Original Article

Renoprotective Effect of Angiotensin-Converting Enzyme Inhibitor Combined with α_1 -Adrenergic Antagonist in Spontaneously Hypertensive Rats with Renal Ablation

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To assess the renal benefits of combined angiotensin-converting enzyme inhibition and α_1 -adrenergic antagonism, we studied the antihypertensive and renoprotective effects of temocapril (TMP) alone and in combination with doxazosin (DOX) in spontaneously hypertensive rats (SHR)/Izumo rats with renal ablation. Five-Sixths-nephrectomized rats were assigned to receive TMP (10 mg/kg/day) (TMP group), TMP plus DOX (2 mg/kg/day) (TMP + DOX group), or vehicle (control group) orally for 12 weeks. Both systolic blood pressure (SBP) and urinary excretion of albumin (UalbV) in the control group progressively increased during the experimental period and were significantly higher than in sham-operated rats. Treatment with either TMP or TMP plus DOX had similar antihypertensive effects in this rat model. Twelve weeks after initiation of treatment, the SBP values in the control, TMP, and TMP + DOX groups were 265 ± 8 , 157 ± 4 , and 163 ± 3 mmHg, respectively, in comparison with 233 ± 4 mmHg in sham-operated rats ($p < 0.0001$ control vs. sham, $p < 0.001$ TMP vs. control, $p < 0.001$ TMP + DOX vs. control). UalbV, serum creatinine (Scr), blood urea nitrogen (BUN), and heart weight/body weight (HW/BW) ratio were significantly lower in the TMP and TMP + DOX groups than in the control group (UalbV: $p < 0.05$; Scr: $p < 0.01$; [BUN, HW/BW ratio]: $p < 0.0001$; and [UalbV, Scr, BUN, HW/BW ratio]: $p < 0.0001$ vs. control, respectively). The index of glomerular sclerosis (IGS) and relative interstitial volume (RIV) were significantly lower in the TMP + DOX group than in the control group (IGS: $p < 0.05$; RIV: $p < 0.01$). Especially, UalbV, IGS, and RIV were significantly better in the TMP + DOX group than in the TMP group ([IGS, RIV]: $p < 0.05$; UalbV: $p < 0.01$). These results suggest that simultaneous administration of TMP and DOX provides greater renoprotective effects than administration of TMP alone.

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Key Words: chronic renal failure, spontaneously hypertensive rats, combination therapy, angiotensin-converting enzyme inhibitor, α_1 -adrenergic antagonist

Introduction

Hypertension is a well-known causative disease of progressive renal injury in both human and experimental animals (1, 2).

In rats, reduction of the functioning renal mass causes a rise in the systemic blood pressure and progressive renal failure. Increased filtration rate per glomerulus has been suggested to be responsible for the progressive aggravation of renal function in this model (3, 4). Antihypertensive therapy

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decreases the rate of decline of renal function in cases of established progressive renal disease (5, 6). Recent evidence suggests that the ability of antihypertensive therapy to protect the kidney relates largely to the glomerular hemodynamic consequences of therapy (7). The available antihypertensive regimens provide varying degrees of renal protection, with agents regulating glomerular hypertension possibly offering the maximal protection against the advance of progressive renal injury (7). Angiotensin-converting enzyme inhibitors (ACEIs) reduce hyperfiltration damage in remnant kidney nephrons in chronic renal failure (CRF) (7, 8), and meta-analyses have shown that these agents have the beneficial effects of reducing proteinuria and preserving renal function (6, 9). However, the antihypertensive and renoprotective effects of ACEI monotherapy in patients with CRF are less than perfect.

α_1 -Adrenergic antagonists are effective agents for the treatment of hypertension (10) and have little or no adverse effect on the glomerular filtration rate or effective renal plasma flow in patients with renal parenchymal disease (11). Moreover, α_1 -adrenergic antagonists do not adversely affect carbohydrate tolerance (12) and have favorable direct effects on serum lipid profiles (13, 14). The sympathetic nervous system is activated in renal failure (15). Since nerve terminals exist in the glomerular afferent and efferent arterioles, the activation of adrenergic α_1 receptors in renal failure may constrict the arterioles and have an unfavorable influence on the renal hemodynamics. On the other hand, α_1 -adrenergic antagonists dilate the arterioles, and may improve the renal hemodynamics. This raises the question of whether an additional renoprotective effect exceeding the effects of ACEI monotherapy could be derived from combination therapy using both classes of agents.

