis [58-63]. To investigate whether or not AM protects the target organs against hypertensive damage *in vivo*, three experimental approaches have so far been taken: long-term administration of AM, virally-mediated overexpression of AM and genetic manipulation of the AM gene. Using the long-term infusion method, Nishikimi and his colleagues showed that in Dahl salt-sensitive hypertensive rats fed a high-salt diet, AM had a minimal effect on blood pressure, but significantly inhibited left ventricular hypertrophy (LVH) and the transition from LVH to heart failure [98]. The infusion also lessened pulmonary congestion and the deterioration of left ventricular function, improving survival in this animal model of hypertension [98]. The inhibitory effect of AM on LVH without a significant reduction of blood pressure suggests a possibly direct action on cardiac myocytes, which was shown previously by our cell culture experiments [45, 46].

Either preload or afterload to the heart results not only in LVH but also in cardiac fibrosis, where excessive fibroblast proliferation and collagen deposition occur, leading to impairment of diastolic dysfunction. To test *in vivo* the anti-fibrotic action of AM observed in the cell culture experiments [46], we infused recombinant human AM into a rat model of acute myocardial infarction (MI) [121, 122]. Consistent with the study *in vitro*, the infusion significantly reduced the myocardial collagen deposition following MI, while the exact mechanism of action remains to be specified [121, 122]. As shown in Fig (**5**), we recently found that long-term administration of AM reduced perivascular fibrosis of the coronary arteries in rats with hypertension induced by chronic infusion of angiotensin II [123]. This effect was accompanied by the suppression of fibroblast activation and transforming growth factor-β1 (TGF-β1) expression, but not by a significant reduction of blood pressure [123], suggesting an AM action independent of hemodynamic factors.

Renoprotective effects of AM have been implied by *in vitro* experiments as mentioned above. Indeed, the long-term infusion of AM has been reported to reduce glomerular and arterio-arteriolar scleroses in Dahl salt-sensitive rats given a high-salt diet and spontaneously hypertensive rats administered deoxycorticosterone acetate and salt (DOCA-salt SHR) [99, 104]. As a result, urinary protein excretion was reduced and creatinine clearance preserved by the AM infusion. The renoprotective effects were observed without a significant reduction of blood pressure, suggesting again direct actions of AM on kidneys [99, 104]. Increased urinary excretion of cAMP and cGMP suggests roles for these cyclic nucleotides in mediating the actions of AM [104], but the mechanisms involved remain to be specified as do those for the effects of AM on the heart. Irrespective of the mechanisms, the plasma levels of exogenous AM were found to be within the physio-

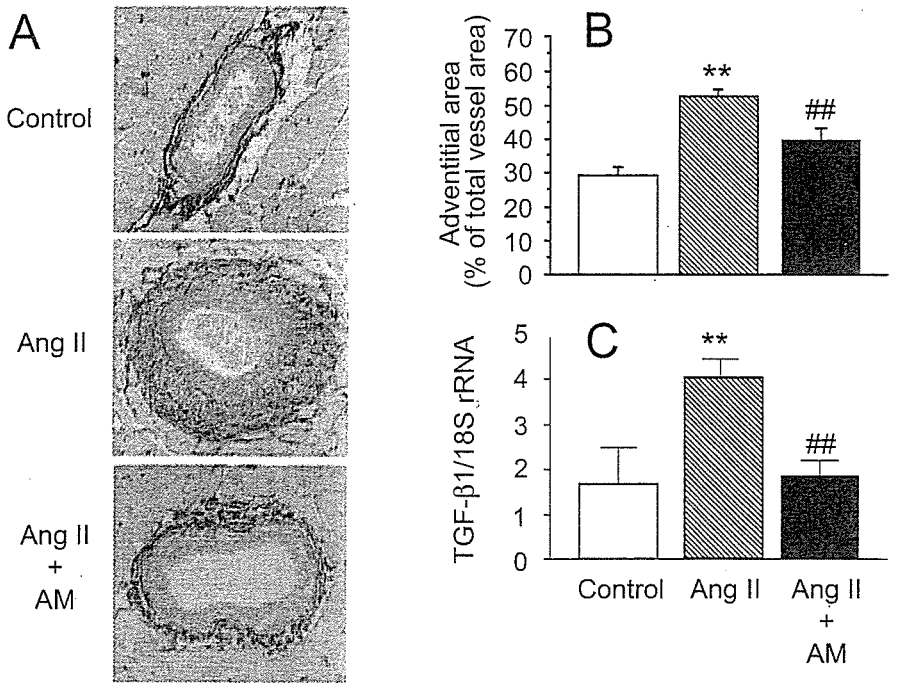

Fig. (5). Effects of angiotensin II (Ang II) and AM on perivascular fibrosis of coronary arteries. The two-week infusion of 200 ng/kg/min Ang II thickened the adventitial area of coronary arteries of rats as determined by Sirius red staining (**A** and **B**). The infusion of AM at 60 ng/kg/min alleviated the adventitial thickening concomitantly with a reduction in TGF-β1 expression (**C**), but did not have a significant effect on blood pressure (Ang II 180+/-7, Ang II+AM 191+/-4 mmHg). Values are the means+/-SEM; **P<0.01, *vs.* control; ##P<0.01, *vs.* Ang II. Reprinted from reference 123 with permission from the European Society of Cardiology.

logical range in the above-mentioned long-term infusion studies, where 0.2 to 1.0 μg/h AM was used [95, 96, 98, 99, 104]. It is therefore possible that endogenous AM functions to inhibit cardiac and renal damage in the rat models of hypertension.

Chao and coworkers demonstrated that adenovirus-mediated systemic delivery of the human AM gene ameliorated cardiac and renal damage in rats with deoxycorticosterone acetate (DOCA) salt-induced and 2K-1C renovascular hypertension and in Dahl salt-sensitive hypertensive rats fed a high-salt diet [124-126]. Similar to the results of the long-term infusion experiments, the overexpression of AM lessened cardiac hypertrophy and fibrosis and glomerular sclerosis, improving urinary protein excretion and creatinine clearance [124-126]. Urinary excretion of cAMP was increased by the overexpression [124, 125]; while systolic blood pressure was reduced by about 20 to 30 mmHg [124-126], therefore blood pressure-dependent mechanisms may have also contributed to the beneficial effects of AM. In addition to the systemic overexpression of the AM gene, the same group reported that the local delivery of AM to balloon-injured carotid artery inhibited neointimal formation in rats [127]. The plasma AM concentration rose beyond the physiological range following the systemic delivery of the AM gene; nonetheless, these overexpression studies suggest the potential of AM as a therapeutic tool inhibiting organ damage resulting from elevations of blood pressure.

