CS-866, a New Angiotensin II Type 1 Receptor Antagonist, Ameliorates Glomerular Anionic Site Loss and Prevents Progression of Diabetic Nephropathy in Otsuka Long-Evans Tokushima Fatty Rats

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Abstract

Background: Diabetic nephropathy is a leading cause of end-stage renal disease in industrialized countries. Previous studies have documented that angiotensin converting enzyme (ACE) inhibitors consistently reduce albuminuria and retard the progression of diabetic nephropathy. However, the involvement of angiotensin II in diabetic nephropathy is not fully understood.
Materials and Methods: In this study we compared the effects of CS-866, a new angiotensin II type 1 receptor antagonist, to that of an ACE inhibitor, temocapril hydrochloride, on the development and progression of diabetic nephropathy using Otsuka Long-Evans Tokushima fatty rats, a type II diabetes mellitus model animal.
Results: High doses of CS-866 or temocapril treatment were found to significantly improve urinary protein and β_2-microglobulin excretions in diabetic rats. In electron microscopic analysis, loss of glomerular anionic sites, one of the causes of glomerular hyperpermeability in diabetic nephropathy, was found to be significantly prevented by CS-866 treatment. Light microscopic examinations revealed that both treatments ameliorated glomerular sclerosis and tubulointerstitial injury in diabetic rats. Furthermore, high doses of CS-866 or temocapril treatment significantly reduced the positive stainings for transforming growth factor-β (TGF-β), vascular endothelial growth factor, and type IV collagen in glomeruli of diabetic rats.
Conclusions: These results indicate that intrarenal angiotensin II type 1 receptor activation plays a dominant role in the development and progression of diabetic nephropathy. Our study suggests that CS-866 represents a valuable new drug for the treatment of diabetic patients with nephropathy.

Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease and can account for disabilities and the high mortality rate in patients with this disease (1,2). Although the mechanisms for the development and progression of diabetic nephropathy are not fully understood, a disturbance of the charge barrier of the glomerular basement membrane is considered to be involved in the pathogenesis of microalbuminuria in the early phase of diabetic nephropathy (3,4). The loss of glomerular anionic sites are followed by mesangial expansion and tubular injury, which in concert may deteriorate renal function in diabetes.

There is a growing body of evidence that angiotensin converting enzyme (ACE) inhibition

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consistently reduces albuminuria and retards the progression of glomerulosclerosis in diabetes (5–7). However, ACE, not a specific enzyme, has a capacity to degrade bradykinin, a potent stimulator of nitric oxide and prostacyclin (8). Therefore, the main involvement of angiotensin II in the pathogenesis of diabetic nephropathy remains to be elucidated. Recently, the development of a specific angiotensin II type 1 receptor antagonist made it possible to differentiate the pathophysiologic properties of angiotensin II from that of bradykinin in diabetic nephropathy. In this study, we compared the effects of long-term administration of CS-866, a new angiotensin II type 1 receptor antagonist, to that of an ACE inhibitor, temocapril hydrochloride, on the development and progression of diabetic nephropathy using Otsuka Long-Evans Tokushima fatty (OLETF) rats, a type II diabetes mellitus model animal. The effects of these drugs on urinary protein and β_2-microglobulin excretions, anionic sites of glomerular basement membrane, and glomerular and tubular morphology were investigated to isolate

the role of the blocking of the angiotensin II type 1 receptor from other factors associated with ACE inhibitor treatment.

Materials and Methods

Drugs

CS-866 and temocapril hydrochloride were generously provided by Sankyo Co. Ltd (Tokyo, Japan).

Animals

Forty-six male OLETF rats, a type II diabetes mellitus model established in 1990 at the Tokushima Research Institute, were obtained from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Eleven male Long-Evans Tokushima Otsuka (LETO) rats served as normal controls. The OLETF rats at aged 20 weeks were randomly divided into four groups; [1] no treatment, [2] treatment with chow containing 0.001% of CS-866 (about 0.3 mg CS-866/d), [3] treatment with chow containing 0.01% of CS-866 (about 3 mg CS-866/d), and [4] treatment with chow containing 0.01% of temocapril (about 3 mg temocapril/d). Each animal was followed to 52 weeks. Rats were kept at approximately 22 ± 3 °C and $50 \pm 20\%$ humidity under a 12-hr light/dark cycle. Experiments in this study were performed in accordance with Guiding Principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society. The ethical committee of New Drug Development Research Center Inc. (Hokkaido, Japan) approved this study.

Clinical Parameters

Animals were placed in metabolic cages and their 24-hr urinary protein and β_2-microglobulin excretions were determined at 4-week intervals. Blood samples were taken from jugular veins at 4-week intervals for the measurement of blood glucose, total cholesterol, and triglycerides. Systolic and diastolic blood pressure (BP) were monitored by the tail-cuff method (UR-5000, Ueda Manufactory, Tokyo, Japan) every 8 weeks.

