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Contribution of renal angiotensin II type I receptor to gene expressions in hypertension-induced renal injury

SHOKEI KIM, KENSUKE OHTA, AKINORI HAMAGUCHI, TAKASHI OMURA, TOKIHITO YUKIMURA, KATSUYUKI MIURA, YOSHIYUKI INADA, TAKEO WADA, YOSHIMASA ISHIMURA, FUMIO CHATANI, and HIROSHI IWAO

Department of Pharmacology, Osaka City University Medical School, and Pharmaceutical Research Division and Pharmaceutical Development Division, Takeda Chemical Industries, Ltd., Osaka, Japan

Contribution of renal angiotensin II type I receptor to gene expressions in hypertension-induced renal injury. Recent evidence indicates that transforming growth factor-β1 (TGF-β1) plays an important role in renal fibrosis via stimulation of extracellular matrix synthesis. The present study was undertaken to investigate the role of angiotensin II type I receptor (AT1 receptor) in hypertension-induced renal injury. Twenty-two-week-old stroke-prone spontaneously hypertensive rats (SHRSP), which had established hypertension and moderate renal damage, were orally given TCV-116, a selective non-peptide AT1 receptor antagonist (0.1, 1 or 10 mg/kg/day), enalapril (10 mg/kg/day) or vehicle once a day for 10 weeks. At the end point of the treatment, we examined renal function, the gene expressions of TGF-β1 and extracellular matrix components in the interstitium [collagen types I (COI) and III (COIII), fibronectin (FN)] and the basement membrane (COIV and laminin), and renal microscopic morphology in rats aged 32 weeks. In vehicle-treated 32 week-old SHRSP with renal dysfunction and nephrosclerosis, renal mRNA levels for TGF-β1, COI, COIII, FN, COIV were all several-fold higher than in WKY. Thus, renal TGF-β1 gene expression was enhanced in SHRSP, which may contribute to the increased renal expressions of COI, COIII, FN, COIV in SHRSP. Treatment with TCV-116 (0.1 mg/kg/day) in SHRSP, in spite of no reduction of blood pressure, decreased renal mRNA levels for TGF-β1, COI, COIII, FN, COIV, being accompanied by the significant decrease in urinary protein and albumin excretion, blood urea nitrogen and plasma creatinine. Treatment with TCV-116 (10 mg/kg/day) in SHRSP decreased mRNAs for TGF-β1, COI, COIII, FN and COIV to almost the same levels as WKY, being associated with normalization of urinary protein and albumin excretion and the prevention of nephrosclerosis, as judged by microscopic histological observations. On the other hand, the effects of enalapril (10 mg/kg/day) on the above mentioned mRNA levels, renal function and renal morphology were weaker than those of TCV-116 (10 mg/kg/day) and were as much as TCV-116 (1 mg/kg/day). These results suggest that independently of hypotensive action, AT1 receptor antagonist has a potent renal protective effect by inhibiting the gene expression of renal TGF-β1 and extracellular matrix components.

Although hypertension is a major risk factor for renal injury [1], the mechanism of development of renal sclerosis in hypertensive patients and animals remains to be elucidated. Stroke-prone spontaneously hypertensive rats (SHRSP), a substrain of spontaneously hypertensive rats (SHR), are considered a useful model of human malignant hypertension. SHRSP characteristically develop severe nephrosclerosis and have been extensively studied as an example of hypertension-induced renal injury [2–4].

Investigations on effects of angiotensin converting enzyme (ACE) inhibitors [5, 6] or an angiotensin II type I (AT1) receptor antagonist [7] on renal function of SHRSP suggest the important role of renin-angiotensin system in renal injury of SHRSP, although the mechanism is not fully understood. While the changes in renal hemodynamics such as the reduction of glomerular capillary pressure are reported to be responsible for the renal protective effects by these drugs [8–11], several recent works have demonstrated the dissociation between the time course of antiproteinuric and hemodynamic effects by ACE inhibitors [12–14]. Recent histological study shows that ACE inhibitors and AT1 receptor antagonist can prevent renal and extrarenal vascular structural damage to some extent in rats with chronic renal failure, independent of their depressor effect on systemic blood pressure or local glomerular pressure [15]. Thus, the renal protective effects by inhibition of renin-angiotensin system appear to be at least in part mediated by unknown mechanisms other than renal hemodynamic effects [12–15]. Furthermore, it is possible that the renal protective effects of ACE inhibitors are in part due to the activation of kinin-kallikrein system and the increased prostaglandins [16, 17].

Transforming growth factor-β1 (TGF-β1) is a multifunctional growth factor and plays an important role in cell proliferation or hypertrophy, chemoattraction of leukocytes and monocytes migration, and synthesis of extracellular matrix [18–20]. Recent evidence [21–24] supports the notion that TGF-β1 is involved in fibrosis in several experimental renal diseases by increasing the synthesis of extracellular matrix. However, the role of TGF-β1 in hypertension-mediated renal injury remains to be determined. Furthermore, it is unknown whether or not angiotensin II (Ang II) contributes to the *in vivo* gene expression of renal TGF-β1.

Our present study was undertaken to examine whether or not TGF-β1 might be responsible for hypertensive renal injury and whether renal injury by Ang II is mediated by TGF-β1. For this purpose, we studied the gene expression of TGF-β1 and extracellular matrix proteins in the kidney of SHRSP and the effects of TCV-116 [25–28], a non-peptide selective AT1 receptor antagonist, on these gene expressions.

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Methods

Animals

SHRSP and Wistar-Kyoto rats (WKY) were donated to Takeda Chemical Industries, LTD. (Osaka, Japan) by Dr. K. Okamoto (Department of Pathology, Kyoto University, Kyoto, Japan) in 1972, and have been maintained by selective mating at Takeda Chemical Industries, Ltd. since 1972 [3, 29]. Male SHRSP and age-matched control WKY [3, 29] were fed a standard laboratory chow (CE2, Clea Japan, Tokyo, Japan) and given tap water *ad libitum*.

Drugs

TCV-116 and enalapril maleate, which were synthesized by Takeda Chemical Industries, Ltd., were suspended with small amount of gum arabic solution for oral administration.

Experimental protocol

Anti-hypertensive drugs are often used in hypertensive patients with renal dysfunction, although the renal protective effect of anti-hypertensive drugs in such patients is still unclear. Thus, it is clinically very important to investigate the renal protective effects of anti-hypertensive drugs in hypertensive rats with established hypertension and renal damage. SHRSP at 22 weeks have established hypertension (systolic BP greater than 200 mm Hg) and moderate renal damage; by 32 weeks such rats have renal fibrosis and renal insufficiency [4, 5, 6, 29]. Therefore, in the present study drug treatment of SHRSP was carried out from the age of 22 to 32 weeks. Twenty-two-week-old male SHRSP were randomly separated into five groups, and there was no difference in blood pressure, heart rate, body weight and urinary protein and albumin excretion among each group before the experiments. The animals were orally given TCV-116, enalapril or vehicle (2 ml/kg) by gastric gavage in the morning once a day. TCV-116 were given to SHRSP at the dose of 0.1, 1 or 10 mg/kg/day. The hypotensive effects of these 3 doses of TCV-116 have been examined in detail in other hypertensive models, including SHR and two-kidney, one-clip hypertensive rats [28]. Preliminary experiments showed that 0.1 mg/kg/day of TCV-116 did not significantly lower blood pressure in SHRSP throughout 10 weeks of treatment, (manuscript in preparation). Therefore, in the present study, treatment with this dose of TCV-116 was performed to study the effect of AT1 receptor antagonist not mediated by systemic blood pressure. Enalapril is the most popular ACE inhibitor and has been well characterized, with respect to the hypotensive effects and the renal protective effects in SHRSP [5, 6] and rats with renal failure [9, 11, 12], which led us to use enalapril as an ACE inhibitor in the present study, to compare with AT1 receptor antagonist. Previous reports show that 10 to 15 mg/kg of enalapril has significant hypotensive and renal protective effects in SHRSP [5, 6]. Therefore, this dose of enalapril was used in the present study. Control SHRSP were given only vehicle (5% gum arabic solution). Treatment with the above mentioned drugs or vehicle were carried out for 10 weeks (from the age of 22 to 32 weeks). Blood pressure, heart rate and body weight were measured periodically throughout drug treatment. The measurements of blood pressure were all carried out at five hours after oral dosing of each drug, because previous studies indicated that the maximal hypotensive effects of TCV-116 occur at 5 hours after oral administration [28]. For measurement of urinary protein and albumin excretion, rats were individually housed in metabolic cages two days before termination of 10 weeks of drug treatment, and urine was collected for 24 hours. After 10 weeks of treatment, the above five groups of SHRSP with the age of 32 weeks and age-matched WKY were killed by decapitation and trunk blood was collected. Kidneys were rapidly excised, weighed, frozen in liquid nitrogen and stored at −80°C until the extraction of total tissue RNA.