Lastly, the cardiovascular and renal protective effects have been suggested by genetic manipulation of the AM gene in mice. Shimosawa *et al.* showed that, when given angiotensin II and excessive salt, heterozygotes of AM knockout mice showed more severe perivascular fibrosis and intimal thickening of coronary arteries, compared with their wild-type littermates, despite a similar elevation of blood pressure between the knockout and wild-type mice [128]. Based on increases in the production of reactive oxygen species (ROS) and in NAD(P)H oxidase expression in the AM knockout mice, the possibility of augmented oxidative stress was raised as the mechanism responsible for the severe vascular lesions [128]. Periarterial cuff-induced intimal thickening of the femoral artery was also found to be more severe in the knockout mice, compared with the control mice [112]. The enhanced neointimal formation was reversed by delivery of the AM gene and by an NAD(P)H oxidase inhibitor or tempol, a superoxide dismutase mimetic, further suggesting augmentation of oxidative stress in the AM knockout mice [112]. More recently, Niu *et al.* subjected heterozygotes of another strain of AM gene-knockout mice to the cardiovascular stress of aortic constriction or angiotensin II infusion, resulting in left ventricular hypertrophy and perivascular fibrosis, changes that were more pronounced in the knockout mice than in the controls [129]. In addition, the renal damage characterized by reduced creatinine clearance and glomerular sclerosis was worse in the knockout mice than in the controls [129]. The precise mechanisms remain to be clarified; though, it appears convincing that AM has protective effects on the progression of the cardiovascular or renal damage associated with elevations of blood pressure.

INSULIN RESISTANCE AND AM

Insulin resistance with resultant hyperinsulinemia is often seen in patients with hypertension, particularly in those with

obesity-related hypertension. Chan *et al.* reported that the blood pressure-lowering effect of AM was attenuated in the Zucker obese rat, an animal model of insulin resistance, compared with the control [130]. A comparable result was recently obtained in a human study by Kita *et al.*, where the effects of intravenous AM infusion were examined in subjects without or with insulin resistance [131]. The infusion reduced not only blood pressure but also pulse wave velocity (PWV), a parameter for arterial stiffness. Interestingly, the reduction in PWV was smaller in the subjects with insulin resistance than those without [131]. It therefore seems likely that insulin resistance blunts the actions of AM in humans as well as in rats. On the other hand, AM deficiency has been shown to be related with insulin resistance by using heterozygotes of AM gene-knockout mice [132, 133]. Shimosawa *et al.* found that AM knockout mice developed insulin resistance on aging with increased urinary 8-iso-prostaglandin F2-α excretion and reactive oxygen species (ROS) production in skeletal muscle. Both the reduced response to insulin and augmented oxidative stress were partially reversed by supplements of AM or tempol, a superoxide dismutase-mimetic compound [132]. The same group also showed that angiotensin II-induced insulin resistance with increased oxidative stress was more prominent in the AM knockout mice than in the controls, and this was reversed by the AM or tempol supplements [133]. These findings from the AM gene-knockout mice suggest that reduced AM actions would result in insulin resistance, probably by augmentation of oxidative stress.

CONCLUSIONS AND PERSPECTIVES

Thirteen years has passed since AM was discovered as a novel vasodilator peptide. During this period, much research, basic and clinical, has been done to clarify its pathophysiological role in hypertension and hypertensive organ damage. As discussed in this review, a substantial amount of data accumulated in this field suggests that AM functions to counteract the elevation in blood pressure and progression of the cardiovascular and renal diseases associated with hypertension. Research on AM now seems to be entering a new phase, with clinical benefits to be examined and specified. AM itself is orally inactive, but the development of either AM analogues or drugs inhibiting AM degradation would provide a new therapeutic tool for treating patients with hypertension or those with the cardiovascular and renal diseases associated with hypertension.

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Soluble Guanylate Cyclase Stimulation on Cardiovascular Remodeling in Angiotensin II–Induced Hypertensive Rats

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Abstract—It is unknown whether long-term pharmacological stimulation of soluble guanylate cyclase (sGC), elevating intracellular cGMP levels, has a beneficial effect on hypertension. The purpose of this study is to investigate the effects of BAY41-2272, an orally available sGC stimulator, on cardiovascular remodeling in hypertensive rats. Eight-week-old male Wistar rats with hypertension induced by angiotensin II infused subcutaneously at 250 ng/kg per minute were treated orally with a low ([L] 2 mg/kg per day) or high ([H] 10 mg/kg per day) dose of BAY41-2272 for 14 days. BAY41-2272-H partially suppressed the rise in blood pressure and reduced the heart weight (4.20±0.34 versus 3.68±0.20 mg/g; $P<0.01$), whereas BAY41-2272-L had no effect. However, both doses decreased the angiotensin II–induced left ventricular accumulation of collagen in the perivascular area (L, −20%, $P<0.05$; H, −30%, $P<0.01$) and myocardial interstitium (L, −21%, $P<0.05$; H, −38%, $P<0.01$), reducing the number of activated fibroblasts surrounding coronary arteries (L, −74%; H, −79%; $P<0.05$). BAY41-2272 downregulated the angiotensin II–induced left ventricular gene expression of type 1 collagen (L, −41%, $P<0.05$; H, −49%, $P<0.01$) and transforming growth factor-β1 (L, −49%, $P<0.05$; H, −65%, $P<0.01$). cGMP levels were elevated by BAY41-2272 not only in the left ventricle, but also in cultured cardiac fibroblasts, resulting in reduced thymidine incorporation into the cells. Thus, stimulation of sGC by BAY41-2272 attenuates fibrosis of the left ventricle in rats with angiotensin II–induced hypertension partly in a pressure-independent manner, suggesting an important role for sGC generating cGMP in inhibiting cardiovascular remodeling. (***Hypertension.* 2006;48:972-978.**)

Key Words: hypertension ■ fibrosis ■ soluble guanylate cyclase ■ cGMP ■ extracellular matrix

Hypertensive heart disease is characterized histologically by left ventricular (LV) hypertrophy and fibrosis. LV hypertrophy is recognized as a risk factor for cardiovascular morbidity and death,[1] and fibrosis surrounding coronary arteries and myocardial fibers decreases the supply of oxygen and nutrients to the myocardium.[2] These histological changes, termed "cardiac remodeling," impair the diastolic function of the LV, often leading to overt heart failure or fatal arrhythmia.[2–5] Cardiac remodeling is caused by hemodynamics and various neurohumoral factors, such as catecholamine, angiotensin II (Ang II), aldosterone, and endothelin-1.[6,7] Notably, activation of the renin—angiotensin–aldosterone system accelerates the process of cardiac remodeling, and, indeed, inhibiting the system has been demonstrated to have beneficial effects.[8] Therefore, not only the reduction of blood pressure but also the suppression of these humoral factors would be important to regulate cardiac remodeling in hypertensive heart disease.

Natriuretic peptides–cGMP signaling has been reported to inhibit cardiac remodeling: atrial natriuretic peptide (ANP) evoked potent antihypertrophic effects on cardiac ventricles,[9,10] and brain natriuretic peptide (BNP) inhibited cardiac fibrosis in vitro[11,12] and in vivo through a cGMP-dependent pathway.[13] Thus, cGMP signaling seems to play a critical role in attenuating cardiac remodeling. Soluble guanylate cyclase (sGC [GC]), a heterodimeric haem protein consisting of α- and β-subunits, is an intracellular effector for NO,[14] converting guanosine triphosphate to cGMP. However, it remains unknown whether pharmacological stimulation of sGC attenuates cardiac hypertrophy and fibrosis in hypertension.

