Combined Treatment With Valsartan and Spironolactone Prevents Cardiovascular Remodeling in Renovascular Hypertensive Rats

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SUMMARY

Treatment with an angiotensin blocker (ARB) and an aldosterone blocker has been shown to have beneficial effects on cardiac remodeling in several cardiac diseases. It is still not clear whether the combination of these drugs is more effective against cardiac remodeling than the use of either agent alone. We examined the effects of combined treatment with valsartan, an ARB, and spironolactone, an aldosterone blocker, on cardiac remodeling in the renovascular hypertensive (RHT) rat. The RHT rats were divided into 4 groups administered valsartan (3 mg/kg/day, ARB group), spironolactone (4 mg/kg/day, SPRL group), both drugs at these doses (combined group), or neither drug (untreated RHT group). After 5 weeks, systolic blood pressure was significantly reduced in the 3 treatment groups, however, there were no significant differences in the extent of blood pressure reduction among the 3 treatment groups. The heart weight/body weight ratio in each of the 3 treatment groups was significantly lower than that in the untreated RHT group. The degree of cardiac and perivascular fibrosis in the SPRL group and the combined group were significantly lower than that in the untreated RHT group. Myocyte remodeling in the ARB group and in the combined group was significantly smaller than that in the untreated RHT group. These results suggest that SPRL treatment prevents cardiac and perivascular fibrosis and ARB treatment suppresses the cellular hypertrophy of myocytes, and that, therefore, combined treatment with both drugs prevents cardiac remodeling by acting against both myocyte hypertrophy and cardiac fibrosis in RHT rats. (Int Heart J 2006; 47: 783-793)

Key words: Cardiac hypertrophy, Cardiac fibrosis, Angiotensin receptor blocker, Valsartan, Aldosterone blocker, Spironolactone, Renin-angiotensin-aldosterone system

CARDIAC hypertrophy is known to be associated with an increased risk of coronary artery disease, heart failure, arrhythmias, and sudden death. The importance of the renin-angiotensin aldosterone system (RAAS) in the patho-
physiology of cardiovascular diseases is now well recognized.\textsuperscript{1}) Direct administration of angiotensin II\textsuperscript{2)} and aldosterone,\textsuperscript{3)} as well as transfection of the angiotensin II gene or aldosterone gene\textsuperscript{4)} have been shown to increase blood pressure and cause cardiac hypertrophy. Several studies to date have shown that local aldosterone is produced locally in nonepithelial tissues such as the brain and the heart, and also that it plays a crucial role in cardiac remodeling.\textsuperscript{5,6)} Many clinical and experimental studies have demonstrated that renin-angiotensin blockade has beneficial effects against cardiac remodeling in hypertensive left ventricular (LV) hypertrophy, postmyocardial infarction heart, and heart failure.\textsuperscript{7-9)} However, long-term treatment with angiotensin-converting enzyme (ACE) inhibitors sometimes causes re-elevation of the serum aldosterone levels, the so-called “aldosterone breakthrough.” In the RALES study,\textsuperscript{10)} the addition of spironolactone (SPRL), an aldosterone blocker, to ACE inhibitor treatment improved the prognosis in patients with severe heart failure. Therefore, combined treatment with SPRL and an ACE inhibitor may be more effective against cardiac remodeling than treatment with an ACE inhibitor alone.

We previously reported that the serum aldosterone level in renovascular hypertensive (RHT) rats was significantly higher during treatment with an angiotensin receptor blocker (ARB) than during treatment with an ACE inhibitor.\textsuperscript{11)} Recently, the occurrence of aldosterone breakthrough was also shown during ARB treatment, with attenuation of the drug’s cardioprotective effects.\textsuperscript{12-14)} Tanabe, \textit{et al}\textsuperscript{15)} reported that the addition of SPRL to ARB treatment prevented cardiac collagen accumulation in stroke-prone spontaneously hypertensive rats. Cardiac hypertrophy generally consists of cellular hypertrophy of myocytes and proliferation of the cardiac connective tissue; therefore, we examined the effects of combined treatment with an ARB and SPRL on myocyte hypertrophy and cardiac fibrosis in RHT rats in comparison with the effects of treatment with either class of drug administered alone.

\textbf{METHODS}

This study was conducted in accordance with the guidelines of the Institutional Guidance Committee of the Jikei University School of Medicine for the use and care of animals. The methods have been described in detail in 2 previous reports.\textsuperscript{11,16)} The main procedures used in the present study were as follows.