To assess the renal benefits of angiotensin-converting enzyme inhibition combined with α_1 -adrenergic receptor antagonism, we studied the antihypertensive and renoprotective effects of temocapril (TMP) alone and in combination with doxazosin (DOX) in an spontaneously hypertensive rat (SHR) remnant kidney model of CRF.

Methods

Twenty-four 5-week-old male SHR/Izumo rats (Funabashi Farm, Chiba, Japan) were subjected to 5/6-nephrectomy by removal of the left and two-thirds infarction of the right kidney under ether anesthesia. The right kidney was exposed *via* flank incision; the two poles were encircled with loops of ligatures and, after tightening the loops, the incision was closed. Two weeks later, the left kidney was exposed *via* flank incision, removed in total, and the flank incision closed. Sham operations were performed in age-matched male-SHR/Izumo rats (sham group; $n = 10$). Rats were housed in a metabolic cage (model ST; Sugiyamagen, Tokyo, Japan) designed to prevent feces-urine contact and kept in a humidity- and temperature-controlled room ($55 \pm$

10% and $22 \pm 2^\circ\text{C}$, respectively) with a 12-h light/dark cycle. The rats were fed a regular diet (0.19 wt% sodium, 0.25 wt% potassium, 20.8 wt% protein; Funabashi Farm) and had free access to tap water.

One week after the last operation, when the rats were 8 weeks old, baseline measurements of body weight (BW), systolic blood pressure (SBP), urine volume (UV) and urinary excretion of albumin (UalbV) were made. The 5/6-nephrectomized rats were then randomly assigned to 1 of 3 groups. The control group ($n = 8$) was given vehicle, the second group ($n = 8$) received TMP (Sankyo Co., Tokyo, Japan) 10 mg/kg/day alone (TMP group), and the third group ($n = 8$) received TMP 10 mg/kg/day plus DOX (Pfizer Pharmaceuticals Inc., Tokyo, Japan) 2 mg/kg/day (TMP + DOX group). TMP and DOX were mixed with 0.5% carboxymethyl cellulose immediately before administration. TMP was administered once daily (9 AM) and DOX twice daily (9 AM/9 PM) by gavage for 12 weeks. The TMP group received vehicle at 9 PM. Oral administration of TMP at 10 mg/kg/day for 21 weeks lowered SBP of conscious SHR significantly from week 1 and throughout the rest of the 21-week observation period (16). Oral administration of DOX at 0.5–5.0 mg/kg/day produced a dose-related fall in the blood pressure of SHR (17).

Twelve weeks after initiation of treatment, the rats were killed by decapitation and trunk blood was collected in polyethylene tubes for the determination of serum creatinine (Scr) and blood urea nitrogen (BUN). The heart was removed and weighed and the heart weight/body weight (HW/BW) ratio calculated. Portions of remnant kidneys removed when the rats were killed were fixed in 10% neutral buffered formalin. Three micrometer-thick paraffin sections were cut, stained with haematoxylin and eosin, periodic acid-Schiff's reagent, Masson's trichrome, and Azan Mallory, and analyzed by an investigator with no prior knowledge of the groups to which the rats belonged. For calculating focal glomerular sclerosis, 150–200 glomeruli from each stained section were examined. High-power fields were used to examine for evidence of focal sclerosis (18). The degree of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4: Grade 0, no change; Grade 1, sclerotic area $\leq 1/4$ of the glomerulus or distinct adhesion present between the capillary tuft and Bowman's capsule; Grade 2, sclerosis of $1/4$ – $1/2$ of the total glomerular area; Grade 3, sclerosis $> 1/2$ of the glomerulus but not global; Grade 4, global sclerosis. The index of glomerular sclerosis (IGS) was then calculated using the following formula (18, 19):

$$\text{IGS} = (1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4) / (N_0 + N_1 + N_2 + N_3 + N_4),$$

where N is the number of glomeruli at each grade of sclerosis.

To estimate the relative interstitial volume (RIV) of the renal cortex, single stained sections from each rat were examined by a color image analyzer (SP500F; Olympus, Tokyo, Japan). The glomerulus, blood vessels, tubules, and medulla

Fig. 1. Sequential systolic blood pressure values in the control, sham, TMP, and TMP + DOX groups during the 12-week experimental period. Values are expressed as the means $\pm$ SEM. $\circ$, control (vehicle); $\bullet$, sham; $\square$, TMP (temocapril alone); $\blacksquare$, TMP + DOX (combination with temocapril and doxazosin). * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; § $p < 0.0001$ vs. control; † $p < 0.001$ vs. control; $\S$ $p < 0.0001$ vs. control.

were avoided; the values of RIV from 20 areas in each rat were measured and the mean value was calculated.