BAY41-2272, developed recently as an orally active sGC stimulator,[15] has been shown to have beneficial effects on hemodynamics in systemic hypertension,[15] heart failure,[16] and pulmonary hypertension.[17] In the present study, to examine whether continuous stimulation of sGC inhibits cardiac hypertrophy and fibrosis, we administered BAY41-2272 to rats with hypertension induced by Ang II. The goal of this study was to better understand the role of sGC in cardiovascular remodeling in Ang II–induced hypertension.

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Methods

Animal Experiment

Eight-week-old male Wistar rats (Charles River Laboratories Japan, Yokohama, Japan) weighing 200 to 250 g were housed in a temperature and light-controlled room (25±1°C; 12/12-hour light/dark cycle) for 1 week before use, with free access to normal rat chow and water. Control rats not infused with Ang II were divided into 3 groups given either a placebo (n=7) or a low or high dose (2 and 10 mg/kg per day) of BAY41-2272 (10 for each). Similarly, rats infused with Ang II were divided into a placebo-treated group (n=13) and low-dose (n=9) and high-dose (n=13) treatment groups. Ang II was infused at 250 ng/kg per minute subcutaneously by miniosmotic pumps (Alzet model 2002) for 14 days as described previously.[18] The BAY41-2272 compound, kindly supplied by Bayer HealthCare, was given orally twice a day for 14 days. Blood pressure was measured while the animal was conscious ≥9 times by tail-cuff plethysmography (Softron, BP-98A) at 2:00 to 3:00 PM.

The doses of BAY41-2272 used in this study were determined based on our preliminary study, where oral administration at a single dose of 1, 5, or 10 mg/kg was tested in Ang II–infused rats (Figure I, available online at http://hyper.ahajournals.org). The blood pressure–lowering effect of 1 mg/kg BAY41-2272 was minimal and insignificant, but we observed similar hypotensive actions lasting for 12 hours after the administration at 5 and 10 mg/kg. Meanwhile, the primary purpose of the present study was to test inhibitory actions of BAY41-2272 on cardiovascular remodeling, so we therefore chose the experiment period of 14 days based on our previous study, in which sufficient cardiac hypertrophy and fibrosis occurred with significant changes of LV gene expressions in this model of hypertension.[18]

At day 14, rats were anesthetized with pentobarbital sodium, and a 2F micromanometer–tipped catheter (Model SPC-721, Millar Instruments) was inserted into the LV through the right carotid artery. LV end-diastolic pressure (LVEDP) was measured using a transducer control unit (Model TCB-500, Millar Instruments) connected to a PowerLab system (ADInstruments Pty Ltd). Then the rats were euthanized by drawing blood from the abdominal aorta. After the whole heart was weighed, LV was frozen in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin wax.

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2006-014). This investigation also conformed with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Histology and Immunohistochemistry

Immunohistochemical staining was performed as described previously.[18] For determining myofibroblastic differentiation, slides were stained with an anti-α-smooth muscle actin (α-SMA) monoclonal antibody (Clone 1A4, DAKO) at a dilution of 1:200 overnight at 4°C. For the detection of collagen fibers, slides were incubated with 0.1% picrosirius red (Direct Red 80, Sigma) dissolved in saturated picric acid for 10 minutes as described previously.[18]

Morphometric Analysis

The morphological evaluation and cell counting of myofibroblasts surrounding intramyocardial coronary arteries were performed in the middle portion of the LV by a single observer in a blinded manner as described previously.[18] Each section immunostained with the antibody against α-SMA was scanned at a magnification of ×400. The number of cells positive for α-SMA surrounding the intramyocardial coronary artery was counted and normalized to the coronary vessel area, encircled by the external elastic lamella. To evaluate the magnitude of perivascular fibrosis, medium-sized intramyocardial coronary arteries with a diameter of 100 to 200 μm were randomly selected from ≥3 different sites, and the ratio of the perivascular fibrotic area to the coronary vessel area was determined using WinROOF (Mitani Co.). Collagen volume fraction in the interstitial space of myocardial fibers was determined by calculating the ratio of the collagen area to the entire area of an individual section. To measure cardiocyte size, cross-sectional areas of ≥50 myocardial fibers were measured at the level of nuclei while omitting longitudinally or obliquely sectioned cells as described previously.[18]

Gene Expression

Gene expression for type 1 collagen and transforming growth factor (TGF)-β1 in the LV was measured by real-time quantitative RT-PCR (ABI Prism 7700 Sequence Detector, Applied Biosystems) as described previously.[18] cDNA reverse transcribed from total RNA was amplified with oligonucleotide primers, forward and reverse, and with probes labeled with 6-carboxy-fluorescencein as reporter fluorescence and 6-carboxy-tetramethyl-rhodamine as quencher fluorescence. The oligonucleotide sequences used are detailed in previous reports.[18,19] The PCR products were of the expected molecular size, and the gene expression levels were normalized relative to the level of 18S ribosomal RNA.

Cell Culture

Cultured cardiac fibroblasts were isolated from ventricles of 1-day-old Wistar rats as described previously.[20] The cells were treated with 1 μmol/L Ang II in the absence or presence of BAY41-2272 for 24 hours. The magnitude of their proliferation was assessed by measuring the amount of [^{3}H]-thymidine incorporated into the cells.[20]

Assays for ANP, BNP, and cGMP

Blood samples were collected at day 14 with 1.5 mg/mL of di-sodium ethylenediamine tetraacetate and 500 kallikrein inactivator units per milliliter of aprotinin, centrifuged at 2000*g* for 15 minutes at 4°C and then stored at −80°C until use. Plasma levels of ANP were measured with a specific radioimmunoassay, as described previously,[21] and those of BNP with a commercially available kit (Peninsula Laboratories Inc). To determine the effect of BAY41-2272 on cGMP levels in the LV, rats were infused with 250 ng/kg per minute of Ang II for 14 days and given orally 2 or 10 mg/kg per day of BAY41-2272 twice a day on days 13 and 14. After the animals were euthanized, the myocardial tissue was immediately collected and stored at −80°C. In the cell culture study, fibroblasts were treated with BAY41-2272 for 10 minutes and immediately collected as described previously.[12] cGMP content was determined using a radioimmunoassay kit (YAMASA Cyclic GMP Assay Kit).

Statistical Analysis

Values shown are expressed as mean±SEM. Differences between groups were assessed using the 1-way ANOVA followed by Scheffe's test, and statistical significance was accepted at $P<0.05$.

Results

Hemodynamics

Figure 1A and 1B illustrate the effects of Ang II and BAY41-2272 on the systolic blood pressure and heart rate. Continuous, subcutaneous infusion of Ang II significantly ($P<0.01$) raised systolic blood pressure from days 3 to 14. The high dose of BAY41-2272 significantly reduced systolic blood pressure during the first 10 days in the Ang II–infused rats; however, the reduction became insignificant at day 14, and the low dose of BAY41-2272 had no effect on blood pressure raised by Ang II (Figure 1A). Heart rates of the Ang II infusion groups without BAY41-2272 or with the low dose of BAY41-2272 were reduced, but the changes were statistically insignificant (Figure 1B). As shown in Figure 1C, LVEDP was significantly ($P<0.05$) raised by the infusion of Ang II and was lowered by the high dose of the drug ($P<0.05$) but not by the low dose.