For renal histological study, 22-week-old male SHRSP were treated with vehicle, TCV-116 (0.1 mg/kg/day), TCV-116 (10 mg/kg/day) and enalapril (10 mg/kg/day) for 10 weeks, as described above, decapitated, and kidneys were removed to examine histology as described below.

Extraction of renal total RNA

Total RNA was extracted from kidneys, according to the method of Chomczynski and Sacchi [30] with a minor modification. In brief, frozen renal tissues were homogenized with a Polytron homogenizer (PCU-11, Kinematica AG, Littau/Luzern, Switzerland) for 60 seconds at speed dial 10 in denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, 0.5% N-lauroylsarcosine). The homogenate was added to 1/10 vol of 2 M sodium acetate (pH 4), 1 vol of water-saturated phenol and 1/2 vol of chloroform, and centrifuged at 10,000 × g at 4°C for 20 minutes. The resulting upper aqueous phase was transferred to a fresh tube and precipitated by addition of 1 vol of isopropanol followed by centrifugation. The precipitate was dissolved in the above denaturing solution, incubated with 3.5 M lithium chloride at 4°C for 18 hours and then centrifuged. After the centrifugation, the RNA pellet was washed with 3 M lithium chloride and then with 70% ethanol. Finally, the RNA pellet was dissolved in 0.1% diethyl pyrocarbonate-treated water and stored at −80°C until use. The RNA concentration was spectrophotometrically determined at 260 nm.

Northern blot hybridization

Thirty micrograms of total RNA from kidneys was denatured by incubating with 1 M deionized glyoxal and 50% dimethyl sulfoxide at 50°C for one hour [31], electrophoresed on a 1% agarose gel at 50 V, and transferred to a nylon membrane (Gene Screen Plus, E.I. du Pont de Nemours & Co., NEN Products, Boston, Massachusetts, USA). Each cDNA probe was labeled with (^{32}P)-dCTP (specific activity 3000 Ci/mM; E.I. du Pont de Nemours) by random primer extension method [32] with a Random Primer DNA Labeling Kit (Takara, Kyoto, Japan). Prehybridization and hybridization were performed according to the manufacturer's instruction. In brief, the membranes were prehybridized in a solution containing 50% formamide, 5 × Denhardt (Ficoll, polyvinylpyrrolidone and bovine serum albumin, 1 mg/ml each), 5 × SSPE (0.75 M sodium chloride, 50 mM sodium phosphate, 5 mM ethylenediaminetetraacetic acid), 1% sodium dodecyl sulfate (SDS) and 200 μg/ml denatured salmon sperm DNA at 42°C for four hours. Then the membranes were hybridized with ^{32}P-labeled cDNA (1 to 2 × 10^6 dpm/ml) at 42°C for 24 hours in fresh hybridization solution which was identical to the prehybridization solution except for the absence of salmon sperm DNA. The membranes were washed in 2 × SSPE at room temperature for 30 minutes, in 2 × SSPE and 2% SDS twice at 65°C for 45 minutes, and in 0.1 × SSPE at room temperature for 30 minutes. After washing, the membranes were exposed to Kodak XAR-5 film between two intensifying screens at −70°C. The nylon membrane

Table 1. Body weight, kidney weight, blood pressure and heart rate in WKY and SHRSP at 10 weeks after drug treatment

		SHRSP				
	WKY	Vehicle	TCV (*0.1 mg*)	TCV (*1 mg*)	TCV (*10 mg*)	Enalapril (*10 mg*)
Body weight *g*	455 ± 12[b]	345 ± 12	383 ± 6	375 ± 7	355 ± 8	376 ± 16
Kidney weight *mg/g body wt*	7.38 ± 0.26[b]	9.76 ± 0.36	8.71 ± 0.15[b]	8.11 ± 0.15[b]	8.25 ± 0.25[b]	8.66 ± 0.35[a]
Blood pressure *mm Hg*	132 ± 2[b]	222 ± 10	208 ± 7	183 ± 4[b]	142 ± 8[b]	178 ± 3[b]
Heart rate *bpm*	354 ± 13	353 ± 20	329 ± 17	387 ± 12	391 ± 20	368 ± 20

Abbreviations are: WKY, Wistar-Kyoto rats; SHRSP, stroke-prone spontaneously hypertensive rats; Vehicle, vehicle-treated SHRSP; TCV (0.1 mg), TCV-116 (0.1 mg/kg/day)-treated SHRSP; TCV (1 mg), TCV-116 (1 mg/kg/day)-treated SHRSP; TCV (10 mg), TCV-116 (10 mg/kg/day)-treated SHRSP; Enalapril (10 mg), enalapril (10 mg/kg/day)-treated SHRSP.

[a] $P < 0.05$, [b] $P < 0.01$ vs. vehicle-treated SHRSP

was stripped off by boiling in 0.1 × SSC solution containing 1% SDS, and then rehybridized with other cDNA probes. All renal RNA samples from six groups of rats were electrophoresed on the same agarose gel, transferred to the same membrane and hybridized with each cDNA probe at the same time, thereby allowing for the comparison of mRNA levels among all six groups of rats.

To evaluate tissue mRNA levels, an optical scanner (EPSON GT-8000, Seoko, Tokyo, Japan) was utilized for digitizing autoradiograms. The autoradiogram bands in the digitized image were measured for their density with the use of the public domain NIH Image program and a computer (Macintosh LC-III, Apple Computer, Inc., USA). For all RNA samples, the density of an individual mRNA band was normalized for that of GAPDH mRNA band, to correct for the difference in RNA loading and/or transfer.

cDNA probes

cDNA probes used were as follows. Rat renin DNA was a 0.70 kb KpnI/KpnI fragment, provided by Dr. A. Fukamizu (Institute of Applied Biochemistry, Tsukuba University, Ibaraki, Japan) [33]. Rat TGF-β1 cDNA was a 1.0 kb HindIII/XbaI fragment from Dr. S.W. Qian (Laboratory of Chemoprevention, NIH, Bethesda, Maryland, USA) [34]. Rat fibronectin cDNA was a 0.27 kb HindIII/EcoRI fragment from Dr. R.O Hynes (Center for Cancer Research, Massachusetts Institute of Technology, Massachusetts, USA) [35]. Rat α1 (I) collagen cDNA was a 1.3 kb PstI/BamHI fragment from Dr. D. Rowe (Department of Pediatrics, University of Connecticut, Connecticut, USA) [36]. Mouse cDNAs for α1 (III) collagen [37], α1 (IV) collagen [38], and laminin B1 chain [39] were the fragments of 1.8 kb EcoRI/EcoRI, 0.83 kb AvaI/PstI, 0.65 BamHI/EcoRI, respectively, from Dr. Y. Yamada (Laboratory of Developmental Biology, National Institute of Dental Research, Bethesda, USA). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was a 1.3 kb PstI/PstI fragment from Dr. P. Fort (Laboratoire de Biologie Moleculaire, Universite De Montpellier II, France) [40].

Measurement of blood pressure

Systolic blood pressure was measured by the tail-cuff method (Riken Kaihatsu PS-8000, Japan).

Measurement of plasma renin concentration

Plasma renin concentrations (PRC) were measured as the rate of Ang I generation from rat plasma angiotensinogen, as described [41]. In brief, rat plasma samples were incubated with 48-hour nephrectomized rat plasma containing a excess of renin substrate (2 μM angiotensinogen) at 37°C in 0.1 M sodium phosphate buffer (pH 7.0) containing protease inhibitors, and the generated Ang I was measured by radioimmunoassay.

