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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

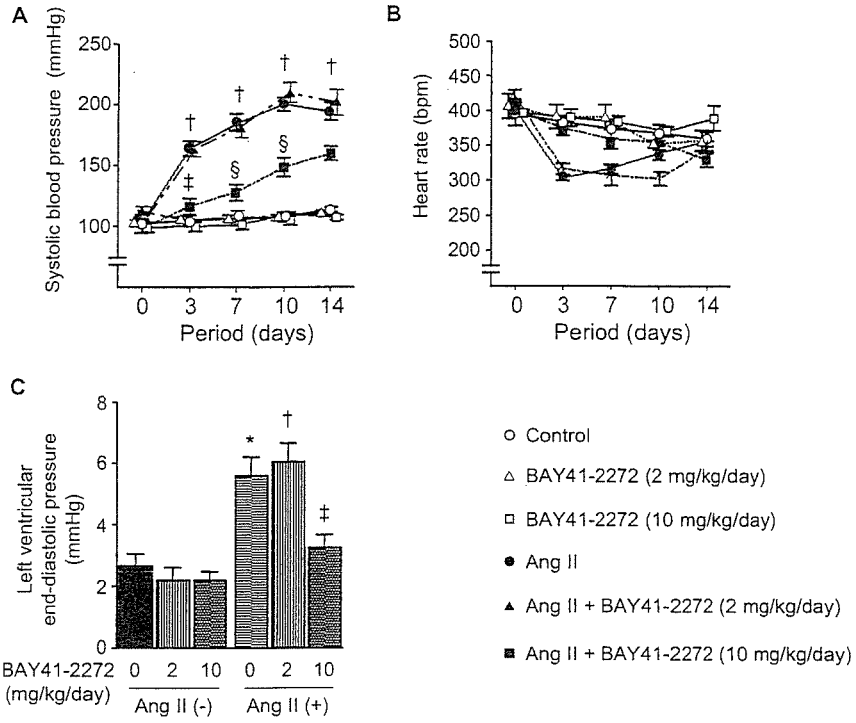

Figure 1. Effects of BAY41-2272 on systolic blood pressure (A), heart rate (B), and LVEDP (C). Values are shown as mean±SEM. *$P<0.05$, †$P<0.01$, vs the control group without Ang II and BAY41-2272; ‡$P<0.05$, §$P<0.01$, vs the Ang II group without BAY41-2272.

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

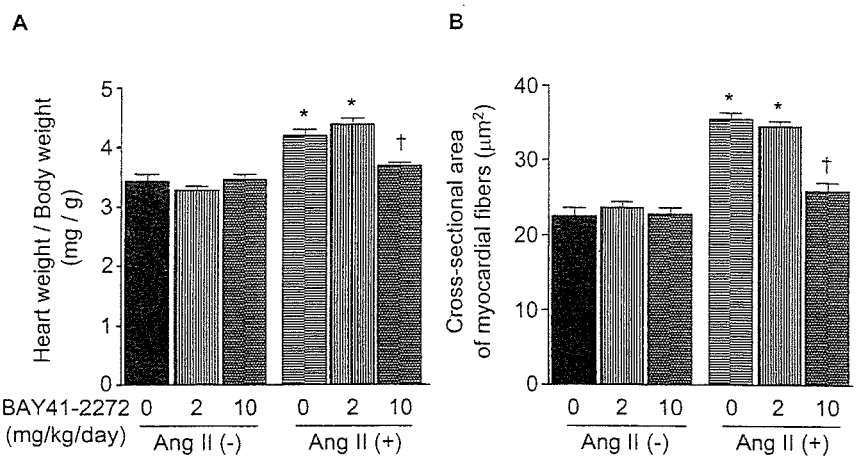

Figure 2. Effects of BAY41-2272 on heart weight:body weight (A) and cross-sectional area of myocardial fibers (B). Values are shown as mean±SEM. *$P<0.01$ vs the control group without Ang II and BAY41-2272; †$P<0.01$ vs the Ang II group without BAY41-2272.

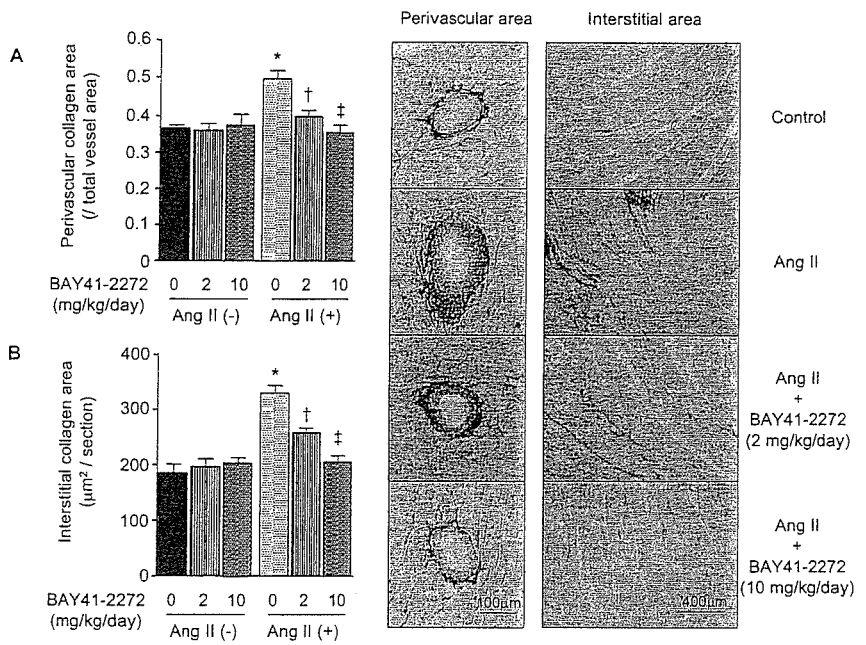

Figure 3. Effect of BAY41-2272 on picrosirius red-positive areas in adventitia of intramyocardial coronary arteries (A) and myocardial interstitium (B). Panels show representative images of the control and Ang II groups with or without BAY41-2272 treatment. The original magnification of the perivascular 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