SBP was monitored every 2 weeks in conscious rats by the indirect tail-cuff method (UR 1000; Ueda Industries Co., Tokyo, Japan) without anesthesia. SBP measured by this method correlates well with that by the direct method (20). UV was measured gravimetrically, and urine was collected every 2 weeks and stored at -30°C immediately after collection. UalbV was determined by enzyme-linked immunosorbent assay (NEPHRAT; Exocell, Inc., Philadelphia, USA). BUN and Scr were measured by a standard auto-analysis technique (Synchron CX-3; Clinical Systems, Beckman Coulter Inc., Fullerton, USA).

Values are expressed as the means $\pm$ SEM. With respect to Scr, BUN, and the HW/BW ratio, comparisons between the different groups of rats were performed using one-way analysis of variance (ANOVA) and Bonferroni/Dunn test. For SBP, UalbV and BW, comparisons between the different groups of rats were performed by ANOVA with repeated measures over the duration of the study. Statistically significant differences on each day were assessed between groups by the Bonferroni/Dunn test, with values of $p < 0.05$ being considered statistically significant. With respect to the IGS and RIV, comparisons between the different groups of rats were performed using the Kruskal-Wallis test and Bonferroni/Dunn test. The statistical analysis in the present study was performed using the statistical software package STATVIEW 5.0 (Abacus Concepts Inc., Berkeley, USA).

This study conforms to the principles for the use of live animals outlined in the Declaration of Helsinki and those of the ethics committee of Tohoku University Graduate School of Medicine.

Fig. 2. Urinary excretion of albumin during the 12-week experimental period. Values are expressed as the means $\pm$ SEM. $\circ$, control (vehicle); $\bullet$, sham; $\square$, TMP (temocapril alone); $\blacksquare$, TMP + DOX (combination with temocapril and doxazosin). * $p < 0.05$ vs. control; ** $p < 0.01$ vs. control; § $p < 0.0001$ vs. control; † $p < 0.05$ vs. TMP; A $p < 0.05$ vs. TMP + DOX; B $p < 0.01$ vs. TMP + DOX; C $p < 0.001$ vs. TMP + DOX; D $p < 0.0001$ vs. TMP + DOX.

Results

SBP in the control group progressively increased to 265 ± 8 mmHg during the 12-week experimental period and was significantly ($p < 0.05$) higher than in the sham group (233 ± 4 mmHg) at 12 weeks (Fig. 1). SBP in the TMP and TMP + DOX groups (157 ± 4 and 163 ± 3 mmHg, respectively) was significantly ($p < 0.0001$) decreased compared with that in the control group at 12 weeks (Fig. 1). SBP in the TMP group was not significantly different from that in the TMP + DOX group.

UalbV in the control group progressively increased during the experimental period and was significantly ($p < 0.0001$) higher than in the sham group (Fig. 2). UalbV was significantly decreased in the TMP and TMP + DOX groups compared with that in the control group ($p < 0.01$ and $p < 0.0001$ vs. control, respectively). Furthermore, UalbV in the TMP + DOX group was significantly lower than that in the TMP group ($p < 0.05$) and significantly higher than that in the sham group ($p < 0.0001$).

The control group demonstrated focal and segmental glomerular structural lesions and increased cortical interstitial volume. These lesions were milder in the TMP and TMP + DOX groups than in the control group. Furthermore, these lesions in the TMP + DOX group were slighter than those in the TMP group (Fig. 3).

Similar patterns were seen with respect to Scr, BUN, IGS, RIV, and the HW/BW ratio. All values were significantly ([Scr, BUN, HW/BW ratio]: $p < 0.0001$; [IGS, RIV]: $p < 0.01$) higher in the control group than in the sham group (Figs. 4–6). Scr, BUN, and the HW/BW ratio were significantly

Fig. 3. Light micrographs of the kidneys from rats of the sham, control (vehicle), TMP (temocapril alone) and TMP + DOX (combination with temocapril and doxazosin) groups (periodic acid-Schiff, $\times 200$).