RHT rats were produced by constricting the left renal artery with a silver clip (two-kidney one-clip: 2K1C) in male Wistar rats weighing 130 to 150 g. One week after the operation, rats with a systolic blood pressure (SBP) of over 140 mmHg were considered as representing the RHT model. These rats were divided into 5 groups, as follows; control group, age-matched control rats (\(n = 6\));
untreated RHT (un-RHT) group, untreated RHT rats (n = 6); ARB group, RHT rats treated with valsartan, 3 mg/kg/day (n = 6); SPRL group, RHT rats treated with SPRL, 4 mg/kg/day (n = 6); and combined group, RHT rats treated with both drugs (n = 6). After 5 weeks of the 2K1C procedure, the rats were perfused with phosphate buffer and the heart was excised. Cross sections were cut at the mid-ventricular level and fixed with 10% formalin for histological analysis.

**Histological examination:** The fixed cardiac tissues were sectioned into 4 μm-thick slices, which were stained with Masson's trichrome for evaluation of cardiac fibrosis. To estimate the degree of myocardial fibrosis, photomicrographic figures of the sections were digitized and the fibrotic areas were assigned numerical values using NIH image software. Vascular tissue changes were evaluated according to the method described by Ito, et al.

**Measurement of cell size:** The cell length and width of the cardiac myocytes were measured using the method reported by Obayashi, et al. An immunohistochemical analysis was performed using anti-Connexin 43 antibody (Santa Cruz Biotechnology, USA) and a streptavidin-biotin staining kit (Nichirei Biosciences Tokyo). The myocyte volume was calculated based on the assumption of a cylindrical configuration. In this study, the values for 150-200 myocytes were measured for each group.

**ANP gene expression:** Total DNA was collected by the conventional method using Trizol (Gibco, USA), and reverse-transcribed using M-MLV reverse transcriptase (Gibco). RT-PCR was then performed using Taq synthetase (Takara, Kyoto).

**Statistical analysis:** Statistical analyses were performed using Statview 5 software (SAS Institute Inc., North Carolina, USA). Values are expressed as the mean ± 1 standard error (SE). Differences in each of the parameters examined, such as blood pressure and the expression level of ANP mRNA, among the groups were tested by one-way ANOVA combined with Scheffe's post hoc test. P values < 0.05 were considered to indicate statistical significance.

**RESULTS**

The changes in SBP in each group are shown in Figure 1. In the untreated RHT group, the SBP increased markedly from 1 week after the operation until 5 weeks. SBP in the ARB, SPRL, and combined treatment groups tended to be lower than that in the untreated RHT group. The Table shows the hemodynamic and heart weight/body weight ratio data at 5 weeks after the operation. SBP in each treatment group was about 20% lower than that in the untreated RHT group, however, the difference was not statistically significant. Also, there were no significant differences in the extent of SBP reduction among the 3 treatment groups.
The heart weight/body weight ratio was markedly higher in the untreated RHT group than in the control group, and only the value in the combined treatment group was significantly lower than that in the untreated RHT group.

ANP mRNA expression in each group as an indicator of cardiac hypertrophy is shown in Figure 2. ANP mRNA expression was up-regulated in the untreated RHT group as compared with that in the control group, and this up-regulation was significantly suppressed only in the combined treatment group.

Micrographs of cardiac sections stained with Masson's trichrome are shown in Figure 3. Marked proliferation of fibrous tissue in the interstitial and perivas-
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Figure 2. A: Typical expression of ANP mRNA. B: Amplified ANP gene expressions were compared after adjustment with GAPDH gene expressions. * P < 0.05 versus control, # P < 0.05 versus untreated RHT group.

Figure 3. Photomicrographs of the cardiac sections stained with Masson’s trichrome method in each group. A: control group, B: untreated RHT, C: ARB group, D: SPRL group, E: Combined treatment group. Bar is 100 µm.

C: control group was observed in the untreated RHT group (Figure 3B). These changes were attenuated in the SPRL group (Figure 3D) and the combined treatment group (Figure 3E). Quantification of the cardiac fibrosis in the cross-sections of the heart in each group is shown in Figure 4. The degree of cardiac fibrosis was significantly lower in the SPRL group and the combined treatment group than that in the untreated RHT group. Micrographs of the regions around the vascular
tissues are shown in Figure 5. Marked proliferation of fibrous tissue in the interstitial and perivascular areas was observed in the untreated RHT group (Figure 5A), but was attenuated in the SPRL group (Figure 5D) and the combined treatment group (Figure 5E). Figure 6 shows that the ratio of the thickness of the arterial smooth-muscle layer to the diameter of the vascular lumen was significantly

![Graph](image)

**Figure 4.** The ratio of cardiac fibrosis area to cross-sectional area of the heart in each group.
* *P* < 0.05 versus control group, # *P* < 0.05 versus untreated RHT group.