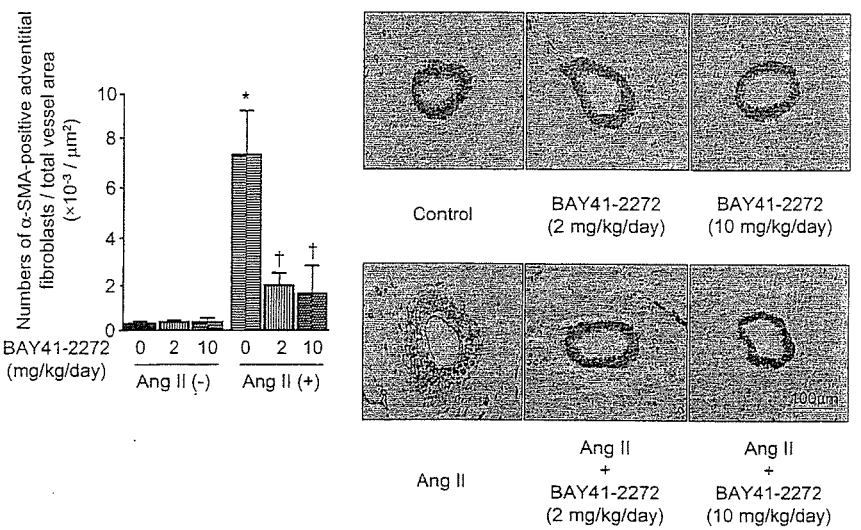

Figure 4. Effect of BAY41-2272 on numbers of adventitial fibroblasts positive for α-SMA. The number of cells positive for α-SMA surrounding intramyocardial coronary arteries was standardized with the coronary vessel area. Panels show representative histological sections stained with α-SMA antibody at 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.

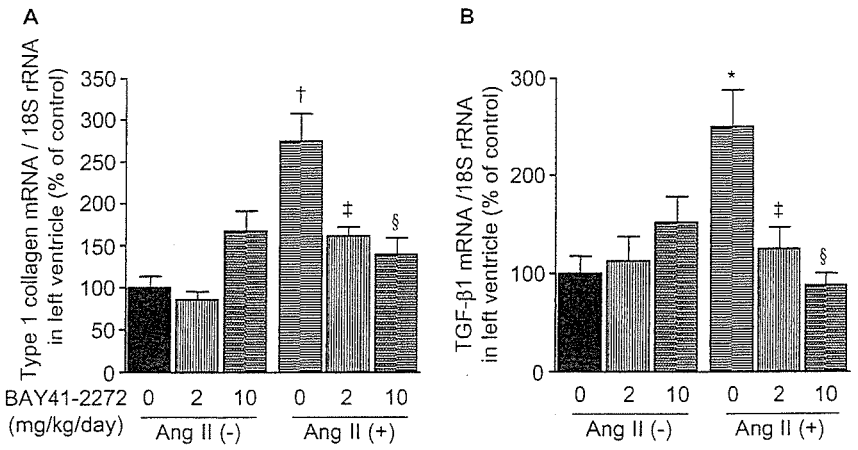

Figure 5. Effects of BAY41-2272 on mRNA levels for type 1 collagen (A) and TGF-β1 (B) in left ventricle. Values are shown as mean±SEM. *$P<0.05$, †$P<0.01$, vs the control group without Ang II and BAY41-2272; ‡$P<0.05$, §$P<0.01$, vs the Ang II group without BAY41-2272.

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.

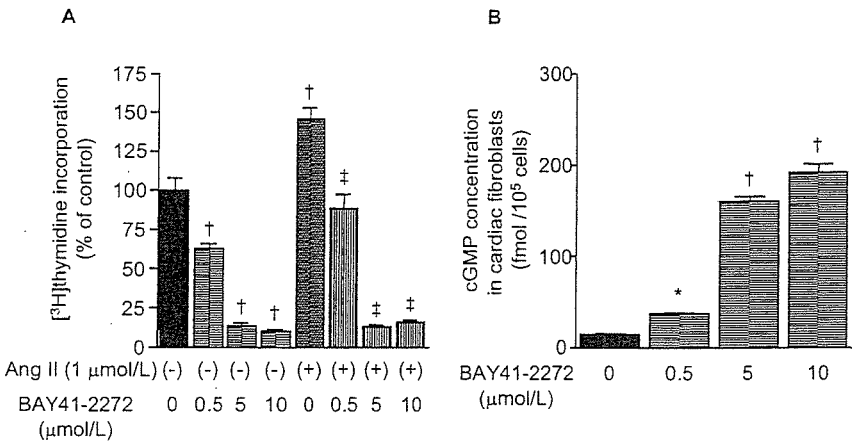

Figure 6. Effects of BAY41-2272 on [^{3}H]-thymidine incorporation (A) and intracellular cGMP production (B) in cultured cardiac fibroblasts. Values are shown as mean±SEM of 7 (A) and 12 (B) samples examined. *P<0.05, †P<0.01, vs the control group without Ang II and BAY41-2272; ‡P<0.01 vs the Ang II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

H.M. and T.T. have received a research grant from Bayer HealthCare, and J-P.S. is an employee of that company. BAY41-2272 was synthesized at Bayer HealthCare as a research tool but not for use in humans.

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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-