Histological examination

SHRSP, subjected to oral administration of vehicle, 0.1 or 10 mg/kg/day of TCV-116 or 10 mg/kg/day of enalapril for 10 weeks (from the age of 22 to 32 weeks), as described above, and age-matched WKY were decapitated, kidneys were removed, fixed in 10% phosphate-buffered formalin, embedded in paraffin, cut into 4 μm-thick sections, and Azan staining of the sections were carried out to examine renal fibrosis. Semiquantitative assessment was carried out in standard transverse sections through the hilum of each kidney to include the apex of the pyramid, the corticomedullary junction and cortex. Parameters scored were as follows: fibrinoid necrosis in glomerulus; atrophy of the epithelium, hypertrophy of basement membrane and hyaline cast with dilatation in renal tubules; fibrinoid necrosis and hypertrophy in arterioles; hyalinoid hypertrophy of intima in artery; interstitial fibrosis. The criteria scoring was as follows: score 0, intact; score 1, very mild; score 2, mild; score 3, moderate; score 4, severe.

Miscellaneous measurements

Urinary protein was measured using a A/G-B test (Wako, Osaka, Japan) based on Biuret reaction, and urinary albumin was measured using an Albumin-B test with bromcresol green (Wako). Blood urea nitrogen (BUN) and plasma creatinine were also measured using their respective kit (Wako).

Statistical analysis

All values were expressed as mean ± SEM. Statistical significance was determined using ANOVA and Duncan's multiple range test, except for the use of Kruskal-Wallis ANOVA and Wilcoxon's test for histological data. Differences were considered statistically significant at a value of $P < 0.05$.

Results

Effects of TCV-116 and enalapril on body weight, kidney weight, blood pressure and heart rate

As shown in Table 1, body weight of SHRSP was unchanged by treatment with TCV-116 or enalapril. However, kidney weight in SHRSP, which was larger than WKY ($P < 0.01$), was significantly reduced by 10 weeks of treatment with three doses of TCV-116 ($P < 0.01$) or enalapril ($P < 0.05$).

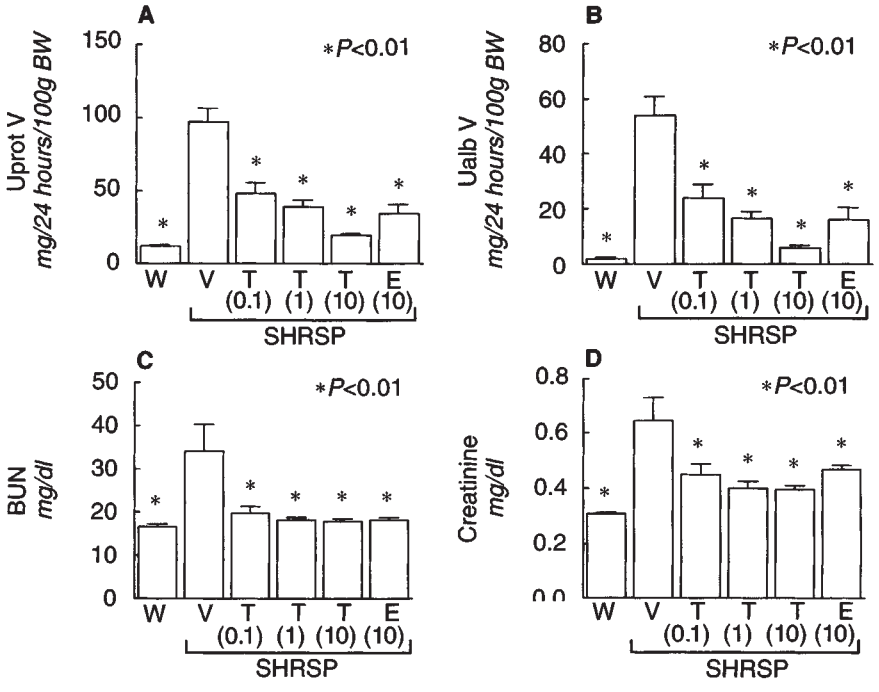

Fig. 1. *Urinary protein excretion (A), albumin excretion (B), BUN (C) and plasma creatinine (D) in WKY and SHRSP after 10 weeks of treatment with vehicle, TCV-116 or enalapril.* Abbreviations are: W, WKY; V, vehicle-treated SHRSP; T (0.1), TCV-116 (0.1 mg/kg/day)-treated SHRSP; T (1), TCV-116 (1 mg/kg/day)-treated SHRSP; T (10), TCV-116 (10 mg/kg/day)-treated SHRSP; E (10), enalapril (10 mg/kg/day)-treated SHRSP; $U_{Prot}V$, urinary protein excretion; $U_{Alb}V$, urinary albumin excretion; BUN, blood urea nitrogen. Each bar represents the mean ±SE. Each group included 5 animals. * $P < 0.01$ vs. V (vehicle-treated SHRSP).

Table 1 also shows systolic blood pressure at 10 weeks after treatment with vehicle, TCV-116 or enalapril, and the measurements of blood pressure were all carried out at five hours after oral administration. Systolic blood pressure in vehicle-treated SHRSP (222 ± 10 mm Hg) was higher than age-matched WKY (132 ± 2 mm Hg; $P < 0.01$). Ten weeks of treatment with TCV-116 at 0.1 mg/kg/day failed to decrease blood pressure in SHRSP. On the other hand, treatment with 1 and 10 mg/kg/day of TCV-116 and 10 mg/kg/day of enalapril lowered blood pressure ($P < 0.01$). There was no significant difference in blood pressure between WKY and TCV-116 (10 mg/kg/day)-treated SHRSP. Heart rate was affected by neither TCV-116 nor enalapril.

Effects of TCV-116 and enalapril on renal function

As shown in Figure 1A and B, urinary protein and albumin excretion (96.9 ± 9.6 and 53.9 ± 6.9 mg/24h/100 g body weight, respectively) in vehicle-treated SHRSP were 8.0-fold and 25.7-fold, respectively, greater than those in WKY. Ten weeks of treatment with TCV-116 (0.1, 1 and 10 mg/kg/day) and enalapril (10 mg/kg/day) in SHRSP significantly decreased both urinary protein and albumin excretion ($P < 0.01$ vs. vehicle-treated SHRSP). Urinary protein and albumin excretion (19.0 ± 1.5 and 5.8 ± 1.0 mg/24 hr/100g body wt, respectively) in TCV-116 (10 mg/kg/day)-treated SHRSP were not significantly different from those in WKY, while there was a significant difference in urinary protein and albumin excretion between WKY and enalapril (10 mg/kg/day)-treated SHRSP ($P < 0.05$). Figure 1C and D indicate that BUN (34.0 ± 6.2 mg/dl) and plasma creatinine concentration (0.648 ± 0.085 mg/dl) in SHRSP were significantly higher than those in WKY (16.6 ± 0.5 and 0.304 ± 0.009 mg/dl, respectively; $P < 0.01$). TCV-116 at the three doses or enalapril significantly lowered BUN and plasma creatinine concentrations in SHRSP ($P < 0.01$).

Effects of TCV-116 and enalapril on PRC and renal renin mRNA

As shown in Figure 2A, the mean value of plasma renin concentration in vehicle-treated SHRSP (86 ± 16 ng Ang I/hr/ml) was 5.4-fold higher than that in WKY ($P < 0.01$). Ten weeks of treatment with TCV-116 (0.1 or 1 mg/kg/day) or enalapril (10 mg/kg/day) did not change plasma renin levels in SHRSP. However, TCV-116 (10 mg/kg/day) increased PRC in SHRSP by 5.1-fold ($P < 0.01$). Renal renin mRNA levels, corrected for GAPDH mRNA levels, in vehicle-treated SHRSP was 2.8-fold higher compared with WKY (Figs. 2B and 3). TCV-116 (0.1 mg/kg/day) or enalapril (10 mg/kg/day) did not alter renal renin mRNA levels in SHRSP, while TCV-116 at the dose of 1 and 10 mg/kg/day increased renal renin mRNA in SHRSP 1.6-fold ($P < 0.05$) and 3.6-fold ($P < 0.01$), respectively.

Effects of TCV-116 and enalapril on renal mRNA levels for TGF-β1 and extracellular matrix components in the interstitium and the basement membrane

Figure 4 shows that renal TGF-β1 mRNA levels in SHRSP were 3.4-fold higher than WKY ($P < 0.01$). Ten weeks of treatment with TCV-116 at the three doses and with 10 mg/kg/day of enalapril significantly decreased renal TGF-β1 mRNA levels in SHRSP, whose levels were all similar to those in WKY.

As shown in Figure 5, renal mRNA levels for types I and III collagen and fibronectin in vehicle-treated SHRSP were 5.6-, 4.3- and 2.8-fold, respectively, higher than WKY ($P < 0.01$). TCV-116 at all three doses used or enalapril significantly decreased mRNA levels for these three extracellular matrix components in SHRSP ($P < 0.01$). Furthermore, there was no significant difference between TCV-116 (1 mg/kg/day) and enalapril (10 mg/kg/day)-treated SHRSP, in renal mRNA levels for these three extracellular matrix components.