![Images](image)

**Figure 5.** Photomicrographs of vascular sections stained with Masson's trichrome method in each group. A: control group, B: untreated RHT, C: ARB group, D: SPRL group, E: Combined treatment group. Bar is 50 μm.
larger in the untreated RHT group than that in the control group, and that the values in the ARB group and the combined treatment group were significantly smaller than the value in the untreated RHT group. The ratio of the thickness of
the perivascular fibrosis layer to the diameter of the vascular lumen was significantly larger in the untreated RHT group than that in the control group, and the values in the SPRL group and the combined treatment group were significantly lower than that in the untreated RHT group.

A micrograph of an immunohistochemically stained section with Connexin 43 is shown in Figure 7A. The cell cross-sectional area (CSA) and the myocyte volume were both significantly smaller in the ARB group and the combined treatment group than the corresponding values in the untreated RHT group (Figure 7C, D). There were no significant differences in cell length between the untreated RHT group and any of the treatment groups.

**DISCUSSION**

In the present study, combined treatment with valsartan and SPRL was more effective in preventing cardiac remodeling in RHT rats than treatment with either drug alone. We demonstrated for the first time that SPRL treatment mainly prevented the proliferation of interstitial and perivascular fibrosis and valsartan treatment mainly prevented the development of cellular hypertrophy, despite similar changes in blood pressure.

Up-regulation of RAAS is closely involved in cardiac hypertrophy in RHT rats. Many clinical and experimental studies have demonstrated the beneficial effects of renin-angiotensin blockade against cardiac remodeling. However, the dose of each renin-angiotensin blocker remains controversial; in particular, it is not clear whether low doses of ARB exert cardioprotective effects independent of BP reduction. In the present study, we administered a low dose of valsartan in order to maintain a similar degree of BP reduction to that observed with SPRL treatment. Although neither treatment affected the cell length of myocytes in the untreated RHT rats, the CSA and myocyte volume were suppressed in the rats administered a low dose of valsartan and in the combined treatment group. Interestingly, SPRL treatment did not prevent myocyte hypertrophy, suggesting that aldosterone may be not be involved in the cellular hypertrophy of myocytes observed in the RHT rats.

As cardiac hypertrophy generally involves both cellular hypertrophy and interstitial fibrosis, it is important to not only prevent cellular hypertrophy but also cardiac fibrosis. SPRL has been shown to suppress cardiac fibrosis in RHT rats and the addition of SPRL to candesartan treatment prevented cardiac collagen accumulation in stroke-prone spontaneously hypertensive rats. These findings suggest that aldosterone plays an important role in the proliferation of cardiac fibrosis in hypertensive animal models. The present study results demonstrate that SPRL and combined SPRL and valsartan treatment prevented the pro-
liferation of interstitial and perivascular fibrosis in the heart without significant BP reduction. On the other hand, the low dose of valsartan in our study did not prevent the proliferation of cardiac fibrosis, suggesting that ARBs may not have any significant effect against aldosterone-mediated cardiac remodeling. However, the dose-dependent effects of valsartan on the prevention of cardiac fibrosis need further evaluation. Also, aldosterone breakthrough has been reported during long-term ARB treatment in clinical and experimental studies.\(^{13,21}\) Regarding the mechanism underlying this phenomenon, the increase in the angiotensin II level during ARB treatment is thought to stimulate aldosterone secretion via the mediation of angiotensin II type 2 (AT\(_2\)) receptors.\(^{15}\) Since RAAS was markedly activated in the untreated RHT rats, it is believed that ARBs strongly block the angiotensin II type 1 receptors, but without sufficient blockade of the mineralocorticoid receptors. Many recent studies have shown that aldosterone is produced locally in nonepithelial tissues, such as the brain and the heart,\(^{22}\) with up-regulation of the aldosterone-synthesizing gene, CYP11B2.\(^{23}\) Local aldosterone production may also be involved in the proliferation of cardiac fibrosis in RHT rats. Therefore, combined treatment with a renin-angiotensin inhibitor and an aldosterone blocker is more useful for the prevention of cardiac hypertrophy in RHT rats than treatment with either of the 2 classes of drugs alone.

ANP is a potent vasoactive peptide that plays important roles in cardiovascular homeostasis, and ANP gene expression has also been reported to be up-regulated in LV hypertrophy.\(^{24}\) The combined treatment in this study strongly suppressed the up-regulation of ANP mRNA expression as compared to treatment with either drug alone. Based on these results of ANP mRNA expression noted in this study, combined treatment is probably more useful to prevent LV hypertrophy in RHT rats.

The present study results suggest that valsartan prevented the progression of cellular hypertrophy and SPRL prevented the proliferation of cardiac fibrosis, and that combined treatment with both classes of drugs was more useful for the prevention of cardiac hypertrophy in the RHT rats. Future studies are needed to clarify the detailed mechanisms of action of these drugs and to examine the association of the drug effects with the AT\(_2\) receptor and/or the local aldosterone production system.

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REFERENCES