Electron Microscopic Analysis

At 52 weeks of age, all animals were deeply anesthetized with an injection of barbital solution, then each kidney was isolated. The renal cortex was cut into small pieces, and then immersed for 30 min in 0.5% polyethyleneimine (PEI) (molecular weight 1800; Wako Pure Chemical Industries Ltd., Osaka, Japan) solution, pH 7.4, according to the method of Schuer et al. (9). After immersion, the blocks were washed with 0.1 mmol/L cacodylate-buffered solution (pH 7.4) and fixed with 0.1% glutaraldehyde and 2% phosphotungstic acid solution (pH 7.4). The blocks were then embedded in Qetol-812 (Nisshin EM Co., Inc., Tokyo, Japan) and ultrathin sections were examined by JEM-100SX transmission election microscopy (JEOL, Tokyo, Japan). Three glomeruli were chosen from each rat, and then three independent areas of each glomeruli were investigated. The mean numbers of PEI particles were shown per 1000 nm of the lamina rara externa of the glomerular basement membrane.

Light Microscopic Analysis

At 52 weeks of age, kidney tissues were fixed in carnoy's fixative and then embedded in paraffin. Tissues were stained with hematoxylin and eosin and periodic acid Schiff. Glomerular sclerosis was assessed using a semiquantitative score previously described (10,11): grade 0, normal appearance; grade 1, sclerotic area less than or equal to 25% of total glomerulus area; grade 2, sclerosis of 25–50% of the area; grade 3, sclerosis of more than 50% of the area but not global; grade 4, global sclerosis. A glomerular sclerosis index was calculated by multiplying the number of glomeruli with a sclerosis score of 1 by one,

Table 1. Comparison of body weight and serum variables at week 52 and BP at week 50 in OLETF and LETO rats

	OLETF rats				LETO rats
	No treatment ($n = 12$)	CS-866 (0.001%) ($n = 11$)	CS-866 (0.01%) ($n = 12$)	Temocapril ($n = 11$)	($n = 11$)
Body weight (g)	748.6 ± 33.0	$865.6 \pm 15.3^{\#\#}$	792.3 ± 10.5	766.7 ± 13.9	$546.8 \pm 6.5^{**}$
Blood glucose (mmol/L)	10.35 ± 0.38	10.53 ± 0.54	10.49 ± 0.38	10.16 ± 0.41	$7.54 \pm 0.15^{**}$
Systolic BP (mmHg)	153.4 ± 4.3	$138.9 \pm 5.0^{\#}$	$132.1 \pm 3.3^{\#\#}$	$124.0 \pm 3.2^{\#\#}$	$128.5 \pm 4.7^{**}$
Diastolic BP (mmHg)	117.3 ± 4.9	112.6 ± 4.7	$100.8 \pm 2.1^{\#\#}$	$100.1 \pm 3.3^{\#\#}$	$98.2 \pm 3.4^{**}$
Cholesterol (mmol/L)	4.37 ± 0.23	3.97 ± 0.20	$2.42 \pm 0.13^{\#\#}$	$2.03 \pm 0.07^{\#\#}$	$1.76 \pm 0.04^{**}$
Triglyceride (mmol/L)	2.63 ± 0.37	2.80 ± 0.27	$1.26 \pm 0.14^{\#}$	$0.96 \pm 0.08^{\#\#}$	$0.28 \pm 0.03^{**}$

$**p < 0.01$ compared with nontreated OLETF rats (F-t test).
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with nontreated OLETF rats (Dunnett's t-test).
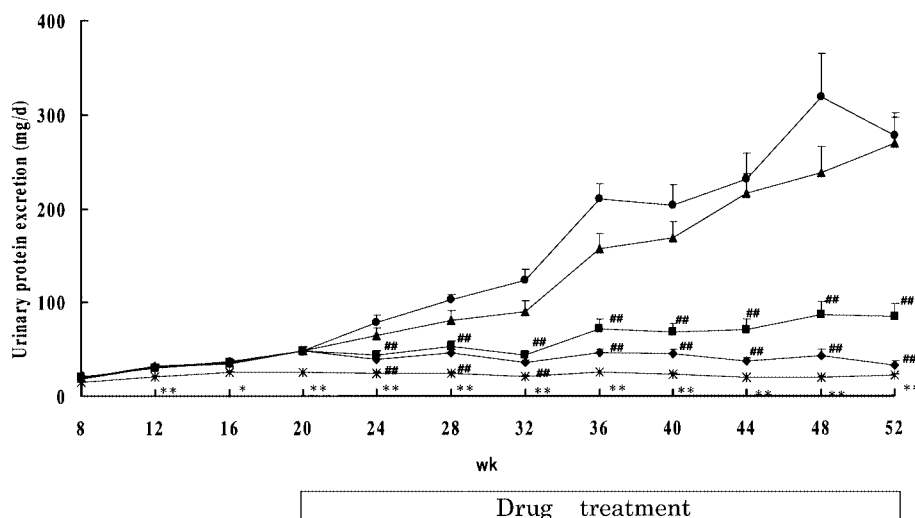
Fig. 1. Effects CS-866 or temocapril treatment on urinary protein excretions in OLETF rats. High doses of CS-866 or temocapril significantly reduced the elevation of urinary protein excretion in OLETF rats. Nontreated OLETF rats (●); 0.001% CS-866–treated OLETF rats (▲); 0.01% CS-866–treated OLETF rats (■); temocapril-treated OLETF rats (◆); LETO rats (*). *$p <$ 0.05. **$p <$ 0.01 compared with nontreated OLETF rats (F-t test). ##$p <$ 0.01 compared with nontreated OLETF rats (Dunnett's t-test).

