“Pulse” Treatment With High-Dose Angiotensin Blocker Reverses Renal Arteriolar Hypertrophy and Regresses Hypertension

Kimiko Ishiguro, Kaori Hayashi, Hiroyuki Sasamura, Yusuke Sakamaki, Hiroshi Itoh

Abstract—One ultimate goal of hypertension therapy is to cause permanent reversal (“regression”) of already established hypertension. Our aim was to examine whether high-dose “pulse” treatment with a renin-angiotensin system inhibitor could cause regression of established hypertension and to link this action to reversal of arteriolar hypertrophy and changes in vascular matrix metalloproteinase activities. First, 16-week-old male spontaneously hypertensive rats (n=60) were pulse treated for 2 weeks with high-dose angiotensin-converting enzyme inhibitor (enalapril), angiotensin receptor blocker (candesartan), calcium channel blocker (nifedipine), or vasodilator (hydralazine) with or without salt restriction, and the long-term effects on blood pressure were examined. Second, spontaneously hypertensive rats were treated with angiotensin receptor blocker or calcium channel blocker, and the effects on renal gene expressions, arteriolar structure, and vascular matrix metalloproteinase were compared. Treatment of spontaneously hypertensive rats with different antihypertensive agents caused apparently similar reductions in blood pressure during the course of the pulse treatment, within the limitations of the tail-cuff method. After cessation of medications, blood pressure in the rats treated with renin-angiotensin system inhibitor remained reduced by >30 to 40 mm Hg for 4 months. No such effect was seen with calcium channel blocker or vasodilator. The 2-week angiotensin receptor blocker treatment induced a marked reversal of the arteriolar hypertrophy specifically in the small (30 to 100 μm) renal arterioles, together with increased expression and activity of matrix metalloproteinase-13. In conclusion, transient high-dose pulse treatment with angiotensin receptor blocker caused changes in vascular matrix metalloproteinase activity, specific reversal of renal arteriolar hypertrophy, and regression of hypertension in spontaneously hypertensive rats. (Hypertension. 2009;53:83-89.)

Key Words: angiotensin receptor blocker ■ calcium channel blocker ■ regression ■ spontaneously hypertensive rat ■ MMP ■ renal arteriolar hypertrophy

It has been estimated that ≈26.4% of the adult world population in the year 2000 had hypertension, and the number was projected to increase to 29.2% by the year 2025.1 Because hypertension is a major risk factor for diseases such as stroke, coronary artery disease, heart failure, kidney disease, and vascular disease, the medical, economic, and social consequences of the current epidemic of hypertension are considerable.2

One strategy for managing this disease is “prevention” of the development of hypertension. Previous studies by Harrap et al,3 Richer et al,4 and other groups, including our own5–7, have shown that treatment of young (4- to 6-week-old) prehypertensive spontaneously hypertensive rats (SHRs) with a renin-angiotensin system (RAS) inhibitor is effective in permanently attenuating the later development of hypertension. In other words, transient administration of a RAS inhibitor, if given before hypertension was fully established, was found to be effective for hypertension prevention in SHR. The feasibility of using transient RAS inhibition to prevent the development of hypertension in human patients has been confirmed recently by Julius et al8 in the landmark Trial of Preventing Hypertension.

A different strategy would be to aim for “regression” of already established hypertension. Importantly, Smallegange et al9 reported that transient treatment of adult SHRs with a high-dose angiotensin-converting enzyme inhibitor (ACEI), together with a low-salt diet, was effective in causing a sustained reduction of blood pressure even if administration of the drug was started at 16 weeks, well after hypertension was established in the SHR model. These results suggested that high-dose RAS inhibition could indeed be effective in the reversal or regression of already established hypertension. Potentially, this could have a great clinical benefit, because it could mean that patients with established hypertension could well be “cured” by appropriate transient therapy.