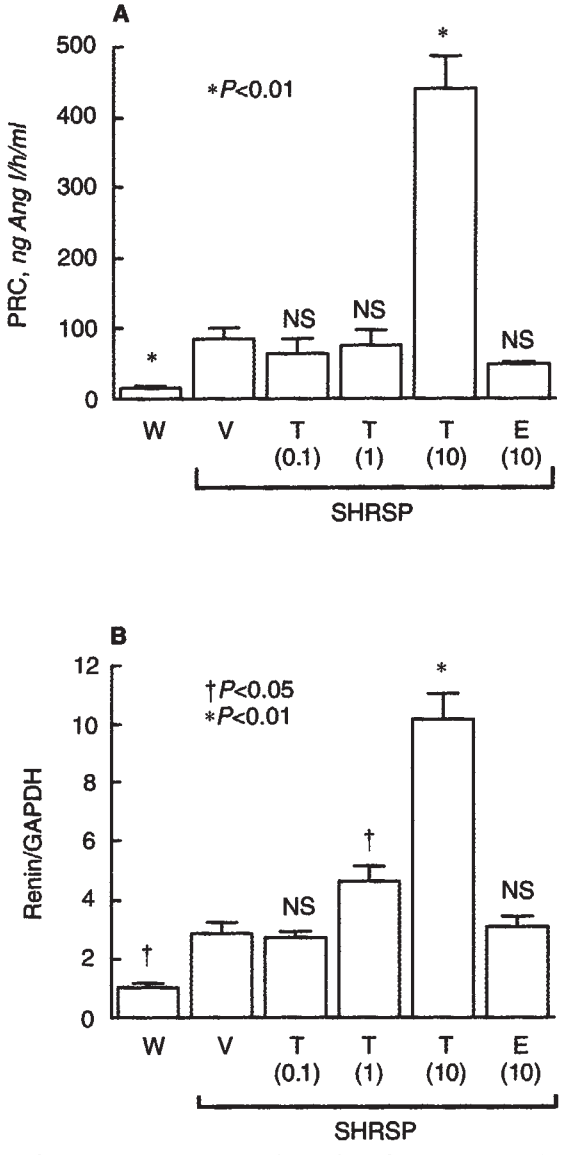

Fig. 2. *Plasma renin concentration (A) and renal renin mRNA (B) in WKY and SHRSP after 10 weeks of treatment with vehicle, TCV-116 or enalapril.* (A) Because the values of plasma renin concentrations showed heteroscedasticity, logarithmic transformation was made for data before statistical analysis. (B) The ordinate shows renal renin mRNA values divided by GAPDH mRNA values. The mean value of renin mRNA corrected for GAPDH mRNA in WKY is represented as 1. PRC, plasma renin concentration. W, V, T (0.1), T (1), T (10) and E (10) indicate the same abbreviations as Figure 1. Each bar represents the mean ±SE. Each group included 5 animals. † $P < 0.05$, * $P < 0.01$ vs. V (vehicle-treated SHRSP). NS, not significant vs. V (vehicle-treated SHRSP).

Figure 6 shows mRNA levels for extracellular matrix components in the basement membrane. Type IV collagen mRNA levels in vehicle-treated SHRSP were 2.1-fold higher than WKY ($P < 0.01$), and were significantly decreased by treatment with TCV-116 or enalapril, to similar levels to WKY(Fig. 6A). On the other hand, mRNA levels for laminin B1 (Fig. 6B) were not different between WKY and SHRSP, and were unaffected by TCV-116 or enalapril.

Histological findings in the kidney

Figures 7 and 8 show photomicrographs of Azan-stained renal sections from WKY and SHRSP treated with vehicle or TCV-116 for 10 weeks, and Table 2 summarizes the score index. In vehicle-treated SHRSP ($N = 5$), fibrinoid necrosis was observed in the glomeruli. The renal tubules showed prominent lesions including atrophy of the epithelium, hypertrophy of the basement membrane, hyaline cast formation and dilatation. The arterioles showed prominent hypertrophy and fibrinoid necrosis, and the significant interstitial fibrosis was observed. Treatment with TCV-116 (0.1 mg/kg/day; $N = 5$) tended to improve all the above-mentioned lesions in SHRSP except for the hypertrophy of the arterioles, as shown by the mean value of the score index, although there was no statistically significant improvement. Enalapril (10 mg/kg/day; $N = 5$) significantly improved fibrinoid necrosis of the glomeruli and hyalinoid hypertrophy of arterial intima, but there was no statistically significant improvement in other parameters. On the other hand, TCV-116 (10 mg/kg/day; $N = 5$) improved the above-mentioned renal lesions in SHRSP more extensively than Enalapril (10 mg/kg/day), as judged by score index. Neither fibrinoid necrosis of the glomeruli and arterioles nor hypertrophy of the arteries and arterioles were seen in the kidney of SHRSP treated with TCV-116 (10 mg/kg/day; Table 2 and Fig. 7e, f). Furthermore, compared with vehicle-treated SHRSP, the score in TCV-116 (10 mg/kg/day)-treated SHRSP was significantly smaller for hypertrophy of the basement membrane, hyaline cast formation and dilatation, and atrophy of the epithelium in the renal tubules (Fig. 8). Interstitial fibrosis tended to be improved by TCV-116 (10 mg/kg/day), although there was no statistically significant difference. The score in the kidney of WKY was 0 for all parameters.

Discussion

In the present study, we examined the *in vivo* gene expression of TGF-β1, and extracellular matrix components in the kidney of SHRSP rats in the malignant phase, and also examined the long-term effects of an AT1 receptor antagonist and an ACE inhibitor on the gene expression. We found that the gene expression of renal TGF-β1, types I and III collagen, fibronectin and type IV collagen are enhanced in the malignant phase of SHRSP rats. Blockade of AT1 receptor by TCV-116 led to the significant inhibition of these gene expressions as well as the improvement of renal function. Furthermore, in spite of no decrease in blood pressure, treatment with 0.1 mg/kg/day of TCV-116 in SHRSP rats significantly reduced the above mentioned gene expressions, being accompanied by the renal protective effects. Thus, the present study demonstrates that intrarenal AT1 receptors play an important role in renal injury of SHRSP rats and that the enhanced gene expression of renal TGF-β1 in SHRSP rats is at least in part mediated by renal AT1 receptor, independent of systemic blood pressure.

SHRSP rats, a substrain of SHR, are considered a useful model of human malignant hypertension and characteristically develop extensive end-organ damage, including severe nephrosclerosis [2–4]. However, the mechanism of renal sclerosis in hypertension is not known. Furthermore, it is unclear which factors can contribute to the progression of nephrosclerosis in hypertension. TGF-β1, a multifunctional growth factor, participates in tissue