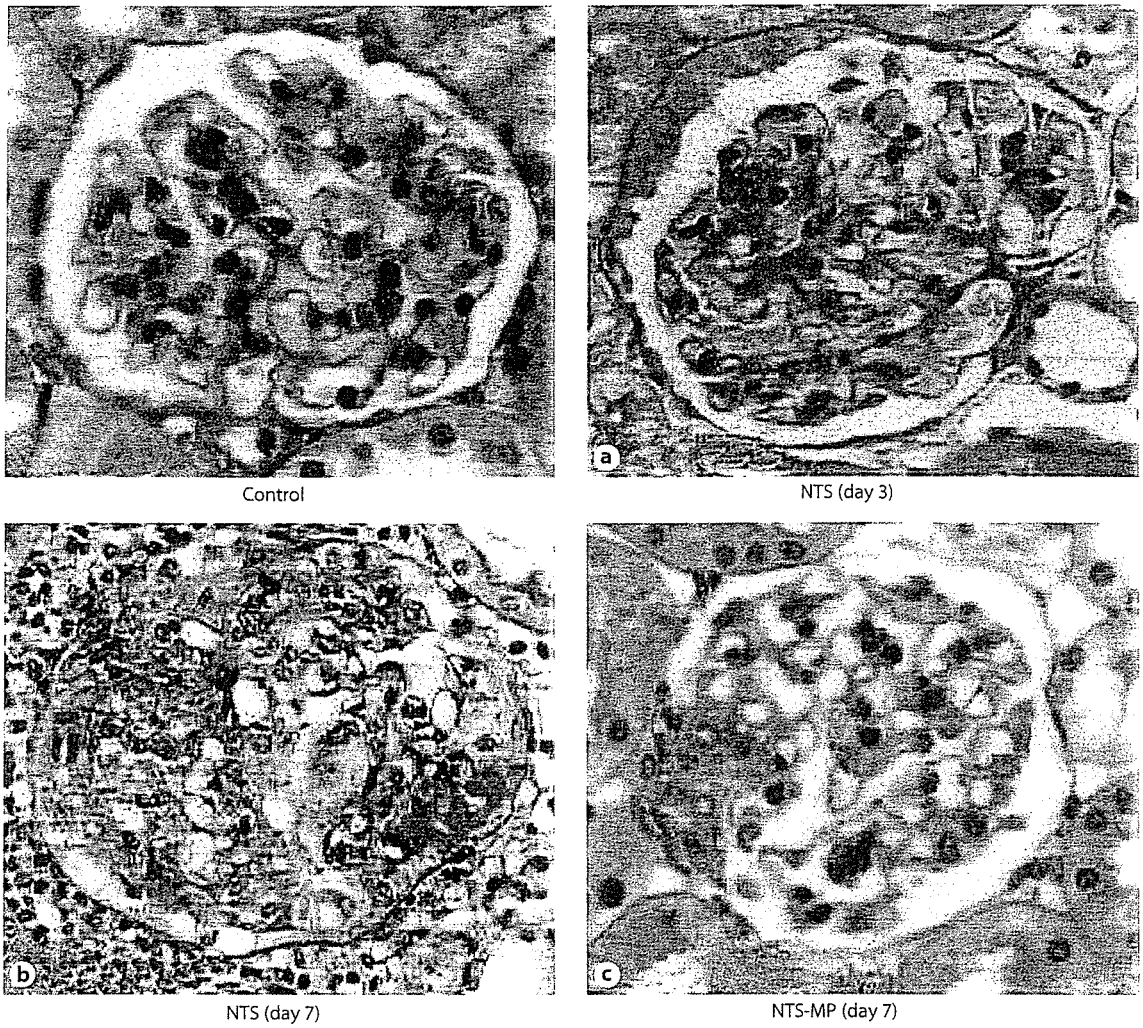


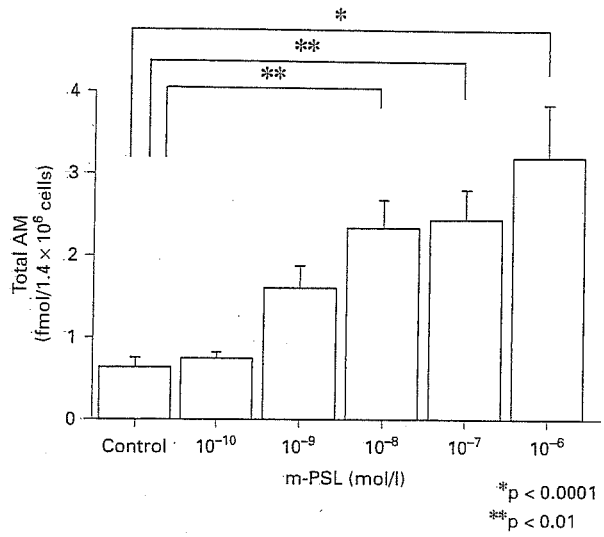
Fig. 1. (PAS staining). Renal histology. **a** Segmental mesangial cell proliferation on day 3 in rats with anti-GBM glomerulonephritis (NTS rats). **b** Diffuse mesangial cell proliferation and crescent formation on day 7 in NTS rats. **c** Improvement of glomerular lesions on day 7 in NTS rats treated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



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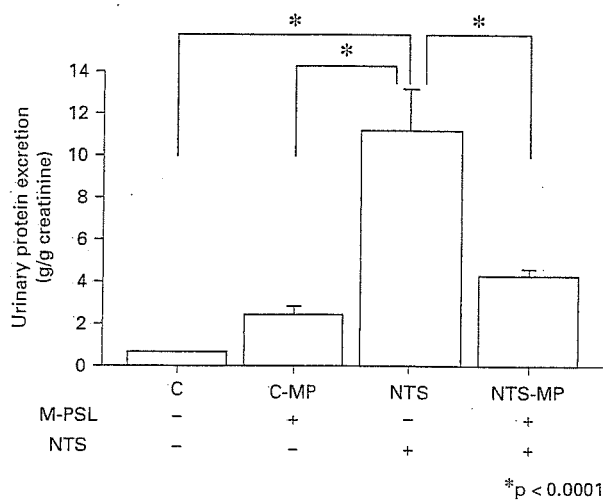

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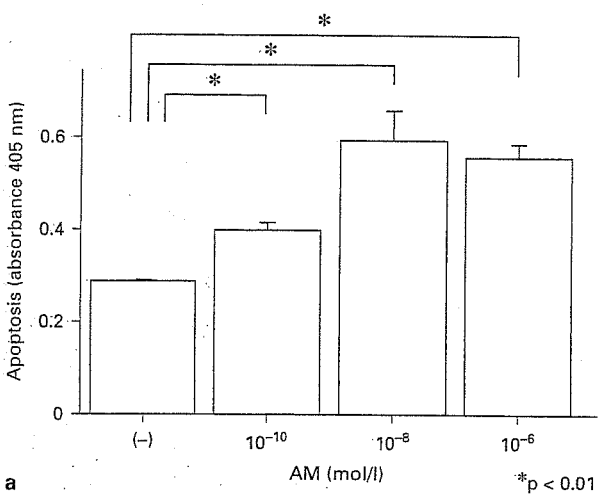