At present, it is unclear whether regression of hypertension is an effect that is specific to RAS inhibitors or is generally
seen with high doses of other antihypertensive agents, such as calcium channel blockers (CCBs) or vasodilators. The molecular mechanism is also undefined, in particular, the relationship with renal arteriolar hypertrophy, which is thought to play an important role in the maintenance of hypertension in SHRs.\textsuperscript{10,11}

The objective of this study was, therefore, to test the hypothesis that high-dose pulse treatment with a RAS inhibitor but not a CCB would cause regression of established hypertension and to link this action to reversal of arteriolar hypertrophy and changes in vascular matrix metalloproteinase (MMP) activities. The specific aim of the first experiment was to compare the efficacy of a total of 8 antihypertensive pulse regimens (with and without a low-salt diet) in inducing regression. In the second experiment, we examined whether high-dose pulse treatment could affect the renal arteriolar hypertrophy found in SHRs, as well as cause changes in activities of the vascular matrix metalloproteinases (MMPs). Our results suggest that high-dose pulse treatment with a RAS inhibitor causes changes in arteriolar MMP activity, leading to reversal of arteriolar hypertrophy and, ultimately, to regression of hypertension in the SHR model.

Methods

Animal Treatment Protocols

The studies were conducted using 16-week-old male Wistar-Kyoto (WKY) rats (WKY/Izm) and SHRs (SHR/Izm) obtained from Sankyo Laboratory Services (Tokyo, Japan). All of the experiments were approved by the institutional review committee and performed in accordance with the Keio University School of Medicine Animal Experimentation Guidelines.

Experiment 1

SHRs were randomly divided into 10 groups as follows (n=6 per group). Rats in group 1 were control SHRs. Rats in groups 2 to 5 were treated from 16 to 18 weeks with the ACEI enalapril maleate in drinking water (20 mg/kg per day), the ARB candesartan cilexetil dissolved in the drinking water\textsuperscript{7} (50 mg/kg per day), the vasodilator hydralazine (25 mg/kg per day), or the CCB nifedipine in chow (50 mg/kg per day). Rats in groups 6 to 10 were treated identically to groups 1 to 5 but were also treated from 16 to 18 weeks with a low-salt diet (0.05% Na). All of the interventions were discontinued at age 18 weeks, and the rats were observed without any medication for a further 18 weeks, then euthanized at age 36 weeks.

Experiment 2

WKY rats and SHRs were randomly divided into 4 groups as follows (n=6 per group). Rats in group 1 were control WKY rats. Rats in group 2 were control SHRs. Rats in groups 3 and 4 (ARB and CCB groups) were treated with either the ARB candesartan (50 mg/kg per day) or the CCB nifedipine (50 mg/kg per day), as described above, then euthanized at the end of the 2-week pulse treatment at age 18 weeks.

Assays

The systolic blood pressure and heart rate of awake animals were measured by tail-cuff plethysmography using a Natsume KN-210 manometer (Natsume, Inc). Twenty-four-hour urine collection was performed in metabolic cages, and urine albumin excretion was measured by a direct competitive ELISA (Nephrat). Other biochemical assays are described in the online data supplement (available at http://hyper.ahajournals.org).

Histological Studies

The kidneys and thoracic aortas were removed and fixed in 4% paraformaldehyde, then embedded in paraffin blocks. In experiment 2, tissue samples were also obtained from the mesentery, heart, and brain, for the examination of mesenteric, cardiac, and cerebral arterioles. Details of the histological assessment are described in the online data supplement.

Preparation of RNA and Real-Time RT-PCR and Microarray Analysis

Kidney RNA was prepared for real-time RT-PCR and microarray analysis, as described in detail in the online data supplement.

In Situ Zymography and Immunofluorescence Staining

High-resolution, high-sensitive zymography was performed using the protocol of Ahmed et al,\textsuperscript{12} with minor modifications. Immunofluorescence staining of vascular MMP expression was performed using standard protocols (for details, see the online data supplement).

Statistics

Results were expressed as the means±SEMs. Statistical comparisons were made by ANOVA, followed by Scheffe’s posthoc test. P values <0.05 were considered statistically significant.