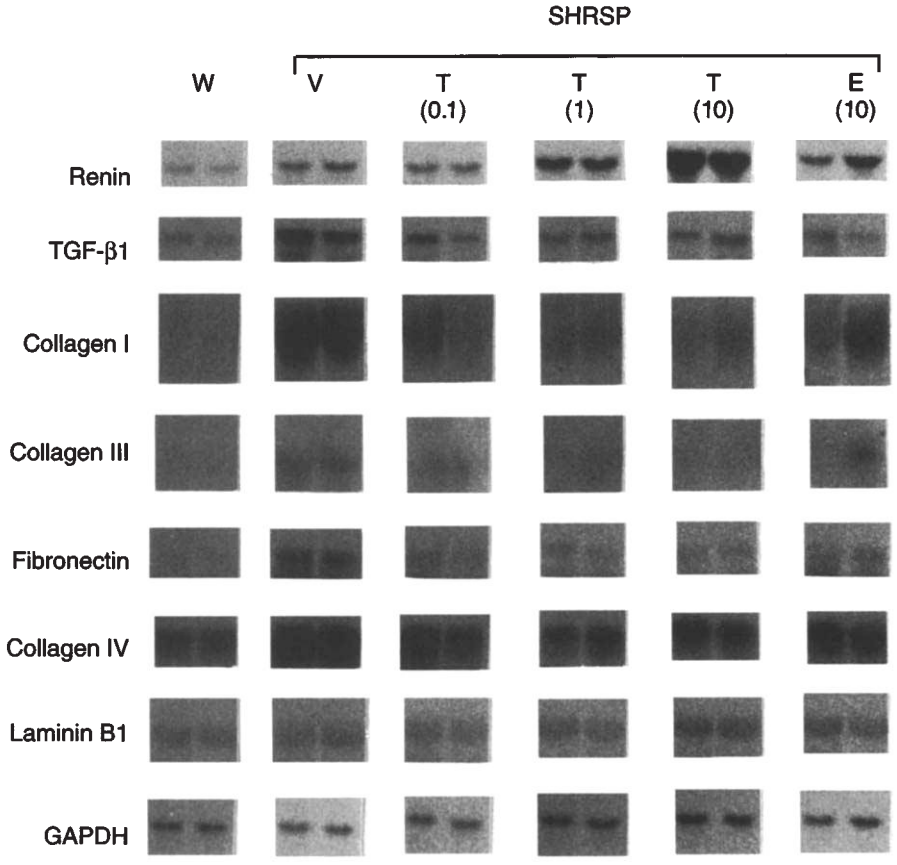

Fig. 3. *Autoradiograms of northern blot analysis of mRNA for renin, TGF-β1, type I collagen (collagen I), type III collagen (collagen III), fibronectin, type IV collagen (collagen IV), laminin B1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in WKY and SHRSP after 10 weeks of treatment with vehicle, TCV-116 or enalapril.* Each blot was a single blot (The northern blot analysis of all RNA samples was carried out on the same blot). The autoradiograms of 2 typical samples of 5 different samples from each group were used as the representative autoradiograms. W, V, T (0.1), T (1), T (10) and E (10) indicate the same abbreviations as Figure 1.

repair and regeneration following tissue injury [18], and is reported to participate in fibrotic diseases in various organs including the kidney, by markedly stimulating synthesis of extracellular matrix proteins [19–24]. Border et al [21] have reported that glomerular TGF-β1 expression and extracellular matrix accumulation are increased in the mesangial proliferative glomerulonephritis, produced by a single injection of antithymocyte serum into rats, and that the administration of anti-TGF-β1 antibody into such glomerulonephritic rats significantly suppresses glomerular extracellular matrix deposition. Elevated expression of TGF-β1 has also been reported in crescentic glomerulonephritis of rabbits, induced by injecting antibody against antigens of the glomerular basement membrane [22], and in obstructive nephropathy of rats with unilateral ureteral ligation [23], which possibly promotes renal fibrosis. Furthermore, Isaka et al, who introduced TGF-β gene into the rat kidney via the renal artery using *in vivo* transfection technique, have found that the overexpression of TGF-β1 in the kidney induces glomerulosclerosis characterized by the accumulation of extracellular matrix proteins, thereby supporting that the increase in TGF-β1 is responsible for glomerulosclerosis *in vivo* [42]. Thus, TGF-β1 has been shown to contribute to glomerulosclerosis and fibrosis in several renal diseases. However, the gene expression and possible role of TGF-β1 in hypertension-induced renal injury remain to be determined.

In the present study, we found that the steady state mRNA levels for TGF-β1 are increased in the kidney of SHRSP. To examine whether the enhanced expression of renal TGF-β1

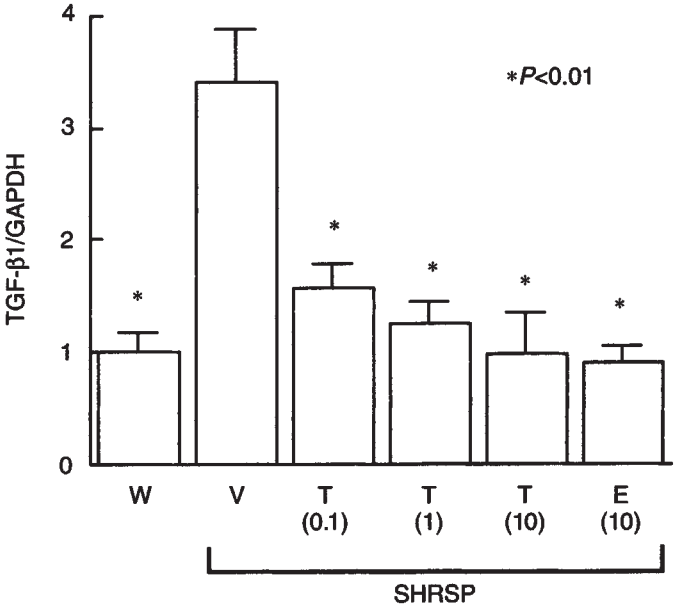

Fig. 4. *TGF-β1 mRNA levels in WKY and SHRSP after 10 weeks of treatment with vehicle, TCV-116 or enalapril.* The ordinate shows TGF-β1 mRNA levels corrected for GAPDH mRNA levels. The mean value of TGF-β1 mRNA corrected for GAPDH mRNA in WKY is represented as 1. W, V, T (0.1), T (1), T (10) and E (10) indicate the same abbreviations as Figure 1. Each bar represents the mean ± SE. Each group included 5 animals. * $P < 0.01$ vs. V (vehicle-treated SHRSP).

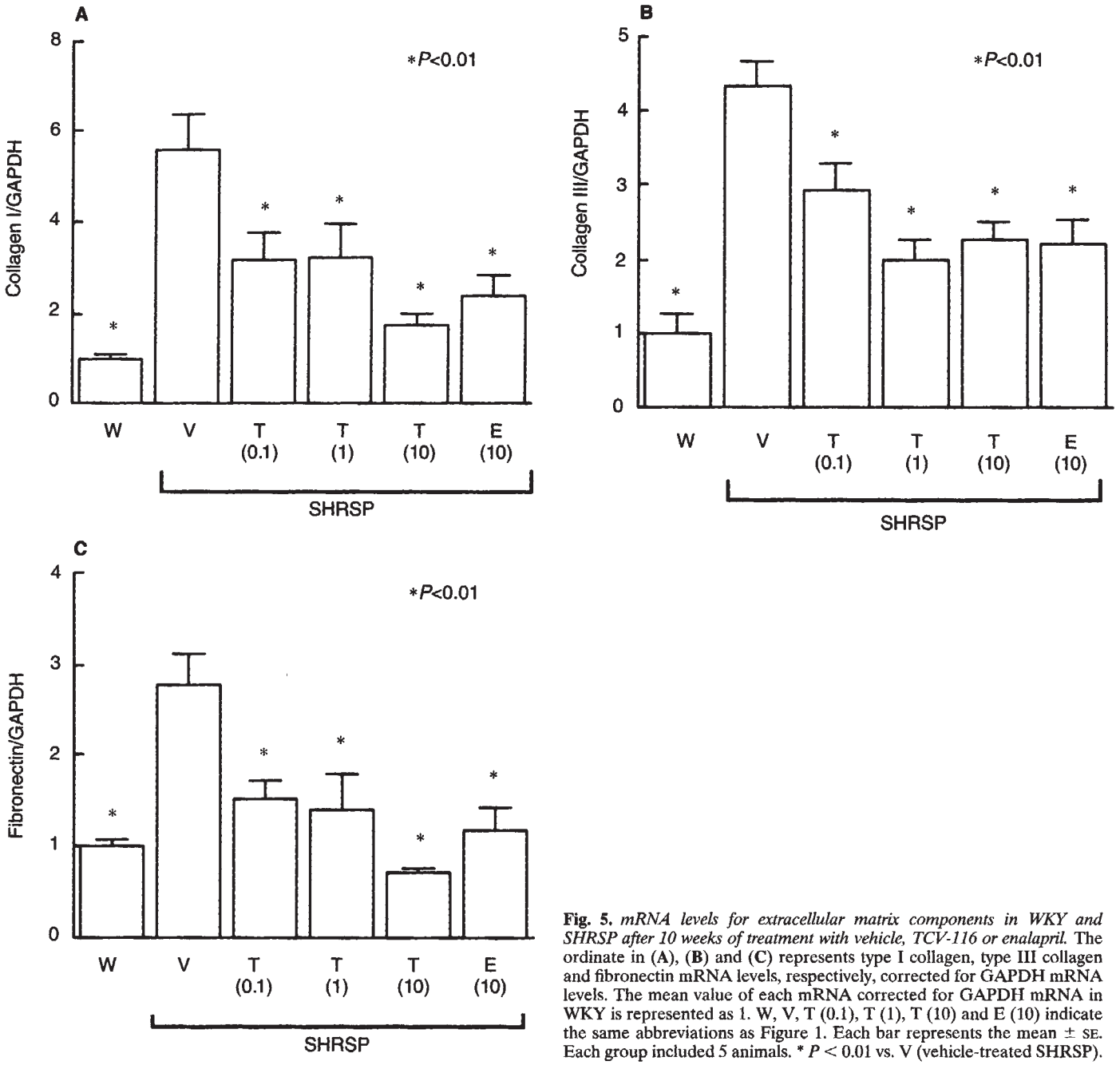

Fig. 5. *mRNA levels for extracellular matrix components in WKY and SHRSP after 10 weeks of treatment with vehicle, TCV-116 or enalapril.* The ordinate in (**A**), (**B**) and (**C**) represents type I collagen, type III collagen and fibronectin mRNA levels, respectively, corrected for GAPDH mRNA levels. The mean value of each mRNA corrected for GAPDH mRNA in WKY is represented as 1. W, V, T (0.1), T (1), T (10) and E (10) indicate the same abbreviations as Figure 1. Each bar represents the mean ± SE. Each group included 5 animals. * $P < 0.01$ vs. V (vehicle-treated SHRSP).