Fig. 4. (a) Serum creatinine (Scr) and (b) blood urea nitrogen (BUN) in the sham, control (vehicle), TMP (temocapril alone), and TMP + DOX (combination with temocapril and doxazosin) groups at 12 weeks. Values are expressed as the means $\pm$ SEM. Scr: ** $p < 0.01$ vs. control, § $p < 0.0001$ vs. control, † $p < 0.01$ vs. sham. BUN: § $p < 0.0001$ vs. control, * $p < 0.05$ vs. sham, ** $p < 0.01$ vs. sham. BUN in the TMP group was not significantly different from that in the TMP + DOX group.

lower in the TMP and TMP + DOX groups compared with those in the control group (Scr: $p < 0.01$, [BUN, HW/BW ratio]: $p < 0.0001$; [Scr, BUN, HW/BW ratio]: $p < 0.0001$ vs. control, respectively). IGS and RIV were significantly lower in the TMP + DOX group than in the control group (IGS: $p < 0.05$; RIV: $p < 0.01$). Furthermore, IGS and RIV were significantly ($p < 0.05$) lower in the TMP + DOX group than in the TMP group.

Scr, IGS, and RIV in the TMP + DOX group were not sig-

nificantly different from those in the sham group. The HW/BW ratio in the TMP group was significantly higher than that in the sham group ($p < 0.001$). The BUN and HW/BW ratio in the TMP + DOX group were significantly ($p < 0.05$) higher than those in the sham group. The HW/BW ratio in the TMP group was not significantly different from that in the TMP + DOX group.

BW in the control group was significantly lower than that in the sham group ($p < 0.0001$) (Fig. 6a) and those in the

Fig. 5. (a) Index of glomerular sclerosis (IGS) and (b) relative interstitial volume (RIV) of the renal cortex in the sham, control (vehicle), TMP (temocapril alone), and TMP + DOX (combination with temocapril and doxazosin) groups at 12 weeks. Values are expressed as the means $\pm$ SEM. IGS: * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control, † $p < 0.05$ vs. sham, ‡ $p < 0.05$ vs. TMP. RIV: ** $p < 0.01$ vs. control, † $p < 0.05$ vs. sham, ‡ $p < 0.05$ vs. TMP.

Fig. 6. (a) Body weight (BW) over the 12-week treatment period and (b) heart weight/body weight (HW/BW) ratio at 12 weeks in the sham, control (vehicle), TMP (temocapril alone), and TMP + DOX (combination with temocapril and doxazosin) groups. Values are expressed as the means $\pm$ SEM. BW: * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control. HW/BW ratio: † $p < 0.0001$ vs. control, * $p < 0.05$ vs. sham, ** $p < 0.01$ vs. sham. , control, , sham; , TMP; , TMP + DOX.

TMP and TMP + DOX groups (both $p < 0.01$). BW was not significantly different among the TMP, TMP + DOX, and sham groups.

Discussion

In the present study, a model combining SHR with renal ablation was chosen because functional and structural nephropathy is known to develop faster in this model than in a normotensive model with reduced renal mass (21). In intact SHR, the glomerulus is protected from high systemic blood pressure by afferent arteriolar vasoconstriction. Despite severe systemic hypertension, the superficial nephrons of SHR exhibit relatively low glomerular capillary plasma flow rates and normal glomerular capillary pressure and single-nephron glomerular filtration rates, and are relatively resistant to focal and segmental glomerular sclerosis (2). However, renal ablation in SHR results in lowering of afferent arteriolar resistance in the remnant kidney, allowing transmission of systemic hypertension and elevation of the glomeru-

lar capillary pressure (2). This hemodynamic alteration is associated with a sharp increase in proteinuria and acceleration of focal and segmental glomerular sclerosis (2). In the present study, we observed focal and segmental glomerular sclerosis and increased cortical interstitial volume associated with progressive increase in albuminuria in nephrectomized SHR, which findings are consistent with the development of hypertension.

Both TMP alone and TMP plus DOX blocked the development of hypertension, blunted rises in albuminuria, and reduced Scr and BUN in this model. These results indicate that the test regimens preserve renal function and lessen renal damage in this model. As far as the investigators are aware, these results provide the first evidence for renoprotective effects of combination therapy using TMP together with DOX.