Cardiac Hypertrophy and Collagen Deposition

As shown in Figure 2A and 2B, the infusion of Ang II significantly ($P<0.01$) increased the ratio of heart weight:body

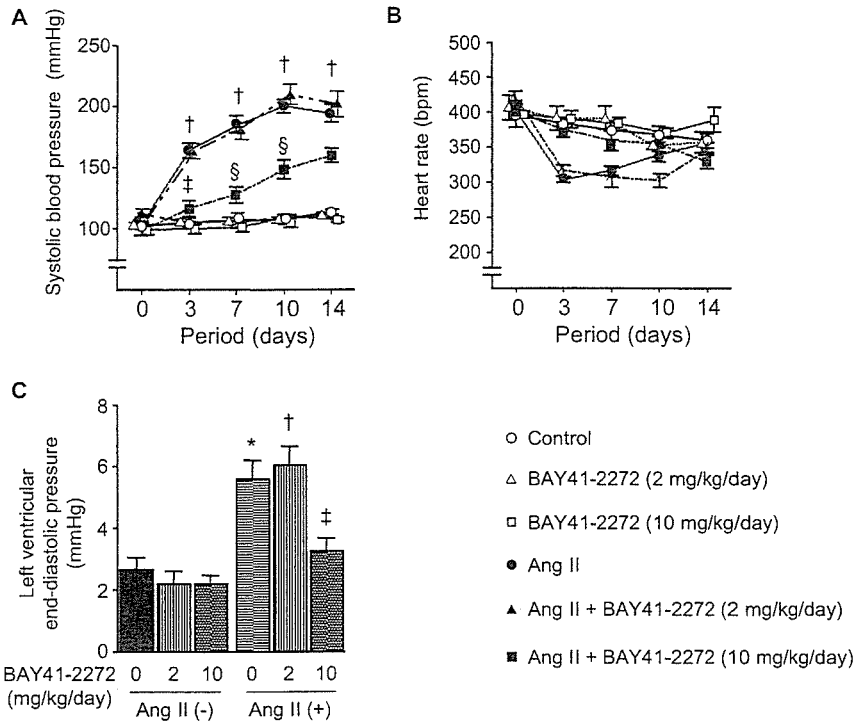

Figure 1. Effects of BAY41-2272 on systolic blood pressure (A), heart rate (B), and LVEDP (C). Values are shown as mean±SEM. *$P<0.05$, †$P<0.01$, vs the control group without Ang II and BAY41-2272; ‡$P<0.05$, §$P<0.01$, vs the Ang II group without BAY41-2272.

weight and cross-sectional area of myocardial fibers, compared with the control, at day 14. The low dose of BAY41-2272 had no effect on the Ang II–induced increase in heart weight:body weight and cardiocyte size, but the high dose significantly ($P<0.01$) reduced both. Figure 3A shows the effect of BAY41-2272 on collagen deposition in the perivascular area of intramyocardial coronary arteries. Ang II significantly ($P<0.01$) increased the deposition, but the low and high doses of BAY41-2272 significantly reduced it by 20% ($P<0.05$) and by 30% ($P<0.01$), respectively. Similarly, Ang II–induced collagen deposition in the myocardial interstitial area was reduced by both the low (−21%; $P<0.05$) and high (−38%; $P<0.01$) dose of BAY41-2272 (Figure 3B).

Myofibroblastic Differentiation

Figure 4 illustrates numbers of fibroblasts positive for α-SMA, a marker for myofibroblastic differentiation, in the perivascular area surrounding intramyocardial coronary arteries. Ang II increased the number of α-SMA–positive cells ($P<0.05$) compared with controls; however, the low and high doses of BAY41-2272 similarly decreased the number by 74% and 79% ($P<0.05$), respectively.

LV Gene Expression

As shown in Figure 5A and 5B, Ang II significantly augmented the LV gene expression of type 1 collagen ($P<0.01$) and TGF-β1 ($P<0.05$). Coadministration of BAY41-2272 significantly reduced the Ang II–induced increases in mRNA for type 1 collagen (low dose: −41%; high dose: −49%) and TGF-β1 (low dose: −49%; high dose: −65%). Although statistically insignificant, slight elevations of both mRNA levels were observed in the high-dose treatment group without Ang II infusion.

Measurements of ANP, BNP, and cGMP

The Ang II infusion significantly ($P<0.01$) increased the plasma level of ANP, but this was not the case for those of

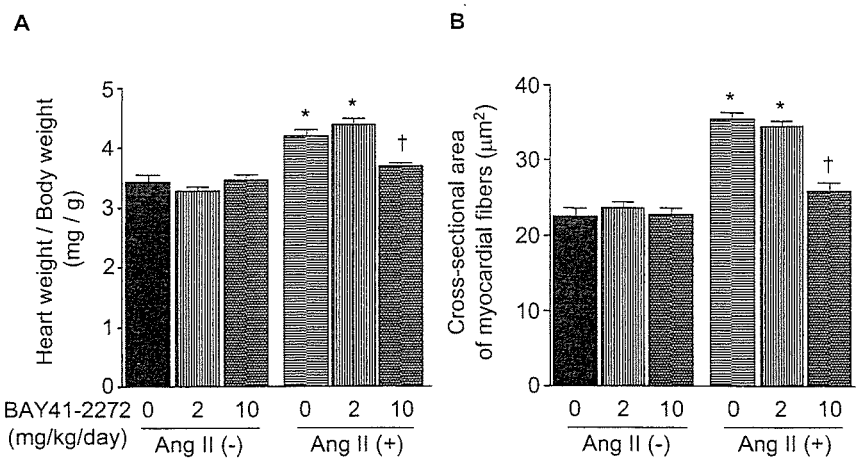

Figure 2. Effects of BAY41-2272 on heart weight:body weight (A) and cross-sectional area of myocardial fibers (B). Values are shown as mean±SEM. *$P<0.01$ vs the control group without Ang II and BAY41-2272; †$P<0.01$ vs the Ang II group without BAY41-2272.