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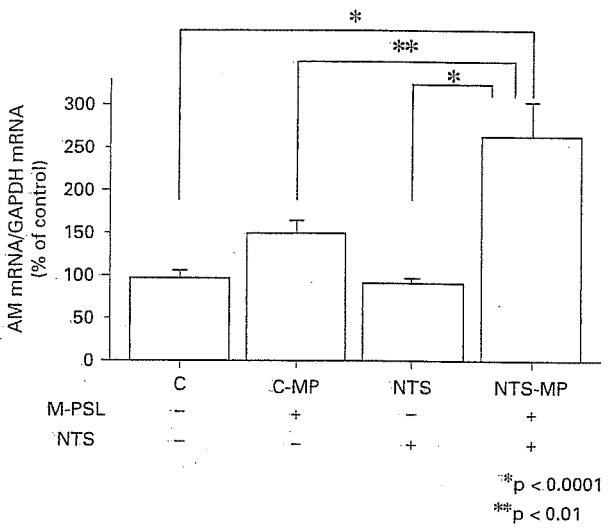

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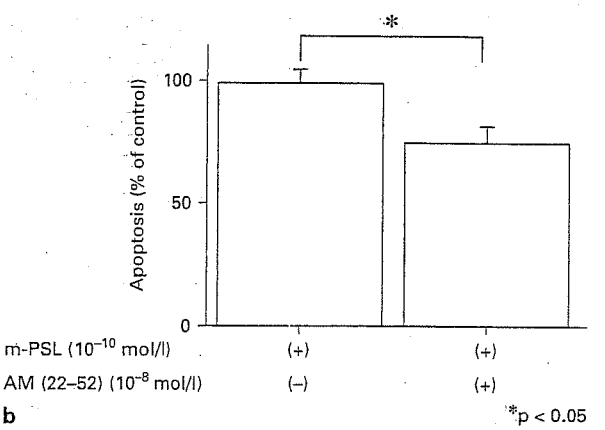

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Fig. 2. Changes of adrenomedullin secretion from the rat cultural mesangial cells by methylprednisolone treatment. Values are expressed as the mean ± SEM (n = 6). * $p < 0.0001$, ** $p < 0.01$.

Fig. 3. a Changes of mesangial cell apoptosis by adrenomedullin treatment. Values are expressed as the mean ± SEM (n = 3). * $p < 0.01$, compared to control cells. **b** Effect of adrenomedullin receptor antagonist AM(22-52) on the apoptosis of rat mesangial cells. Values are expressed as the mean ± SEM (n = 4). * $p < 0.05$

Fig. 4. Urinary protein excretion in the control (C), control treated with methylprednisolone (C-MP), anti-GBM glomerulonephritis model (NTS) and NTS rat groups treated with methylprednisolone (NTS-MP). Values are expressed as the mean ± SEM (n = 6). * $p < 0.0001$.

Fig. 5. Glomerular adrenomedullin expression in the control (C), control treated with methylprednisolone (C-MP), anti-GBM glomerulonephritis model (NTS) and NTS rat groups treated with methylprednisolone (NTS-MP). Values are expressed as the mean ± SEM (n = 6). * $p < 0.0001$, ** $p < 0.01$.

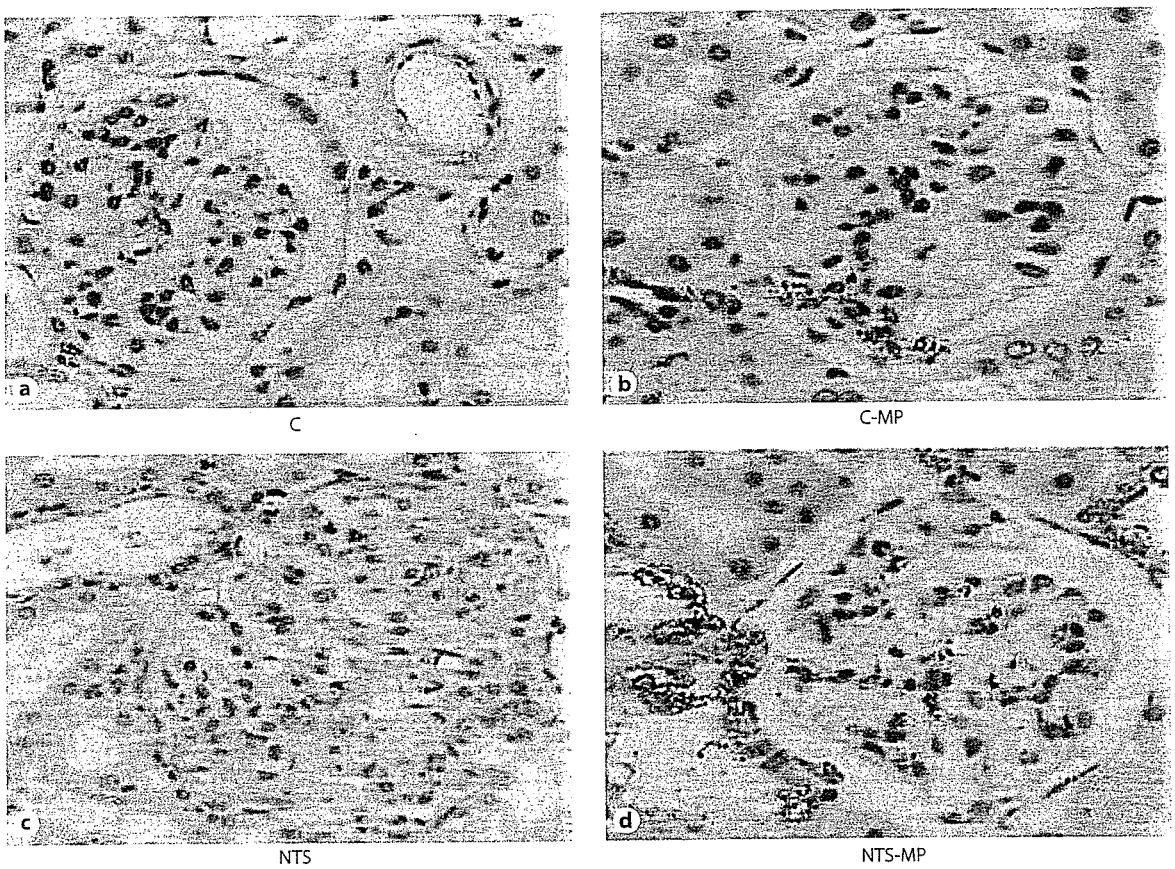

Fig. 6. Immunohistochemical staining of adrenomedullin (browny spot) in rat kidneys from the control (C), control treated with methylprednisolone (C-MP), anti-GBM glomerulonephritis model (NTS), and NTS rat groups treated with methylprednisolone (NTS-MP).

of 10^{-8} *M* AM(22-52) into the cultured MCs compared to the control (m-PSL plus AM(22-52) vs. m-PSL alone, 76 vs. 100%, $p < 0.05$, respectively) (fig. 3b).