TMP is a long-acting ACEI without a sulfhydryl group and is the ester prodrug of the pharmacologically active diacid metabolite temocaprilat. TMP is excreted mainly into the feces and exhibits sustained potent action in animal experiments (22). The main mechanism underlying the reno-

protective effects of TMP may be reduction in intraglomerular capillary pressure. Although we did not assess intraglomerular hyperfiltration in the present experiment, angiotensin II is known to constrict the efferent arterioles and thus help maintain glomerular capillary pressure and glomerular filtration (23–25). Removal of angiotensin II by ACEIs reduces capillary pressure and the glomerular filtration rate (26). In addition to efferent vasoconstriction, angiotensin II has many other actions in the kidney, including stimulating effects on mesangial cell contraction and macromolecular uptake through the mesangium (27), reduction of glomerular permeability to proteins (28), mediating tubular sodium reabsorption (29), and regulation of vasa recta blood flow (30), all of which might modulate the progression of intrarenal pathology in renal failure. Therefore, ACEI-induced inhibition of angiotensin II production may cause many changes other than those seen in glomerular hemodynamic variables, and some of these changes may also contribute to the renoprotective effects of ACEIs (31, 32).

In the present study, both TMP alone and TMP plus DOX similarly decreased SBP compared with that in the control group. The result that TMP alone and TMP plus DOX each induced a similar and significant decrease in the HW/BW ratio compared with the control group supports this finding. On the other hand, TMP plus DOX retarded the progression to glomerulosclerosis, and inhibited the increase of cortical interstitial volume. UalbV in the TMP + DOX group was significantly lower than that in the TMP group. Accordingly, TMP plus DOX had greater renoprotective effects than TMP alone. These results indicate that the TMP plus DOX regimen may have specific tissue effects that are not related to reduction of arterial blood pressure. In the present study, we measured SBP only at 2-week intervals. The use of 24-h blood pressure monitoring with a telemetry system similar to that of Bakris *et al.* (33) may have yielded better blood pressure values. In the present study, TMP + DOX did not enhance the antihypertensive effects of TMP monotherapy. The reason why the combination therapy did not produce an additional significant reduction in systemic blood pressure is unclear. Although a limitation of this study is that the antihypertensive effect of DOX monotherapy could not be estimated in this model, DOX may have had little additive effect on systemic blood pressure in this severe hypertension model when already being treated with TMP.

The mechanisms of the protection against renal injury in this model afforded by the combination of TMP plus DOX are unclear. DOX is a highly selective α_1 -adrenergic receptor antagonist structurally related to and more slowly eliminated than prazosin. DOX is metabolized by the liver and eliminated in the feces. Less than 1% of the drug is excreted unchanged in the urine (11). DOX may reduce renal injury by mechanisms different than those of TMP. Activation of adrenergic α_1 receptor constricts both the afferent and efferent arterioles. Since nerve terminals are much more abundant in the afferent than in the efferent arterioles, increased sym-

pathetic nerve activity would be expected to constrict the afferent arterioles more than the efferent arterioles (34). Therefore, DOX may dilate the afferent arterioles more than the efferent arterioles. Consequently, TMP + DOX may have dilated both pre- and postglomerular vessels and thereby have reduced the glomerular capillary pressure more effectively than treatment with TMP alone. Further investigations will be needed to examine the relation between the effect that DOX may have on glomerular hemodynamics and the renoprotective effect of DOX. Tojo *et al.* (35) reported that increased glomerulosclerosis scores in *N*^ω-nitro-L-arginine methyl ester (L-NAME)-induced hypertensive rats were significantly lowered by treatment with DOX. The decreased nitric oxide synthase (NOS) activity in the kidney of L-NAME-induced hypertensive rats was significantly increased by DOX, and this increase may play a role in the prevention of glomerulosclerosis. Furthermore, Tsai *et al.* (36) reported that DOX inhibits both serum-stimulated and platelet-derived growth factor-induced rat mesangial cell proliferation, and Jyothirmayi *et al.* (37) reported evidence that treatment with DOX may prevent leakage of albumin through the glomerular basement membrane in diabetic rats. Further studies will be needed to confirm the efficacy of combination therapy using an ACEI with an α_1 -adrenergic antagonist.

In conclusion, the antihypertensive and renoprotective effects of TMP were demonstrated in an SHR remnant kidney model of chronic renal failure. Moreover, combination treatment with TMP and DOX had additional renoprotective effects in this rat model. Although both ACEIs and α_1 -adrenergic antagonists are used for the treatment of hypertension with CRF, the combined use of an ACEI and an α_1 -adrenergic antagonist to protect against the progression of CRF might yield greater benefits than treatment with an ACEI alone.

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