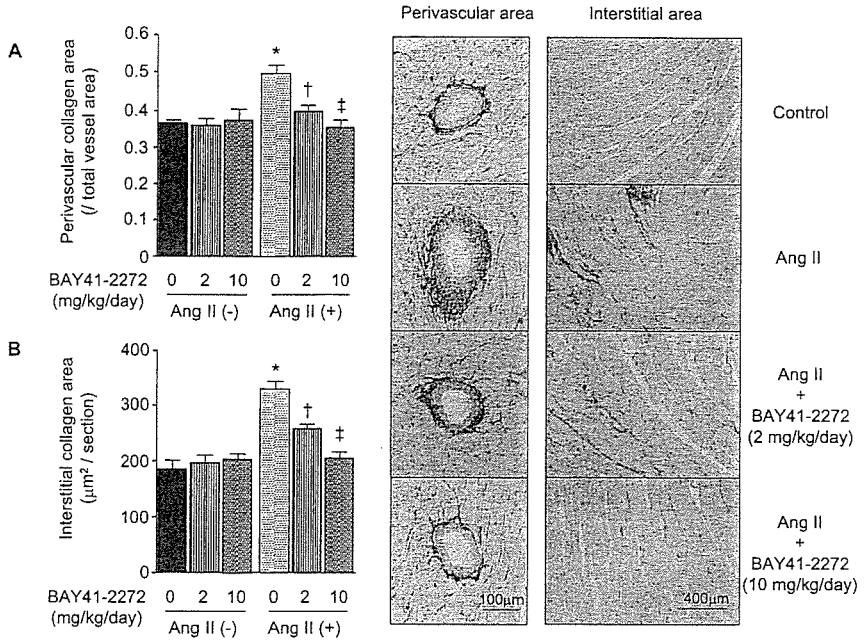

Figure 3. Effect of BAY41-2272 on picrosirius red-positive areas in adventitia of intramyocardial coronary arteries (A) and myocardial interstitium (B). Panels show representative images of the control and Ang II groups with or without BAY41-2272 treatment. The original magnification of the perivascular area and myocardial interstitium is ×400 and ×100, respectively. Values are shown as mean±SEM. *$P<0.01$ vs the control group without Ang II and BAY41-2272; †$P<0.05$, ‡$P<0.01$, vs the Ang II group without BAY41-2272.

BNP, where both the low and high doses of BAY41-2272 had no significant effects on their plasma levels (Table 1). As shown in Table 2, tissue cGMP concentrations in the LV were significantly increased by treatment with the low and high doses of BAY41-2272 with or without the infusion of Ang II.

Cell Culture Study

Figure 6A and 6B illustrate the effects of Ang II and BAY41-2272 on proliferation and intracellular cGMP in cultured cardiac fibroblasts. As shown in Figure 6A, BAY41-2272 significantly ($P<0.01$) attenuated not only basal but also Ang II–stimulated [^{3}H]-thymidine incorporation. The inhibition of fibroblast proliferation was accompanied by a significant rise in the intracellular cGMP level (Figure 6B).

Discussion

Both mechanical load and humoral activation have been shown to cause cardiac hypertrophy and fibrosis in hypertensive heart disease.[22,23] Accordingly, not simply controlling systemic blood pressure but also pharmacological approaches to managing cardiovascular remodeling are necessary for treatment of hypertensive patients. The direct sGC stimulator BAY41-2272 has been shown to lower arterial pressure and peripheral resistance and to increase cardiac output and renal blood flow by raising intracellular cGMP levels[15–17]; however, it remained to be elucidated whether this compound affects cardiac remodeling. In the present study, the continuous stimulation of sGC with BAY41-2272 attenuated LV hypertrophy and fibrosis in rats with Ang II–induced hypertension, suppressing the phenotypic change of fibroblasts and the expression of extracellular matrix–related genes. Thus, this study suggests the importance of the activation of sGC and subsequent rise in the intracellular concentration of cGMP in attenuating adverse cardiac remodeling associated with hypertension.

Two isotypes of GC, particulate GC and sGC, are widely distributed in various tissues and organs including the heart

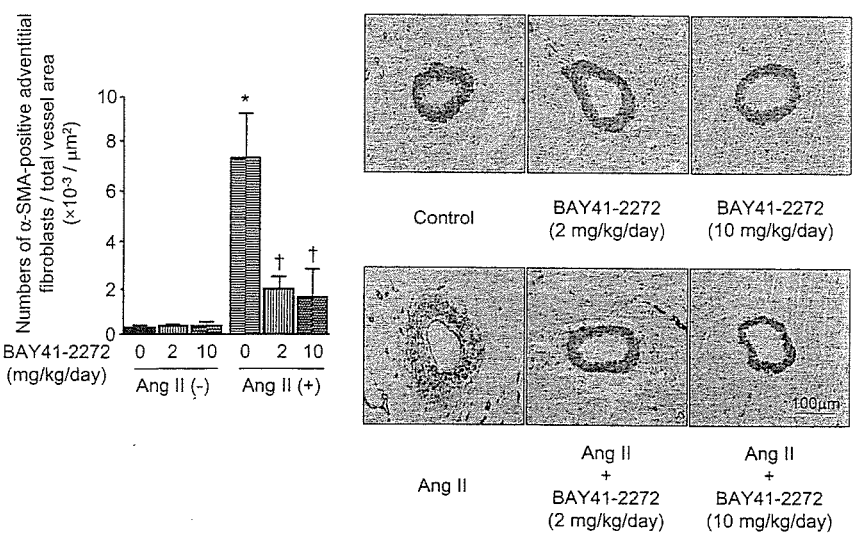

Figure 4. Effect of BAY41-2272 on numbers of adventitial fibroblasts positive for α-SMA. The number of cells positive for α-SMA surrounding intramyocardial coronary arteries was standardized with the coronary vessel area. Panels show representative histological sections stained with α-SMA antibody at an original magnification of ×400. Values are shown as mean±SEM. *$P<0.05$ vs the control group without Ang II and BAY41-2272; †$P<0.05$ vs the Ang II group without BAY41-2272.

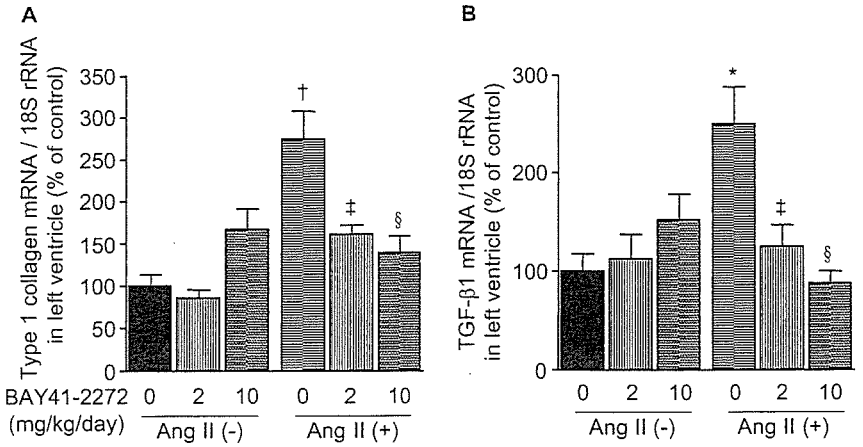

Figure 5. Effects of BAY41-2272 on mRNA levels for type 1 collagen (A) and TGF-β1 (B) in left ventricle. Values are shown as mean±SEM. *$P<0.05$, †$P<0.01$, vs the control group without Ang II and BAY41-2272; ‡$P<0.05$, §$P<0.01$, vs the Ang II group without BAY41-2272.

and kidneys.[24] Although 2 isotypes of the cGMP-generating enzymes share some structural homology, their enzymatic activity is regulated differentially: natriuretic peptides stimulate particulate GC, whereas NO evokes the activity of sGC.[24] Most of the actions of natriuretic peptides, including the suppression of cardiac hypertrophy and fibrosis, are assumed to be mediated by intracellular cGMP. It has been shown that the direct sGC stimulator BAY41-2272 also exerts its effects by raising intracellular cGMP levels,[15–17] and consistent with this, BAY41-2272 increased cGMP levels in the LV myocardium tissue and in cultured cardiac fibroblasts in the present study. Because a question may arise over activity of the natriuretic peptide particulate GC system during activation of sGC by BAY41-2272, we measured plasma ANP and BNP levels in the study groups. The subcutaneous infusion of Ang II indeed increased the plasma level of ANP, but BAY41-2272 had no effect, suggesting that natriuretic peptides were unlikely involved in the increased cGMP levels in the LV.