Effect of m-PSL on NTS Rats

Urinary protein excretion was remarkably increased in NTS rats, while its level was significantly lower in NTS-MP rats than in NTS rats (fig. 4). Histologically, MCs proliferation and crescent formation were reduced in NTS-MP rats (fig. 1c). In immunofluorescence staining, m-PSL did not affect the rabbit IgG deposition in glomeruli, but reduced the glomerular deposition of rat IgG in NTS-MP rats.

AM Expression and Immunohistochemistry

Glomerular AM expressions were not different among groups C, C-MP and NTS. On the other hand, the AM mRNA levels were significantly increased in NTS-MP rats compared to NTS or C-MP rats (NTS-MP rats vs. control rats, C-MP rats, NTS rats, 270 vs. 100, 153, 94%, $p < 0.0001$, $p < 0.01$, $p < 0.0001$, respectively) (fig. 5). In immunohistochemistry, AM staining was obscure in group C, C-MP and NTS (fig. 6a–c). On the other hand, in the NTS-MP rats, AM was significantly increased in the mesangial area (fig. 6d). The area staining positive for AM, determined by a color imaging morphometric analysis system, was significantly increased in NTS-MP rats compared with the control, C-MP or NTS rats (NTS-MP rats vs. control rats, C-MP rats, NTS rats, 1.85 vs. 0.18, 0.34, 0.54%, $p < 0.0001$) (fig. 7).

Discussion

In studies using cultured cells, such as vascular wall cells, fibroblasts and macrophages, AM secretion was found to be generally stimulated by inflammatory cytokines, lipopolysaccharide and hormones [20, 25]. The se-

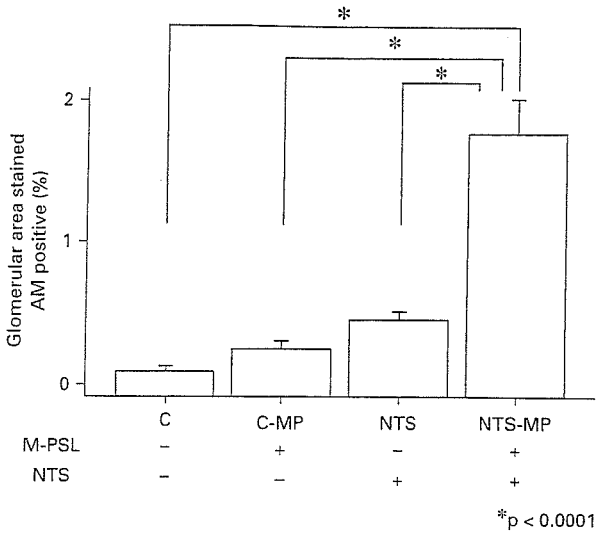

Fig. 7. Glomerular adrenomedullin production evaluated in the control (C), control treated with methylprednisolone (C-MP), anti-GBM glomerulonephritis model (NTS) and NTS rat groups treated with methylprednisolone (NTS-MP). Values are expressed as the mean ± SEM (20 glomeruli per kidney section). * $p < 0.0001$.

cretion of AM was also increased in the cultured endothelial cells, vascular smooth muscle cells, and cardiac fibroblasts by treatment with glucocorticoid, while it was suppressed in macrophages by that of dexamethasone [19, 20, 25]. Our study showed that treatment with m-PSL induced an increase in AM secretion from cultured rat MCs in a concentration-dependent manner. AM inhibited the MCs proliferation stimulated by PDGF in a concentration-dependent manner, as previously reported [4, 6–10]. In addition, it was confirmed that AM increased the MCs apoptosis, while AM(22-52) inhibited the MCs apoptosis, as in a previous study [12]. Based on these findings obtained from the culture studies, we next examined the relationships among the regulation of AM, renal histological lesions and the treatment of m-PSL in the rat model of GN.

We started the treatment with m-PSL for anti-GBM GN rats on day 3, when MCs had slightly proliferated m-PSL therapy inhibited the subsequent MCs proliferation, crescent formation and proteinuria associated with the increased expression and production of AM in glomeruli. Considering the present culture studies, these findings suggest that the glomerular AM increased by m-PSL therapy might inhibit the MCs proliferation and the progression of anti-GBM GN, while it might induce the MCs apoptosis and the resolution of anti-GBM GN. Some reports show the favorable actions of AM on renal damage [13–18], but there are few in vivo studies investigating the role of glomerular AM in GN. Recently, Plank et al. [18] showed that exogenous AM injection reduces the MC number in the state of MC proliferation in a model of mesangioproliferative GN. This report is consistent with our present study in the point that AM plays renoprotective roles in GN, partly via the inhibition of MC proliferation. M-PSL administration, at a dose of more than 10^{-8} *M*, induced the increase in AM secretion from the cultured rat MCs, but m-PSL treatment did not upregulate the expression of glomerular AM in the control rats in our in vivo study. The discrepancy may be caused by the differences in m-PSL concentrations and other circumstances in local areas. On the other hand, m-PSL treatment upregulated the expression and production of glomerular AM in NTS rats. The different state of mesangial cells, such as steady or recovery phases, may be related to these results.

There are also human studies suggesting the relationships between AM in the kidney and renal disease. The urinary excretion of AM, probably derived from the kidney, but not from the blood stream, is negatively correlated with urinary protein excretion and the severity of glomerular lesions [26–28]. Immunohistochemical studies performed by Kuo et al. [29] show that the glomerular production of AM is decreased in IgA nephropathy patients without impaired renal function. Similar to our present study, these findings indicate a protective role of AM against mesangial proliferative glomerular damage. The progression of glomerular disease is characterized by MCs proliferation, and then the accumulation of mesangial extracellular matrix. MC apoptosis has been proposed to be an important mechanism of resolution in mesangial proliferative GN [11]. Glucocorticoid therapy sometimes improves these glomerular lesions, and ameliorates clinical abnormalities. Considering the increased production of AM in MCs following m-PSL treatment, and the antiproliferative and apoptotic effects of AM on MCs, the renoprotective effect of glucocorticoids on GN might occur partly through the AM produced by MCs. Further studies, including experimentation of AM administration of anti-GBM GN rats, will be necessary to determine the role of AM in the improvement of anti-GBM GN.