Results

Experiment 1

Effects of Pulse Treatment With Antihypertensive Agents on Systolic Blood Pressure in SHRs

The changes in systolic blood pressure in the different groups are shown in Figure 1. At age 16 weeks, before the initiation of the pulse treatment, hypertension had been fully established, ie, the blood pressure in the different groups had reached the plateau value of ~220 mm Hg. Treatment with the different antihypertensive agents caused a decrease in blood pressure to ~150 mm Hg during the duration of the 2-week pulse therapy. After discontinuation of the antihypertensive medication, the blood pressure rapidly reverted to control values in the CCB- and vasodilator-treated groups. In clear contrast, the blood pressure in the ACEI- and ARB-treated groups were maintained at values of ~180 mm Hg (a difference of >30 to 40 mm Hg). Essentially similar results were found in groups 6 to 10, which had been exposed to a low-salt diet from age 16 to 18 weeks (Figure 1B).

Effects of Pulse Treatment With Antihypertensive Agents on Cardiovascular Hypertrophy, Parameters of Renal Function, and Plasma Renin Activity/Plasma Aldosterone Concentration at Age 36 Weeks in SHRs

As expected from the sustained decrease in blood pressure, the heart weight:body weight ratios, aortic media:lumen ratios, and renal arteriolar media:lumen ratios were decreased at the end of the study (age 36 weeks) in the rats previously treated with pulse ACEI or ARB (data not shown). No significant differences in blood urea nitrogen, plasma creatinine, plasma renin activity, plasma aldosterone concentration, or the oxidative marker plasma lipid peroxides were found in the different groups at age 36 weeks.

Experiment 2

Short-Term Effects of Pulse Treatment With ARB or CCB on Cardiovascular Hypertrophy and Parameters of Renal Function at Age 18 Weeks in WKY Rats and SHRs

In experiment 2, we examined the short-term effects of the 2-week pulse treatment on cardiovascular hypertrophy and
parameters of renal function in 4 groups of rats: normotensive WKY rats, control SHRs, and SHRs treated with either ARB or CCB for 2 weeks, and data were obtained immediately at the end of the pulse treatment (age 18 weeks). Pulse treatment with ARB was associated with a small decrease in heart weight:body weight ratios compared with CCB, but the results did not attain statistical significance. Similarly, aortic media:lumen ratios were not significantly changed in the rats treated with ARB or CCB. In contrast, the media:lumen ratios in the small (30 to 100 μm) arterioles were markedly reduced by the ARB pulse treatment but not by the CCB (Figure 2). Of interest, the decreases in media:lumen ratios were found to be specific for renal small arterioles and were not found in larger renal arterioles (100 to 300 μm) or arterioles from other tissues, namely, the mesentery, heart, and brain (Table S1). Urine albumin excretion was significantly decreased in the SHRs treated with ARB (WKY: 7.2 ± 2.6 mg/d; SHR: 9.2 ± 3.0 mg/d; SHR+ARB: 0.8 ± 0.4 mg/d [P<0.05 vs SHR]; SHR+CCB: 3.6 ± 1.2 mg/d), but no significant differences in blood urea nitrogen, plasma creatinine, or plasma lipid peroxides were found in the different groups.

**Microarray Analysis of Differences in Renal Gene Expressions in SHRs Treated With Pulse ARB or CCB for 2 Weeks**

The differences in expression of a total of 28,000 genes in the kidneys of SHRs treated with ARB or CCB were examined using the Affymetrix rat 230 2.0 gene expression array. A total of 1345 genes were elevated in the ARB-treated rats compared with the CCB-treated rats, whereas 5671 were reduced. Several extracellular matrix–related genes, including type IV procollagen and MMP-15, were elevated in the ARB-treated rats, whereas MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-2, and TIMP-3 gene expressions were decreased.
were decreased in the ARB-treated group (Table S2). Among the genes of the RAS, only renin mRNA was increased in the ARB-treated group, which was expected as a feedback response to inhibition of the RAS.