BAY41-2272 has been reported to be quickly oxidized after oral administration, although Straub et al[25] showed that the oxidized metabolite exerted a stronger and longer pharmacological effect than BAY41-2272 itself in vivo. In contrast to nitroglycerin, which activates sGC by releasing NO, drug tolerance has been reported to hardly occur for BAY41-2272.[14] In the present study, the blood pressure–lowering effect of the high dose of BAY41-2272 in the Ang II–infused rats had been significant during the first 10 days, but it became insignificant at day 14. Despite the incomplete reduction of blood pressure, BAY41-2272 substantially alleviated cardiomyocyte hypertrophy and collagen accumulation surrounding the intramyocardial coronary arteries and in the myocardial interstitium, reducing LVEDP, in the Ang II–infused rats. The reduction in collagen deposition was accompanied by suppression of the phenotypic change of fibroblasts into myofibroblasts and by lowering of the mRNA levels of type 1 collagen and TGF-β1. Because the phenotypic change of fibroblasts to myofibroblasts by Ang II or TGF-β1 has been found critical in stimulating fibroblast proliferation and producing extracellular matrix,[26] suppression of this process is important for attenuating cardiac fibrosis. Notably, the low dose of BAY41-2272 had no effect on blood pressure or cardiac hypertrophy but substantially suppressed fibroblastic activation, LV gene expression, and collagen deposition, raising the LV cGMP level.

Because the natriuretic peptides–particulate GC system has been shown to suppress cardiac hypertrophy and fibrosis independently of blood pressure,[9,13,27] we further investigated whether BAY41-2272 has the direct effects in vitro on the cultured cardiac fibroblasts. In the cell culture study, BAY41-2272 inhibited the proliferation of cardiac fibroblasts, elevating the intracellular cGMP level, supporting a direct inhibitory action of this compound on cardiac fibrosis observed in vivo. On the other hand, alleviation of the cardiomyocyte hypertrophy was observed in the high-dose group but not in the low-dose group in the present study, and we found that BAY41-2272 had little effect on hypertrophy in the cultured cardiomyocytes (data not shown). Thus, we speculate that the improvement of cardiomyocyte hypertrophy observed in this study is largely dependent on mechanical load rather than a direct effect of BAY41-2272.

TABLE 1. Plasma Levels of ANP and BNP

Group	ANP, fmol/mL	BNP, fmol/mL
Control	13±2	12±1
BAY41-2272, 2 mg/kg per day	15±1	10±2
BAY41-2272, 10 mg/kg per day	20±2	9±1
Ang II	28±3*	11±1
Ang II+BAY41-2272, 2 mg/kg per day	29±2*	12±2
Ang II+BAY41-2272, 10 mg/kg per day	28±2*	13±1

Values are shown as mean±SEM, n=7 to 13 in each group.
*$P<0.01$ vs controls.

TABLE 2. Concentration of cGMP in the Left Ventricle

Group	cGMP, fmol/mg Protein
Control	509±163
BAY41-2272, 2 mg/kg per day	831±150*
BAY41-2272, 10 mg/kg per day	1070±56†
Ang II	603±75
Ang II+BAY41-2272, 2 mg/kg per day	899±53‡
Ang II+BAY41-2272, 10 mg/kg per day	924±197‡

Values are shown as mean±SEM, n=4 in each group.
*$P<0.05$, †$P<0.01$ vs controls; ‡$P<0.05$ vs Ang II.

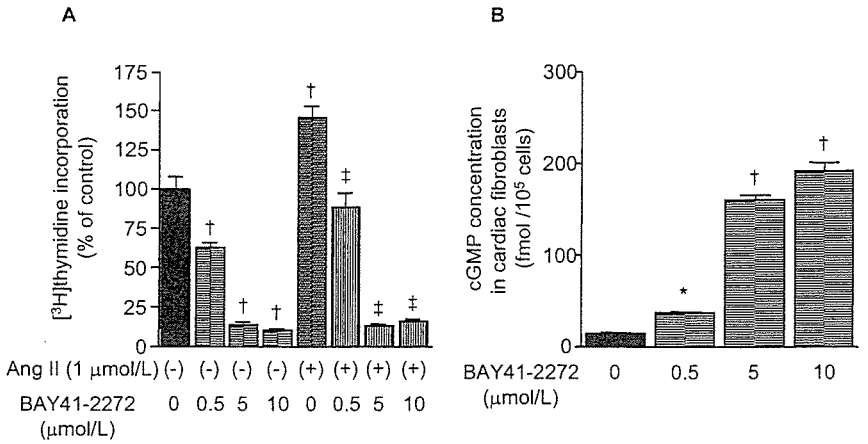

Figure 6. Effects of BAY41-2272 on [^{3}H]-thymidine incorporation (A) and intracellular cGMP production (B) in cultured cardiac fibroblasts. Values are shown as mean±SEM of 7 (A) and 12 (B) samples examined. *P<0.05, †P<0.01, vs the control group without Ang II and BAY41-2272; ‡P<0.01 vs the Ang II group without BAY41-2272.

In conclusion, this study demonstrated that the continuous stimulation of sGC with BAY41-2272 for 2 weeks ameliorated Ang II–induced cardiac remodeling in rats, and the effects on the extracellular matrix may have been exerted partially via cGMP, independently of blood pressure. Thus, sGC generating cGMP would be a therapeutic target for reducing the adverse cardiovascular remodeling associated with hypertension.

Perspectives

Given the significance of myocardial fibrosis and hypertrophy in the process of cardiac remodeling in hypertensive subjects, the present findings may have important implications with regard to pharmacological stimulation of sGC for attenuating the remodeling process of the LV. In this study, we have shown that the orally available compound BAY41-2272, a direct sGC stimulator, would be useful not only in reducing blood pressure but also in attenuating cardiac remodeling. In addition, no adverse effects of BAY41-2272 on the liver or kidneys were detected at least in data of the serum aminotransferases and creatinine levels (Table I, available online at http://hyper.ahajournals.org). Because of the limited clinical use of human recombinant ANP and BNP because of their short half-lives, the present study suggests a potential usefulness for this compound in the treatment of hypertension, warranting further studies, such as administration to other models of hypertension or treatment for longer time periods.