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Original Article

Long-Term Anti-Hypertensive Therapy with Benidipine Improves Arterial Stiffness over Blood Pressure Lowering

Toshihiro KITA, Yoshihiko SUZUKI, Tanenao ETO, and Kazuo KITAMURA

Pulse wave velocity (PWV) reflects arterial stiffness and is an independent predictor of cardiovascular mortality and morbidity. However, because it is closely related to blood pressure (BP), PWV is an imperfect measure for evaluating the effects of anti-hypertensive drugs on arterial wall properties. To clarify the effect of benidipine on arterial properties, we first derived the regression line between BP and PWV changes in a short-term experiment. Using this line, we evaluated the long-term effect of benidipine on PWV changes. In the short-term experiment, 29 participants were intravenously administered nicardipine for 90 min. Maximum decreases of brachial-ankle PWV (baPWV) were plotted against the corresponding decreases in BP. In the long-term experiment, 9 hypertensive patients were treated with benidipine for 1 year, during which BP and baPWV were monitored. After 1 year, benidipine was suspended for 2 weeks, and BP and baPWV were reevaluated. In the short-term experiment, PWV was dependent on BP only, and the equation of the regression line was ΔPWV (cm/s) =10.114 × ΔMBP (mmHg) (r=0.913) or ΔPWV (%) =0.719 × ΔMBP (%) (r=0.926). In the long-term therapy, benidipine treatment achieved stable BP control within 3 months; the real PWV decreases (r-PWV) were almost identical to the PWV decrease estimated (e-PWV) from BP lowering at 3 months. However, r-PWV exceeded e-PWV after 6 months. Relative BP and PWV improvements compared to the control were maintained 2 weeks after suspension of benidipine. In conclusion, long-term benidipine administration improves arterial wall properties beyond what can be accounted for by changes in BP. (*Hypertens Res* 2005; 28: 959–964)

Key Words: **pulse wave velocity, blood pressure, benidipine, nicardipine, human**

Introduction

Arterial stiffness has been recognized as an important indicator of increased cardiovascular risk. Several studies have clearly demonstrated that increased arterial stiffness, as determined by pulse wave velocity (PWV), is a strong risk factor for cardiovascular morbidity and mortality (*1–3*). PWV measurement has been rapidly deployed in medical practice since a convenient automated pulse wave analyzer became available commercially in Japan. Brachial-ankle PWV (baPWV) derived using the automated analyzer is also closely related to risk factors and/or the organ damage characteristic of cardiovascular diseases (*4–9*). Moreover, significant improvements in baPWV have been observed after anti-hypertensive treatments with different classes of drugs (*10–12*), and thus

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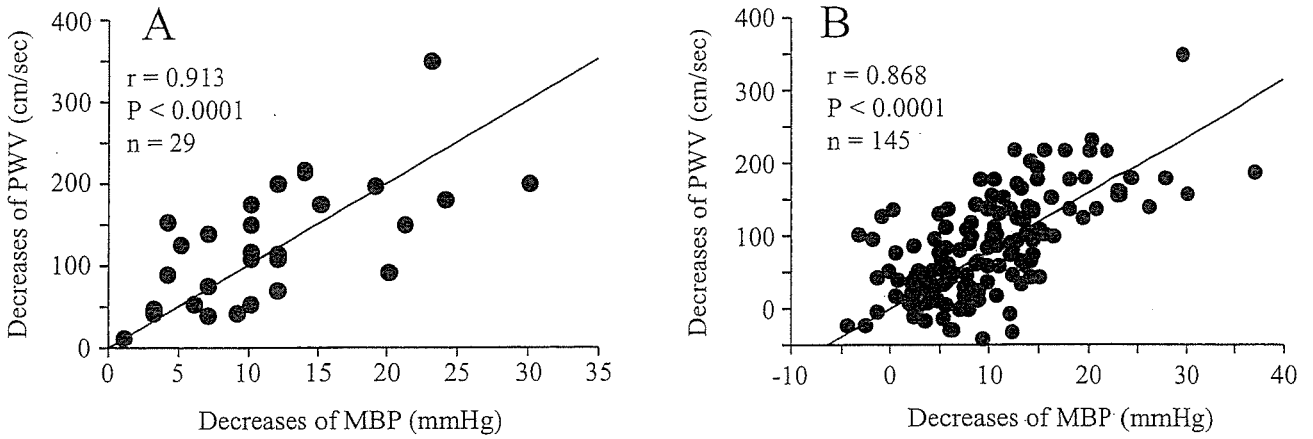

Fig. 1. *A: The correlation between maximum changes of mean blood pressure (MBP) and concomitant changes of pulse wave velocity (PWV) after nicardipine administration. B: The correlation between the changes of MBP and PWV in all-data analysis.*

Table 1. Time-Dependent Effects of Benidipine on the Indicated Parameters

	Control	Treatment with benidipine			2 week after suspension
		6 months	12 months	*p* in trend	
SBP (mmHg)	158.8±3.6	127.1±3.3**	128.8±3.0**	<0.0001	146.0±4.5##
DBP (mmHg)	96.7±4.0	80.6±3.1**	79.9±3.2**	0.0012	87.7±4.0##
MBP (mmHg)	117.4±3.4	96.1±2.9**	96.2±2.2**	<0.0001	107.1±4.4##
HR (bpm)†	69.1±2.7	69.3±3.2	69.1±3.4	1.00	63.8±1.6
PWV (cm/s)	1,768±100	1,464±59*	1,449±69**	0.012	1,631±77#
CRP (ng/ml)	1,163±279	651±108	520±101*	0.05	716±232
t-AM (fmol/ml)	12.8±0.8	12.2±0.3	12.5±0.4	0.78	14.0±0.7
m-AM (fmol/ml)	1.68±0.16	1.66±0.15	1.59±0.10	0.90	1.75±0.23
ET-1 (pg/ml)	2.04±0.16	1.79±0.19	1.84±0.17	0.57	1.56±0.11
PRA (ng/ml/h)	1.06±0.20	1.84±0.23	1.69±0.35	0.11	0.86±0.14
PAC (pg/ml)	92.7±9.1	104.8±9.8	100.4±10.6	0.68	103.4±14.0
IRI (μU/ml)	13.7±6.1	11.9±6.0	8.8±1.8	0.79	9.4±3.3

SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; PWV, pulse wave velocity; CRP, C-reactive protein; t-AM, total adrenomedullin; m-AM, mature adrenomedullin; ET-1, endothelin-1; PRA, plasma renin activity; PAC, plasma aldosterone concentration; IRI, immunoreactive insulin. *$p<0.05$, **$p<0.01$, compared to control (ANOVA); #$p<0.05$, ##$p<0.01$, compared to control (paired *t*-test). †HR was measured by ECG.

baPWV may be a surrogate marker of the efficacy of anti-hypertensive therapy. However, baPWV, like authentic PWV, is itself closely dependent on blood pressure (BP); thus it has been difficult to evaluate whether improvements in baPWV with anti-hypertensive therapy really reflect an improvement in arterial properties.

Ca^{2+} channel blockers (CAB) have been reported to possess anti-sclerotic properties, and CAB have improved cervical artery and coronary artery sclerosis in randomized controlled trials (*13–15*). Benidipine has additional beneficial actions on endothelial function and inhibits intimal thickening in animal studies (*16–18*); thus benidipine could be a more effective treatment for arterial sclerosis in hypertensive patients. To clarify the effect of benidipine on arterial properties, we first derived the regression line between BP and baPWV changes in a short-term experiment using nicardipine. We then used this regression line to evaluate the long-term effect of benidipine on baPWV changes separately from BP changes in hypertensive patients.

Methods

Short-Term Experiment

Participants

Twenty-nine male participants, mean age 46.0±9.2 years, were enrolled in this study. All participants received screening examinations including urine and blood tests, ECG, chest

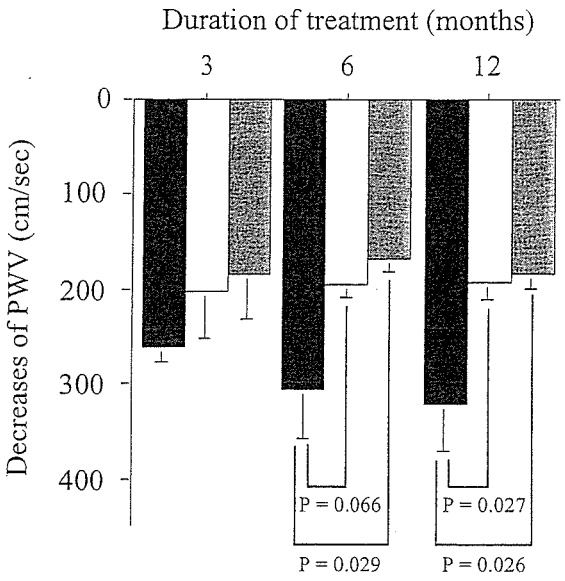

Fig. 2. *Comparison between real pulse wave velocity (PWV) changes (closed column) and estimated PWV changes (open column: analysis with maximum changes; gray column: all-data analysis) after benidipine treatment in hypertensive patients (mean±SEM, n=9). Estimated PWV changes were calculated from blood pressure changes as described in the text.*

X-ray, and echocardiography. They were diagnosed as normotensive (n=11), hypertensive (n=11), or diabetic (n=8), and one patient had both hypertension and diabetes mellitus. They were not receiving any medication, nor was there any evidence of heart failure (left ventricular ejection fraction <50%), renal failure (serum creatinine >1.0 mg/dl), peripheral artery disease, or a history of cardiovascular accidents. The study was approved by the ethics committee of our institute, and all participants gave written informed consent.

Study Protocol

All experiments were started at 9 AM in the fasting state. One 20-gauge cannula was inserted into the forearm vein for infusion of 0.9% saline. Saline was infused at a rate of 100 ml/h throughout the experiments. Baseline measurements were obtained after a 60 min equilibration period. Nicardipine (1 μg/min/kg) was then administered intravenously for 90 min, followed by a 90 min saline only infusion. After 45 min, the dosage of nicardipine was increased to 1.5 μg/min/kg to generate a 10% reduction of systolic BP (SBP), but was not increased above 1.5 μg/min/kg for safety reasons, even when a small BP reduction occurred. For convenience, BP was monitored every 10 min using an automated hemodynamometer with a brachial cuff. At 15 min intervals, PWV, BP and heart rate were measured using an automatic waveform analyzer (form PWV/ABI, BP-203RPE; Colin Medical Technology Co., Komaki, Japan) as reported previously (*4*). Maximum reductions in PWV were observed at 60–90 min after nicardipine infusion. Maximum reductions in PWV and concomitant BP reductions were plotted (linear regression), and the regression formula obtained. All data obtained during nicardipine infusion (5 points per person) were also analyzed by multivariate regression analysis.

Long-Term Treatment

Patients

Twelve patients with untreated essential hypertension were enrolled in this study. However, two patients dropped out within 3 months and one patient had uncontrollable hypertension. The remaining nine patients (53.0±13.3 years of age, 6 men and 3 women) completed the study and were used for the analysis. All patients received screening examinations, including urine and blood tests, ECG, chest X-ray, and echocardiography. No patient was receiving medication for hypertension, and no patient had heart failure, renal failure, peripheral artery disease, or a history of cardiovascular accidents. Three patients had hypercholesterolemia (total cholesterol >240 mg/dl), but no patient had diabetes mellitus. The study was approved by the ethics committee of our institute, and all patients gave written informed consent.

Protocol

Nine patients were treated with benidipine 4 to 8 mg/day only and all patients achieved BP <140/90 mmHg within 3 months. BP and pulse rate were measured in our outpatients office every month, and PWV was monitored at 0, 3, 6, and 12 months with an automatic waveform analyzer. After completion of 1-year of treatment, benidipine was suspended for 2 weeks in all patients with informed consent. BP and PWV were reevaluated after suspension. Not only PWV, but also BP and heart rate were measured by an automatic waveform analyzer for all data analyses.