participates in renal fibrosis in SHRSP, we measured mRNA levels for extracellular matrix components in the interstitium (types I and III collagen and fibronectin), and the basement membrane (type IV collagen). Northern blot analysis indicated that the renal mRNA levels for the above-mentioned extracellular matrix components were significantly elevated in SHRSP, compared with WKY. Furthermore, our present histological observations by Azan staining of renal sections showed the increased interstitial collagen fibers (composed of types I and III collagen) and thickening of tubular basement membranes (mainly composed of type IV collagen) in SHRSP (Table 2), thereby supporting that the increased expressions of collagen mRNAs led to the increased collagen proteins. Thus, TGF-β1 may play an important role in nephrosclerosis in SHRSP, by stimulating the gene expression of extracellular matrix components in the interstitium and the basement membrane.

Accumulating evidence show that renin-angiotensin system is responsible for the pathophysiology of various renal diseases [6–15, 43]. Very recently, using the methods of *in vitro* autoradiography [44], reverse transcription-polymerase chain reaction (RT-PCR) [45] or *in situ* hybridization [46, 47], several groups of investigators have reported that AT1 receptor is widely distributed in the glomeruli, blood vessels and tubules within the kidney, although the pathophysiological role of renal AT1 receptor is not

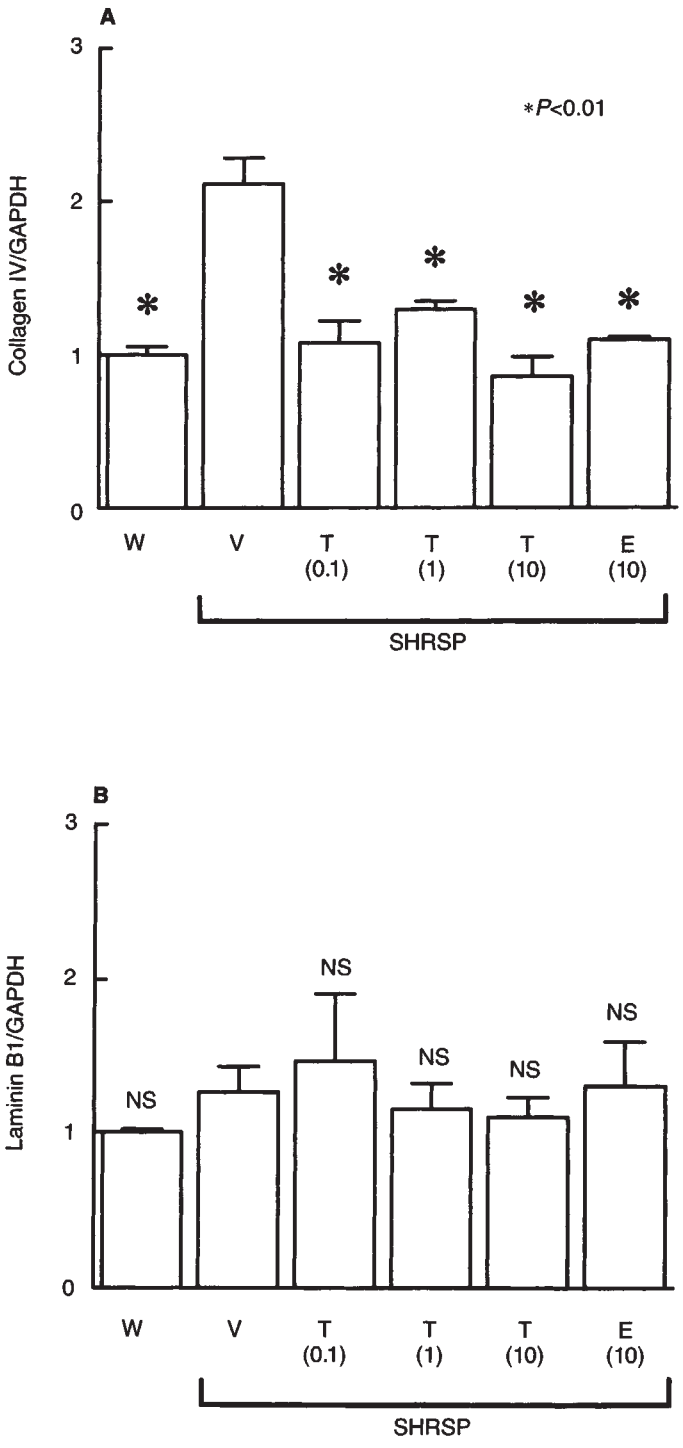

Fig. 6. *mRNA levels for renal extracellular matrix components in the basement membrane in WKY and SHRSP after 10 weeks of treatment.* The ordinate in (**A**) and (**B**) represents type IV collagen and laminin B1 chain mRNA levels, respectively, corrected for GAPDH mRNA levels. The mean value of each mRNA corrected for GAPDH mRNA in WKY is represented as 1. W, V, T (0.1), T (1), T (10) and E (10) indicate the same abbreviations as Figure 1. Each bar represents the mean ±SE. Each group included 5 animals. * $P < 0.01$ vs. V (vehicle-treated SHRSP). NS, not significant vs. V (vehicle-treated SHRSP).

fully determined. Furthermore, *in vitro* study using cultured vascular smooth muscle cells indicates that Ang II stimulates TGF-β1 gene expression, which play an important role in cellular hypertrophy or proliferation [48]. However, the possible contribution of Ang II to TGF-β1 expression *in vivo* is still unclear.

In the present study, we investigated whether or not AT1 receptor *in vivo* is involved in renal TGF-β1 gene expression in SHRSP. Of note are the observations that in spite of no decrease in blood pressure, 10 weeks of treatment with 0.1 mg/kg/day of TCV-116 significantly inhibited the gene expression of renal TGF-β1 in SHRSP, accompanied by the significant decrease in extracellular matrix gene expressions. These inhibitory effects were associated with the renal protective effects, as shown by the significant decrease in renal weight, urinary protein and albumin excretion, BUN, and plasma creatinine concentrations. Furthermore, in separate experiments, we examined the effect of TCV-116 on the gene expression of TGF-β1 and extracellular matrix proteins in the kidney of deoxycorticosterone acetate (DOCA)-salt hypertensive rats, and found that three weeks of treatment with TCV-116, without lowering blood pressure throughout the treatment, significantly reduced renal TGF-β1 mRNA levels of DOCA-salt hypertensive rats, being accompanied by the suppression of extracellular matrix gene expressions [49]. Thus, the present study, taken together with our data on DOCA-salt hypertensive rats [49], support the notion that the inhibition of renal TGF-β1 gene expression by TCV-116 is not mediated by systemic blood pressure but by the direct inhibition of renal AT1 receptor. AT1 receptor may be responsible for the accumulation of extracellular matrix in the kidney of SHRSP, by stimulating TGF-β1 gene expression. However, the suppression of renal types I and III collagen mRNA levels by TCV-116 in SHRSP was not complete but partial, thereby indicating that not only TGF-β1 but also other unknown factors play an important role in the enhancement of these collagen gene expressions in SHRSP.