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Disclosures

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Increased Production of Adrenomedullin in Glomeruli from Anti-Glomerular Basement Membrane Glomerulonephritis Rats Treated with Methylprednisolone

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Key Words
Adrenomedullin · Mesangial cells · Anti-GBM GN rats · Methylprednisolone

Abstract
Background/Aims: Adrenomedullin (AM) has anti-proliferative and apoptotic effects on mesangial cells (MCs). Both effects play an important role in the progression of glomerulonephritis (GN). Glucocorticoids are widely used for the treatment of GN; however, the relationship between AM regulation in MCs or glomeruli and glucocorticoid treatment has not been clarified. ***Methods:*** Using the cultured rat MCs, AM secretion induced by methylprednisolone (m-PSL), and MC proliferation and apoptosis caused by AM were examined. In addition, the role of AM receptor antagonist, AM(22-52), was also investigated. Then, we made an anti-glomerular basement membrane (GBM) GN rat model and compared the AM expression and production in each glomeruli obtained from the control or m-PSL-treated anti-GBM GN rats. ***Results:*** In the cultured rat MCs, AM secretion was increased by m-PSL. MC proliferation was inhibited, while MC apoptosis was increased by AM. MC apoptosis was inhibited by the addition of AM(22-52). M-PSL therapy ameliorated the progression of anti-GBM GN rats. AM expression and production were increased in the glomeruli from m-PSL-treated rats compared to the controls. ***Conclusion:*** Considering the anti-proliferative and apoptotic effects of AM on MCs, increased AM in the glomeruli might participate in the improvement of glomerular lesions in anti-GBM GN rats treated with m-PSL.

Introduction

Adrenomedullin (AM), a vasorelaxant peptide isolated from pheochromocytoma, is now known to possess multiple biological properties, and is expressed on and secreted from a wide variety of cells and tissues [1, 2]. In the kidney, AM is immunoreactively seen in cortical distal tubules, medullary collecting duct cells and glomeruli, where AM is present in microvascular areas, mesangial cells (MCs) and podocytes [3–5]. AM has anti-proliferative and apoptotic effects on MCs. Both effects play an important role in the progression/resolution of mesangial proliferative glomerulonephritis (GN) [6–12]. Recently, the renoprotective effects of AM were reported in in vivo studies using experimental rat models. These preferable results are considered to be caused by the vasorelaxant, natriuretic and antiproliferative effects of AM [13–18]. On the other hand, several in vitro studies have shown the effects of various substances on AM secretion from certain cultured cells. For example, in cultured endothe-

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lial cells, vascular smooth muscle cells and cardiac fibroblasts, the increased secretion of AM was observed by glucocorticoid treatment, which is widely administered for GN in humans [19, 20]. However, AM regulation in the glomeruli with GN and cultured MCs by glucocorticoid treatment has not been observed.

We first, using the cultured rat MCs, examined the relationships between glucocorticoid and AM secretion, and then between proliferation or apoptosis of MCs and AM. Subsequently, we investigated the changes of AM in glomeruli obtained from anti-glomerular basement membrane (GBM) GN under glucocorticoid therapy.

Materials and Methods

Cell Culture Experiments

Rat MCs were obtained from the glomeruli of the kidney cortex isolated from 170 g of male Sprague-Dawley rats (Charles River Breeding, Yokohama, Japan) by differential sieving. Cells were allowed to grow at 37°C in a humidified 5% CO_2 atmosphere. The identity of MCs was confirmed using standard criteria. Passages between 3 and 5 were used for the experiments. MCs were grown in RPMI medium with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 5 μg/ml insulin, 5 μg/ml transferring and 5 ng/ml sodium selenite.

To examine the effect of glucocorticoid on AM secretion from MCs, the cells were plated in 6-well culture plates. After reaching confluence, the cells were incubated in serum free-media with or without m-PSL (Sigma, St. Louis, Mo., USA) for 24 h. After 24 h, more than 90% of the cells were viable under these conditions. The total AM in the supernatants of the cultured rat MCs was measured using a two-site immunoenzymometric assay, as described previously [21].

To examine the effect of AM on MC proliferation, MCs were seeded at 6×10^3 cells/well in a 96-well plate, and then incubated in serum-free media with or without AM and 20 ng/ml platelet-derived growth factor (PDGF)-BB. After 24 h, 10 μl of the reaction solution was added to 100 μl of culture medium/well. The cells were incubated for an additional 4 h, and then the numbers of cells were determined colorimetrically (absorbance at 450 nm) using the cell proliferation reagent, WST-1 (Roche Diagnostics GmbH, Penzberg, Germany).

To examine the effect of AM on MC apoptosis, MCs were seeded at 1.2×10^4 cells/well in a 96-well plate, and then incubated in the medium containing 1% FBS with or without AM. In addition, to evaluate the role of m-PSL induced AM secretion from the MCs, they were also incubated with or without m-PSL and AM receptor antagonist AM(22-52). After 20 h, apoptosis was detected using an ELISA method with the use of Cell Death Detection ELISA Plus (Roche Diagnostics GmbH, Penzberg, Germany), which detected the increased mononucleosomes and oligonucleosomes in the cytoplasm of apoptotic cells. Cells were lysed and 20 μl of the supernatant, which contained the cytoplasmic fraction, was added to a streptavidin-coated plate. Then, a mixture of anti-histone-biotin and anti-DNA-POD was added. After incubation, the unbound components were removed, and the amount of the bound nucleosomes was detected colorimetrically (absorbance at 405 nm) by adding 100 μl/well of 2,2-azino-di-[3-ethylbenzthiazolin sulfonate] (ABTS) solution.

Induction of Anti-GBM GN Model

Rabbit anti-rat GBM serum (nephrotoxic serum, NTS) was prepared as described previously [22]. An anti-GBM GN model (NTS rats) was induced in male WKY 7-week-old rats weighing 170–190 g (Charles River Breeding, Yokohama, Japan) by the intravenous injection of NTS (0.3 ml/100 g body weight). In this model, MC proliferation was found on day 3 (fig. 1a), glomerular necrosis with cellular crescents on day 7 (fig. 1b), and then glomerular sclerosis in a month. As a control, rats were injected with the same dose of normal rabbit serum.

Experimental Design

According to Ou et al. [23], treatment with m-PSL (30 mg/kg/BW i.p.) was started on the control and NTS rats on day 3, and continued for four consecutive days. Rats were divided into the following four groups (n = 6 each): control (C), control treated with m-PSL (C-MP), NTS and NTS treated with m-PSL (NTS-MP). Each rat was sacrificed on day 7. Immediately after the sacrifice, renal tissues were taken from the animals for histological studies, and the glomeruli were isolated by differential sieving for mRNA detection, as described previously [17]. Urinary protein and creatinine were measured on day 7 using the standard methods. The urinary protein data were corrected according to the creatinine concentration (g/g · creatinine).

Measurement of mRNA by Real-Time Quantitative PCR

We measured the rat AM and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using real-time quantitative PCR, and the oligonucleotide primers and probes, as described previously [17]. The PCR products were used as standards and the mRNA levels were compared after normalization relative to those of GAPDH.