Blood samples were taken at 0, 6, and 12 months and used to measure biochemical parameters and vasoactive factors. High-sensitivity C-reactive protein (hsCRP) was measured by latex nephelometry (Dade Behring, Marburg, Germany). Plasma concentration of adrenomedullin was measured with an immunoradiometric assay kit (AM RIA Shionogi, Osaka, Japan), and endothelin-1 levels were measured using a commercially available standard radioimmunoassay kit.

Statistical Analyses

All statistical analyses were performed using StatView-J software (version 4.5; Abacus Concepts, Inc., Berkeley, USA) on a Macintosh computer. After confirmation of normal distributions for all variables, the significance of differences was evaluated by paired t-test or analysis of variance (ANOVA) followed by Fisher's multiple comparison tests. Relationships between variables were analyzed by simple correlation analysis and multivariate regression analysis. Data are expressed as

the mean±SEM and a p value <0.05 was the criterion for statistical significance.

Results

Short-Term Experiment

Nicardipine administration achieved a 10.2±1.3% decrease in SBP and an 11.9±1.3% decrease in diastolic BP (DBP) accompanied by an 8.7±0.8% decrease in PWV and a 13.2±1.5% increase in heart rate. BP and PWV quickly recovered after termination of nicardipine treatment. Maximum decreases in PWV and concomitant BP changes were plotted by linear regression analysis. The changes of SBP and DBP showed a good correlation with the PWV changes, and the equations of the regression lines were $\Delta PWV = 7.258 \times \Delta SBP$ ($r=0.889$) and $\Delta PWV = 10.308 \times \Delta DBP$ ($r=0.912$), respectively. However, mean BP (MBP) changes showed the best correlation with PWV changes. The equation of the regression line was $\Delta PWV = 10.114 \times \Delta MBP$ ($r=0.913$) or $\%\Delta PWV = 0.719 \times \%\Delta MBP$ ($r=0.926$) (Fig. 1A). The changes in MBP and PWV were also well correlated in all-data analysis (Fig. 1B), but the changes in heart rate were not correlated with PWV changes ($r=0.059$, $p=0.48$). The equation of the regression line was $\Delta PWV = 7.901 \times \Delta MBP$ ($r=0.868$). MBP, but not heart rate, was a significant independent variable for PWV in multivariate regression analysis.

Long-Term Treatment

Benidipine treatment achieved stable BP control within 3 months, which was maintained until the end of the study. The average dose of benidipine at 12 months was 6.7 mg/day (4 mg for 3 patients and 8 mg for 6 patients). After 6 months, while PWV was also decreased significantly, heart rate was unchanged throughout the study (Table 1). We compared real (r-PWV) and estimated PWV (e-PWV) changes calculated from the BP decreases using the above regression formula. After 3 months, r-PWV and e-PWV showed almost the same degree of change, but the r-PWV value exceeded the e-PWV after 6 months, and the difference between r-PWV and e-PWV reached significance at 12 months (Fig. 2). The same trend was observed when BP and PWV changes were calculated as % changes (−17.4±2.3% for r-PWV *vs.* −12.3±1.2% for e-PWV; $p=0.028$ at 12 months). The trend became clearer when e-PWV was calculated using another formula derived from analysis of all-data (Fig. 2). Data concerning blood samples are summarized in Table 1. Interestingly, C-reactive protein (CRP) was decreased after benidipine treatment. As indicated in Table 1, other factors were not changed. Blood glucose and lipids including triglyceride, total cholesterol, high-density lipoprotein cholesterol and remnant-like lipoprotein cholesterol were also not changed (data not shown).

After suspension of benidipine for 2 weeks, BP and PWV were increased. However, these BP and PWV values remained significantly lower than the control values (Table 1). CRP was also increased after suspension of benidipine and was no longer significantly different from the control level.

Discussion

The fully automated waveform analyzer (form PWV/ABI) can measure baPWV non-invasively and quickly, and thus has been deployed in clinical practice. The baPWV is composed of a central elastic artery component and a peripheral muscular artery component. The PWV of the peripheral arteries is greater than that of the central arteries; therefore baPWV is considerably faster than authentic carotid-femoral PWV (*7*). baPWV values correlated well with carotid-femoral PWV, but the two parameters did not match completely (*7*). Although there are some differences between the two techniques, baPWV closely correlates with BP and age of subjects and reflects risk or progression of cardiovascular disease, as does carotid-femoral PWV (*4–9*). The most interesting and attractive characteristic of baPWV is, however, its rapid and large response to anti-hypertensive therapy, with significant decreases in baPWV being observed within 1 month after therapy (*12*). Thus, the baPWV is convenient to measure, is well correlated with organ damage and is a suitable index for monitoring arterial wall stiffness. Potentially, therefore, the baPWV may represent a useful new marker of the efficacy of anti-hypertensive therapy.

We examined the acute response of baPWV during hypotension induced by intravenous administration of nicardipine, which is the sole intravenously applicable dihydropyridine CAB in Japan. Acute changes in baPWV are mainly dependent on BP, though they may be affected to a lesser degree by heart rate (*19*), and are independent of arterial sclerosis. Regression analysis indicated that the decrease in baPWV achieved with CAB treatment was 10 times that of MBP, or about 0.7 times the % change in MBP (Fig. 1). The impact of our data is not negligible, because our estimated influence of BP changes on baPWV was much larger than that previously reported by Ichihara *et al.* (*10*). In their study, the change in the ratio of baPWV/MBP in response to various anti-hypertensive drugs was between 3.9 and 5.9, *vs.* 10.1 in our formula. Thus, this formula could account for most of the BP influence on baPWV. However, it has been reported that angiotensin-converting enzyme inhibitors or angiotensin-II receptor antagonists caused larger baPWV reductions than CAB, for a given BP reduction (*11*). Therefore, the formula cannot be applied as an absolute universal standard in cases involving anti-hypertensive treatments, but it does constitute a relative guide for baPWV monitoring. Furthermore, the formula cannot be applied to correction of the influence of BP in untreated hypertensive patients, because anti-hypertensive therapy alters hemodynamic and humoral states, and may alter reactivity of the vascular wall. Indeed, while the importance of raw baPWV values is undeniable, one must be careful in interpreting baPWV changes in anti-hypertensive