In the present study, TCV-116 tended to improve renal interstitial fibrosis in SHRSP, as estimated by the score index of renal histology, although there was no statistically significant improvement. However, TCV-116 at all three doses significantly decreased not only urinary protein excretion but also renal weight, BUN and plasma creatinine levels in SHRSP, thereby confirming that TCV-116 significantly prevented the development of renal injury in SHRSP. Furthermore, in DOCA-salt hypertensive rats, the decrease in renal collagen mRNA levels by TCV-116 was associated with the significant improvement of renal interstitial fibrosis [score index of interstitial fibrosis: 1.1 ± 0.2 (Vehicle group, $N = 14$) vs. 0.6 ± 0.2 (TCV-116 treatment, $N = 14$), $P < 0.05$] [49]. In addition, our present histological analysis by the score index allows for semi-quantitative analysis, but does not for quantitative analysis. These findings, taken together with our results on the partial normalization of types I and III collagen mRNAs by TCV-116, suggest that the technical limitation of our histological analysis might not allow us to detect the partial normalization of interstitial collagen accumulation by TCV-116 in SHRSP. However, the present study cannot exclude the possibility that the decrease in collagen mRNA levels might not lead to the decrease in interstitial collagen accumulation, because the accumulation of interstitial collagen in the tissue may be affected not only by the transcriptional rate of collagen gene but also by the

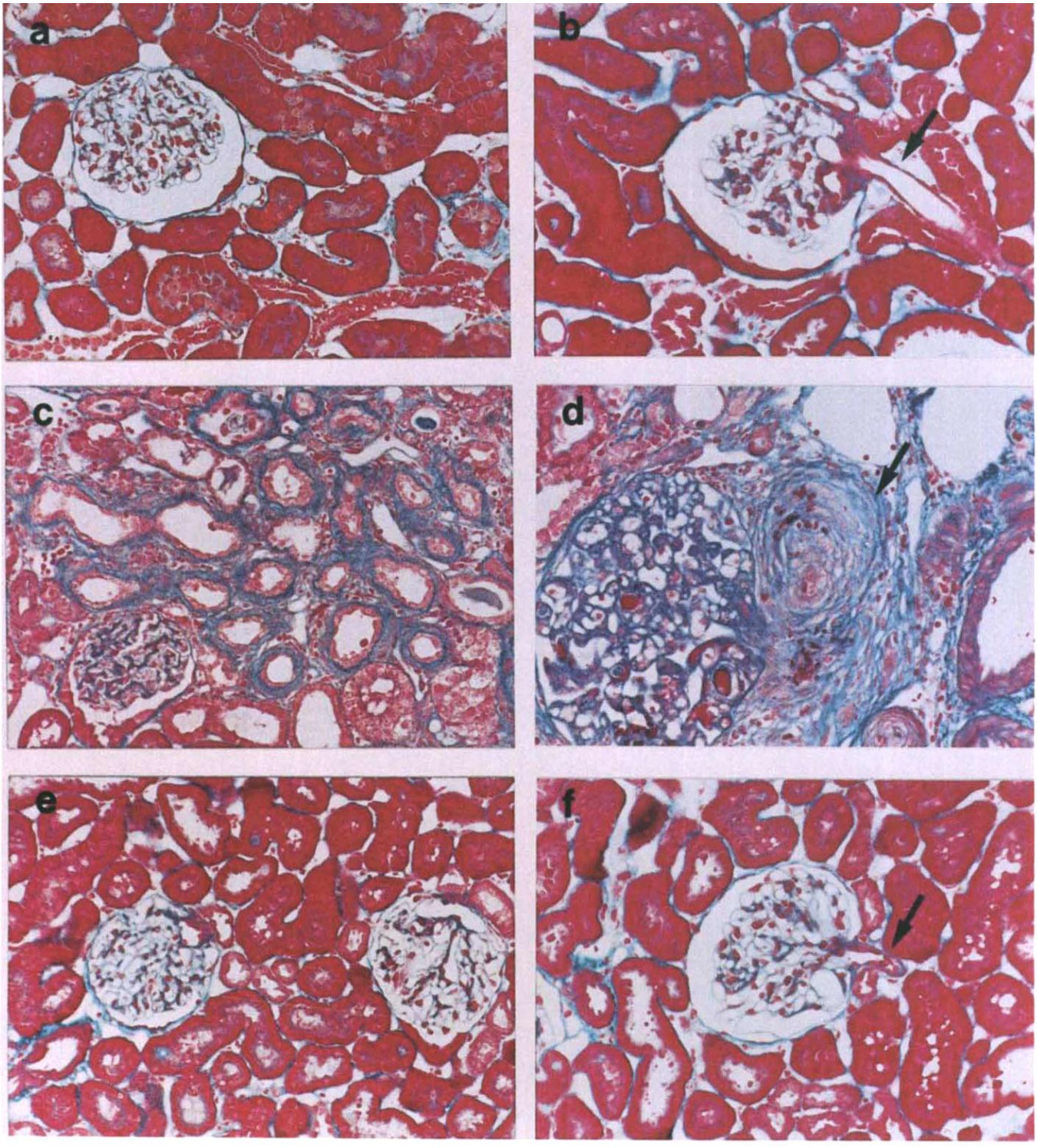

Fig. 7. *Light microscopy of renal cortex from WKY (***a** *and* **b***), vehicle-treated SHRSP (***c** *and* **d***) and TCV-116(10 mg/kg/day)-treated SHRSP (***e** *and* **f***).* The arrows in (b), (d) and (f) show arteriole. In SHRSP treated with vehicle, interstitial fibrosis were observed (c), and prominent hypertrophy of the arteriole was observed (the arrow in d). On the other hand, in TCV-116 (10 mg/kg/day)-treated SHRSP, the renal tubules (e) and the arteriole (the arrow in f) were normal. No interstitial fibrosis was found in 2 of 5 SHRSP treated with TCV-116 (10 mg/kg/day). Azan stain; × 225 (a, c, e), × 300 (b, d, f).

translational rate, posttranslational modification and the degradation rate. Further study is needed to elucidate the regulatory mechanism of collagen accumulation.

Previous reports support that ACE inhibitor or AT1 receptor antagonist reduces intrarenal, especially intraglomerular, blood pressure via blocking the local renin-angiotensin system, and the