Real-Time RT-PCR Analysis of the Short-Term Effects of Pulse Treatment With ARB or CCB on Renal Gene Expressions at Age 18 Weeks in WKY Rats and SHRs

To confirm the results of the microarray analysis, the differences in the gene expression of MMP-2, MMP-9, MMP-13, TIMP-1, TIMP-2, and TIMP-3 were assessed by real-time RT-PCR. As shown in Figure 3, pulse treatment of SHRs with ARB caused a significant decrease in MMP-9 and TIMP-3 mRNA expression, whereas no significant effect was seen with CCB, findings that were consistent with the results of the microarray analysis. MMP-13 is known to be the predominant MMP involved in degradation of type I collagen in the rat, which lacks the MMP-1 gene. The type I collagenolytic activity in the vasculature was inhibited by the MMP-13 inhibitor, confirming that the changes seen reflected MMP-13 activity. In the case of type IV collagenolytic activity, MMP-9–dependent degradation of type IV collagen was decreased in ARB-treated rats but not in the CCB-treated rats. Both type I and type IV collagenolytic activities were completely inhibited by the broad-spectrum MMP inhibitor 1-10 phenanthroline (data not shown). Examination of MMP-13, MMP-2, and MMP-9 expression by immunofluorescence staining showed a similar trend to the results of in situ zymography (Figure 5).

Discussion

The main findings of this study were as follows: (1) pulse treatments with ARB and ACEI (with or without concomitant low-salt diet treatment) were equally effective in causing a long-term reduction in blood pressure; (2) the reductions in blood pressure were accompanied by long-term reductions in cardiac and vascular hypertrophy; (3) the pulse treatment caused a remarkable regression of renal arteriolar hypertrophy in the course of just 2 weeks; and (4) these changes were associated with changes in expression and activity of vascular MMPs in the kidney.

The fact that long-term reductions in blood pressure were seen with an ARB and ACEI but not with the CCB or vasodilator is of interest in view of the widespread use of these agents for the treatment of hypertension. Concerning the dose of ARB or ACEI required to obtain regression, we are performing a companion study to examine the effects of
pulse treatment with different doses of ARB (1 to 50 mg/kg per day) on regression of glomerular hypertrophy and sclerosis and have found that the maximal effect on the regression of glomerular changes is obtained with the high dose (50 mg/kg per day) used in this study (unpublished observation).

One caveat of this study concerns the limitations of the indirect measurement of blood pressure. As in our previous studies, we performed the blood pressure measurements of the different groups on the same day, with the same experienced investigator, after an initial period of training under

Figure 4. Short-term effects of pulse treatment with ARB or CCB on vascular MMP-13, MMP-2, and MMP-9 activities at age 18 weeks in SHRs. A, Representative in situ zymograms of (A through D) total type I collagenolytic activity; (E through H) type I collagenolytic activity in the presence of MMP-13 inhibitor; (I through L) total type IV collagenolytic activity; (M through P) type IV collagenolytic activity in the presence of MMP-2 inhibitor; and (Q through T) type IV collagenolytic activity in the presence of MMP-9 inhibitor. B through D, Quantification of (B) MMP-13, (C) MMP-2, and (D) MMP-9 activities in medial layers of renal arterioles. Abbreviations of groups as in Figure 2. *P<0.05 vs WKY rats; ¶P<0.05 vs SHR/CCB.

Figure 5. Short-term effects of pulse treatment with ARB or CCB on vascular MMP-13, MMP-2, and MMP-9 expression at age 18 weeks in SHRs. Immunofluorescent staining of (A through D) MMP-13 protein; (E through H) MMP-2 protein; and (I through L) MMP-9 protein.
stress-free conditions. Under these conditions, we found a clear-cut reduction of blood pressure (>30 to 40 mm Hg) in the ACEI- and ARB-treated groups every 2 weeks, consistently over several months, compared with the control, CCB-treated, and vasodilator-treated groups. However, it should be recognized that the indirect method is less accurate than direct measurement using an indwelling catheter, and absolute values of blood pressure may not be wholly reliable. Moreover, unlike the telemetry method, the measurements were made at a single point and were not made continuously over multiple days; thus, small differences in blood pressure between the different groups at age 18 weeks cannot be accurately assessed. Because of the limited precision of the tail-cuff method, we cannot conclude whether the effects of ARB at age 18 weeks are entirely independent of blood pressure.

An important finding was that treatment with a high dose of ARB alone was sufficient to cause a remarkable reversal of renal arteriolar hypertension in the course of just 2 weeks. The media:lumen ratios of the small renal arterioles in the ARB pulse-treated rats were decreased almost to the levels found in the normotensive WKY rat, whereas the CCB did not have a significant effect. Several lines of evidence suggest that hypertrophy of the small arterioles in the kidney plays a major role in the pathogenesis of hypertension in the SHR. Therefore, the observed reversal of renal arteriolar hypertrophy is compatible with the regression of hypertension seen in the ARB-treated groups.