Renal Histological Findings and Immunohistochemistry for AM

Renal tissues were obtained from rats, fixed in 10% formalin within 24 h, and embedded in paraffin. Sections of 4 μ*M* in thickness from each block were stained with periodic acid-Schiff (PAS) for morphological examination. Immunofluorescence staining was performed on frozen sections using antibodies against rabbit IgG and rat IgG (Dako, Glostrup, Denmark). Immunohistochemical studies were performed using the standard streptavidin-biotin immunoperoxidase method, with En Vision + (Dako, Carpinteria, Calif., USA). Monoclonal antibody against synthetic AM(12-25) (a ring structure) [24] was used in this study. To evaluate the quantity of glomerular AM, we randomly selected 20 glomeruli per kidney section. Images were automatically analyzed using a color imaging morphometric analysis system (Mac SCORP, Mitani, Fukui, Japan). The average area staining positive for AM was calculated as a percentage of the glomerular area.

Statistical Analysis

All data were expressed as means ± SEM. One-way analysis of variance (ANOVA) was used for statistical analysis, followed by a multiple comparison test (Fisher's Protected Least Significant Dif-

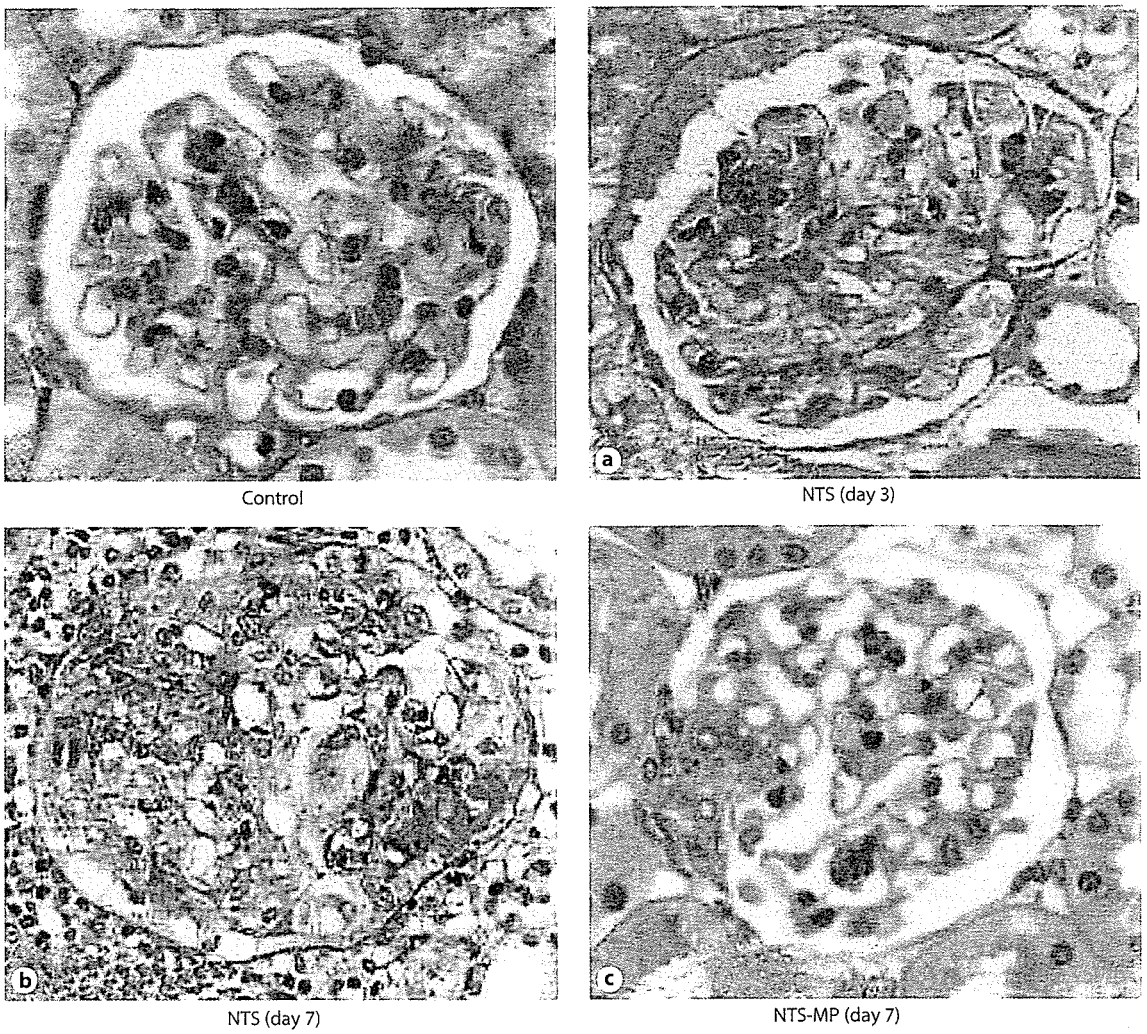

Fig. 1. (PAS staining). Renal histology. **a** Segmental mesangial cell proliferation on day 3 in rats with anti-GBM glomerulonephritis (NTS rats). **b** Diffuse mesangial cell proliferation and crescent formation on day 7 in NTS rats. **c** Improvement of glomerular lesions on day 7 in NTS rats treated with methylprednisolone (NTS-MP rats).

ference test). If the numbers in each group were less than 4, Student's t test was used to compare the two groups. $p < 0.05$ was used as the level of significance.

Results

Cell Culture Experiments

MCs, incubated for 24 h in serum-free medium, secreted 0.6 ± 0.1 fmol/1.4 × 10^5 cells of AM. Adding m-PSL to the culture medium, the AM secretion from MCs was increased in a concentration-dependent manner (control vs. 10^{-8} *M*, 10^{-7} *M*, 10^{-6} *M* fmol/1.4 × 10^5 cells, 0.6 ± 0.1 vs. 2.3 ± 0.3, 2.4 ± 0.3, 3.2 ± 0.6 fmol/1.4 × 10^5 cells, $p < 0.01$, $p < 0.01$, $p < 0.0001$, respectively) (fig. 2). PDGF (20 ng/ml) stimulated the proliferation of MCs (control vs. PDGF alone, 100 vs. 142%, $p < 0.05$), which was inhibited in a concentration-dependent manner by the addition of AM into the cultured MCs (PDGF alone vs. 10^{-11} *M*, 10^{-9} *M*, 100% vs. 54%, 32%, $p < 0.01$, $p < 0.0001$, respectively). MC apoptosis was increased by the addition of AM into the cultured MCs (control vs. 10^{-10} *M*, 10^{-8} *M*, 10^{-6} *M*/1.2 × 10^4 cells, 0.28 ± 0.01 vs. 0.39 ± 0.02, 0.59 ± 0.07, 0.55 ± 0.03/1.2 × 10^4 cells, $p < 0.01$, respectively) (fig. 3a). On the other hand, the MC apoptosis was significantly inhibited by the addition