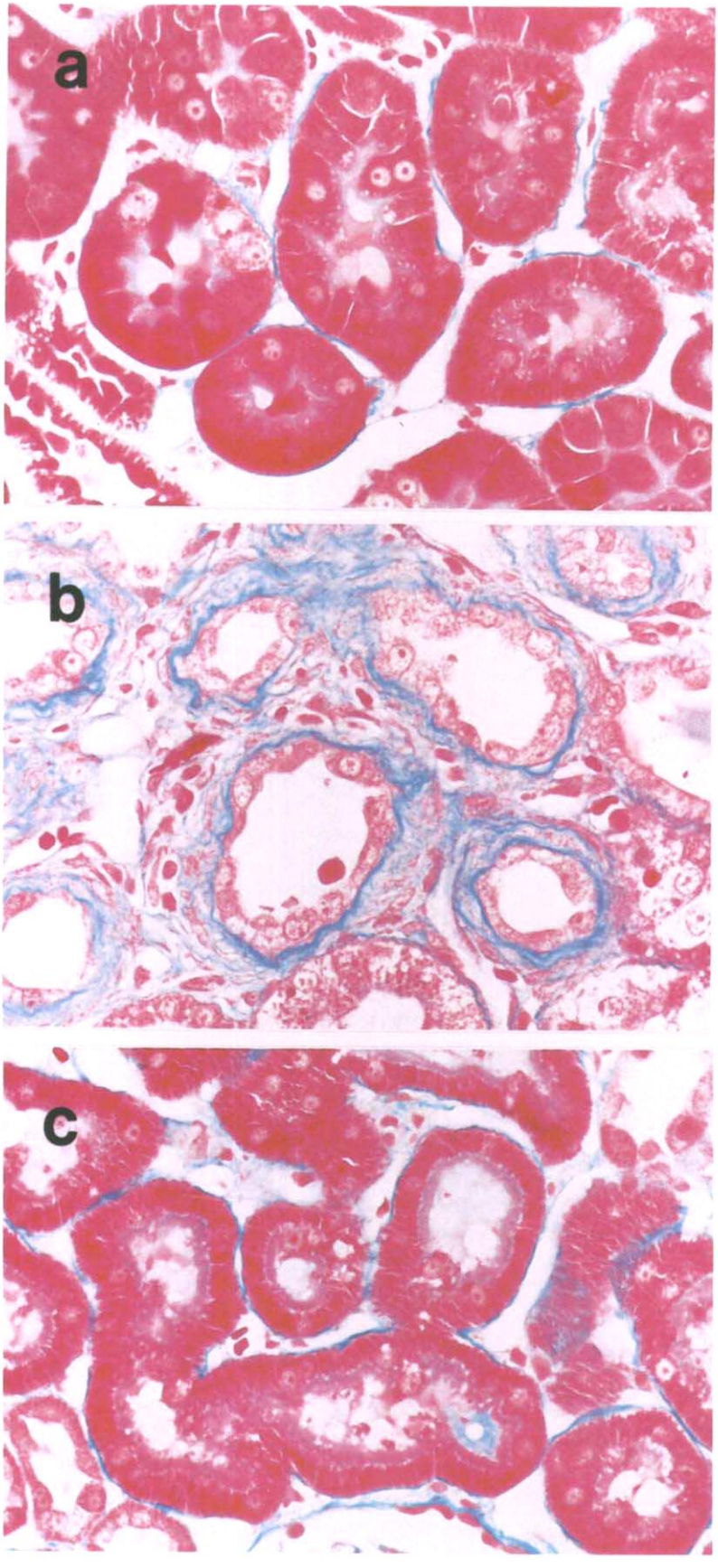

Fig. 8. *High magnifications of light microscopy of renal sections from WKY(***a***), vehicle-treated SHRSP (***b***) and TCV-116 (10 mg/kg/day)-treated SHRSP (***c***).* (b) Atrophy of the epithelium and hypertrophy of the tubular basement membranes were prominent in vehicle-treated SHRSP. (c) TCV-116 (10 mg/kg/day) treatment significantly improved the above-mentioned renal lesions. Azan stain; × 530.

Table 2. Score index of the kidney from SHRSP after 10 weeks of treatment with vehicle, TCV-116 (0.1 mg/kg/day), TCV-116 (10 mg/kg/day) or enalapril (10 mg/kg/day)

	Vehicle	TCV (0.1 mg)	TCV (10 mg)	Enalapril (10 mg)
Glomerulus				
Fibrinoid necrosis	1.4 ± 0.4	1.2 ± 0.6	0 ± 0[a]	0.2 ± 0.2[a]
Renal tubules				
Atrophy	2.2 ± 0.2	1.8 ± 0.4	0.4 ± 0.2[b]	1.8 ± 0.6
Hypertrophy of basement membrane	1.8 ± 0.2	1.2 ± 0.4	0.2 ± 0.2[b]	1.2 ± 0.5
Hyaline cast with dilatation	2.6 ± 0.2	2.2 ± 0.5	0.8 ± 0.2[b]	1.8 ± 0.6
Arteriole				
Fibrinoid necrosis	1.6 ± 0.2	1.2 ± 0.6	0 ± 0[b]	0.6 ± 0.4
Hypertrophy	1.2 ± 0.5	1.4 ± 0.6	0 ± 0[a]	0.8 ± 0.4
Artery				
Hyalinoid hypertrophy of intima	1.2 ± 0.4	0.2 ± 0.2	0 ± 0[a]	0 ± 0[a]
Interstitial fibrosis	1.4 ± 0.4	1.2 ± 0.4	0.8 ± 0.4	1.2 ± 0.5

The values represent the mean ±SE. Each group included 5 animals. Abbreviations are: SHRSP, stroke-prone spontaneously hypertensive rats; Vehicle, treatment with vehicle; TCV (0.1 mg), treatment with TCV-116 (0.1 mg/kg/day); TCV (10 mg), treatment with TCV-116 (10 mg/kg/day); Enalapril (10 mg), treatment with enalapril (10 mg/kg/day).
[a] $P < 0.05$, [b] $P < 0.01$ vs. vehicle-treated SHRSP

renal beneficial effects of these drugs may be at least in part due to the improvement of intrarenal hemodynamics [8, 9, 11, 12]. Riser et al, who examined the effects of glomerular capillary distension and mesangial cell stretching on extracellular matrix synthesis, have suggested that capillary expansion and stretching of mesangial cells by glomerular hypertension provoke increased extracellular matrix production [50]. Therefore, it cannot be ruled out that the suppression of TGF-β1 or extracellular matrix expressions by TCV-116 might be mediated by the intrarenal hemodynamic improvement.

In vivo inhibition of Ang II production by ACE inhibitors increases the biosynthesis and secretion of renal renin [31, 51], because Ang II has a negative feedback effect on renal renin expression. In the present study, 10 mg/kg/day of TCV-116 dramatically elevated renal renin mRNA and plasma renin levels, thereby indicating the major role of AT1 receptor on juxtaglomerular cells [47] in negative feedback regulation of renin expression. Very interestingly, despite the marked reduction of renal TGF-β1 and extracellular matrix components, treatment with 0.1 mg/kg/day of TCV-116 did not alter renal renin mRNA and plasma renin levels. These observations reveal that this AT1 receptor antagonist can inhibit renal gene expression of TGF-β1 and extracellular matrix components at lower concentrations than those that stimulate renin gene expression in SHRSP.

Previously we reported that circulating prorenin (the biosynthetic precursor of renin) is activated within the kidney but not in the blood circulation [52], and suggested that intrarenal activation of prorenin may be enhanced in SHRSP [53]. Moreover, we have obtained the evidence that renal angiotensin II levels, measured by HPLC separation coupled with specific radioimmunoassay, are increased at the malignant phase of SHRSP [54]. These findings support that intrarenal renin-angiotensin system may be activated in SHRSP. In the present study, as shown in Figure 2, renal renin mRNA and plasma renin were increased in SHRSP compared with WKY, indicating the increased synthesis of renal renin in SHRSP. All these findings allow us to assume that the enhancement of renal TGF-β1 gene expression in SHRSP may be due to the activation of intrarenal renin-angiotensin system. Thus, the potent suppressive effect of TCV-116 on TGF-β1 gene expression in SHRSP appears to be in part explained by the inhibition of intrarenal renin-angiotensin system. However, the possible contribution of circulating renin-angiotensin system to TGF-β1 gene expression cannot be excluded, because plasma active renin levels were increased in SHRSP.

The renal source of increased TGF-β1 mRNA in SHRSP was not investigated in this study. Previous report on immunohistochemical localization of TGF-β1 shows that immunoreactive TGF-β1 protein is mainly localized in tubular epithelial cells of normal rat kidney [55]. However, in the kidney of rats with experimental glomerulonephritis, immunoreactive TGF-β1 protein is significantly expressed in glomerular cells as well as epithelial cells [56]. Very recently, using northern blot analysis and in situ hybridization technique, Yamamoto et al have examined the gene expression of renal TGF-β1 in rats with progressive kidney fibrosis which was produced by two injections of anti-mesangial serum [57]. These investigators have found that TGF-β1 mRNA is increased in glomerular cells and interstitial cells in the kidney of such rats, which leads to the increased TGF-β1 protein in glomerular cells and interstitial cells, followed by the development of glomerulosclerosis and tubulointerstitial fibrosis. Therefore, they have proposed that the elevated gene expression of TGF-β1 contributes to the development of glomerulosclerosis and tubulointerstitial fibrosis [57]. However, histological study, using *in situ* hybridization and immunohistochemical techniques, is needed to elucidate the exact source of the increased TGF-β1 expression in SHRSP.

TGF-β1 is secreted mainly in an inactive latent form, which has no biological function including extracellular matrix production [58, 59], thereby suggesting that the activation of the latent form of TGF-β1 may be a crucial regulatory step to determine active TGF-β1 levels. It has been suggested that platelets may play a role in renal glomerular lesions by activating latent TGF-β1 [60]. On the other hand, the investigation on anti-basement membrane glomerulonephritis in the rabbit suggests that some renal cells may secrete directly active TGF-β1 [22]. Thus, the mechanism of activation of latent TGF-β1 *in vivo* remains to be elucidated. The present study did not allow us to determine whether or not the increased TGF-β1 mRNA levels lead to the secretion of the active form of TGF-β1.

In conclusion, we obtained evidence that the gene expression of renal TGF-β1 is increased at the malignant phase of SHRSP, which may contribute to the increased gene expression of renal extracellular matrix components in the interstitium and the basement membrane. AT1 receptor seems to be involved in the increased TGF-β1 expression in SHRSP. Thus, we propose that independent of hypertension, renal AT1 receptor may play an important role in nephrosclerosis in SHRSP.

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Reprint requests to Shokei Kim, M.D., Department of Pharmacology, Osaka City University Medical School, 1-4-54 Asahimachi, Abeno, Osaka 545, Japan.

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