Because we found marked changes in the kidney after just 2 weeks of pulse treatment, we performed a comprehensive survey (microarray analysis) of the differences in gene expression in the kidney of ARB-treated and CCB-treated rats. Interestingly, the microarray analysis did not reveal a major change in components of the RAS, except for an increase in renin mRNA, which could be expected as a feedback response to RAS inhibition. In contrast, expression of several extracellular matrix–related proteins, in particular, MMPs and TIMPs, was differently affected by the 2 treatments, and these results were confirmed by RT-PCR analysis. We focused on these findings, because changes in the expression of these genes could be involved in the remodeling of the renal arterioles. To clarify the changes in the renal arterioles, we assayed tissue MMP activity using a recently developed high-resolution, high-sensitivity in situ zymography technique. Using this method, we found that the ARB treatment had different effects on the vascular MMP system than the CCB treatment and caused an increase in vascular MMP-13 activity and a decrease in MMP-9 activity, which could explain the different effects on regression of renal arteriolar hypertension. These findings were confirmed immunohistologically, by immunofluorescence staining of these MMPs.

It is well established that the MMPs, in particular, members of the collagenase and gelatinase families, play an important role in tissue remodeling by cleaving many structural proteins of the extracellular matrix. MMP-13 is the predominant collagenase in rodents that lack the gene for MMP-1 and is involved in the collagenolysis of types I, II, and III collagens. MMP-13 is known to be expressed in cultured vascular smooth muscle cells and has been implicated in remodeling of the uterine artery during pregnancy, in angiogenesis, and in aneurysm formation. These reports are consistent with the notion that upregulation of vascular MMP-13 activity by high-dose ARB in this model will result in collagenolysis of vascular collagens, favoring regression of renal arteriolar hypertrophy. It has been reported that MMP-9 (gelatinase B) is also expressed in cultured vascular smooth muscle cells, where it is involved in cell migration and is upregulated by angiotensin II. Therefore, the inhibition of MMP-9 by ARB could cause inhibition of compensatory smooth muscle cell migration, further contributing to the vascular changes.

The results of this study extend the work of Smalle et al using ACEI and a low-salt diet. In their studies, they found that cross-transplantation of kidneys from rats treated transiently with ACEI and a low-salt diet to hypertensive rats caused a transfer of the sustained reduction of blood pressure, whereas the untreated kidneys caused an increase in blood pressure. Moreover, the ACEI and low-salt treatment caused a significant decrease in renal vascular resistance. We speculate that the marked regression of renal small arteriolar hypertrophy ("renal microvascular remodeling") reported in this study is intimately involved in the mechanism of hypertension regression found in these models.

Perspectives
Because the animal studies on the prevention of hypertension development were successfully confirmed clinically by the Trial of Preventing Hypertension, we have now designed a multicenter prospective clinical study (Short Treatment With Angiotensin Receptor Candesartan Surveyed by Telemedicine Study) to examine the feasibility of regression of established hypertension (ie, reversal from stage 1 to prehypertension) using transient ARB treatment in patients with essential hypertension. The results of this study lead us to speculate that the development of methods to cause regression (or cure) of hypertension, and the study of the mechanisms of regression, may become one of the central themes in hypertension research this century.

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Disclosures
None.

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3. Harrap SB, Van der Merwe WM, Griffin SA, Macpherson F, Lever AF. Brief angiotensin converting enzyme inhibitor treatment in young spon-


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ONLINE SUPPLEMENT

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*: p<0.01 vs WKY; † p<0.01 vs SHR

Online Supplement Table S1. Values of media/lumen ratios (x 100) of arterioles in the kidney, mesentery, heart, and brain in the different groups at age 18 weeks in Experiment 2. Results shown are means ± SEM. WKY: untreated WKY, SHR: untreated SHR, SHR+ARB: SHR treated with ‘pulse’ candesartan, SHR+CCB: SHR treated with ‘pulse’ nifedipine.