2 by two, 3 by three, and 4 by four (12). These values were summed and divided by the number of glomeruli assessed. The index of tubular atrophy was assessed using a semiquantitative score: grade 0, normal appearance; grade 1, atrophic area less than or equal to 12.5% of total tubules; grade 2, atrophy of 12.5–25% of the total tubules; grade 3, atrophy of 25–50% of the total tubules; grade 4, atrophy of more than 50% of the total tubules. The index of tubular atrophy was calculated by the same method as that of glomerular sclerosis.

Immunohistochemistry

Immunohistochemical staining was performed as described previously (13). Briefly, for transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) immunohistochemical staining, 50 glomeruli were randomly chosen and the number positively stained was counted. Immunohistochemical staining for type IV collagen was semiquantitative by using a scale of 0–2 (0 = no staining, 2 = maximum staining) as described previously (13) and the average score of 100 randomly selected glomeruli was calculated. Negative control staining without antibodies yielded no positive signals in our immunohistochemical analysis. All the histologic examinations were done by observers who were masked to the study groups of origin.

Statistical Analysis

The F-t test and Dunnett's t-test were used for statistical analysis. Values are expressed as mean $\pm$ SEM. p values of $<$ 0.05 were considered statistically significant.

Results

Clinical Characteristics of Animals

Table 1 shows the clinical characteristics of the 46 OLETF rats and 11 LETO rats. OLETF rats were

subdivided into four groups according to the type of treatment. OLETF rats had higher body weights and food consumption than LETO rats and their blood glucose levels were moderately elevated at 12 weeks,
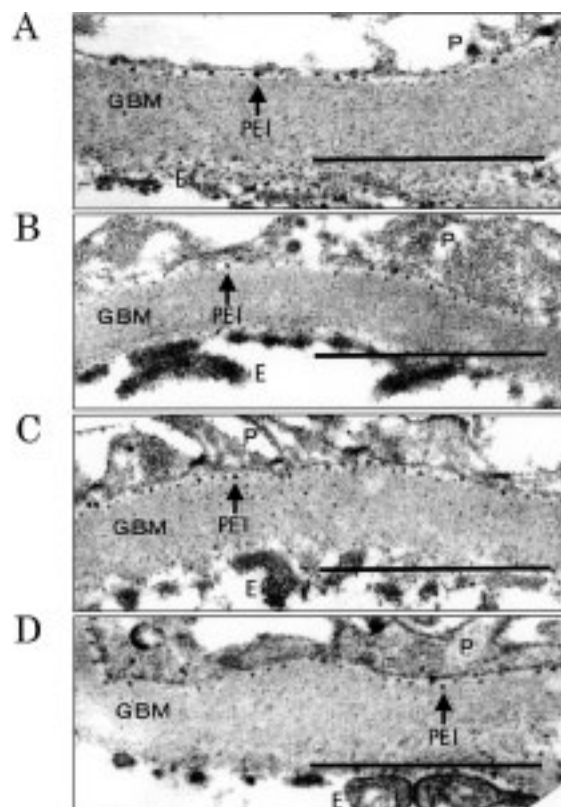

Fig. 2. Electron microscopic photographs of glomeruli. Anionic sites in the glomerular basement membrane of OLETF rats (**A**) No treatment.(**B**) Low-dose (0.001%) CS-866 treatment. (**C**) High-dose (0.01%) CS-866 treatment. (**D**) Temocapril treatment. P, podocyte; E, endothelium; GBM, glomerular basement membrane. Bar, 1000 nm.

Table 2. Effects of CS-866 or temocapril on anionic site loss in glomerular basement membrane

| | OLETF rats | | | | LETO rats (*n* = 11) |
	No treatment (*n* = 12)	CS-866 (0.001%) (*n* = 11)	CS-866 (0.01%) (*n* = 12)	Temocapril (*n* = 11)	
Anionic sites	8.8 ± 0.7	9.0 ± 0.6	12.6 ± 0.8[##]	10.6 ± 0.4	12.4 ± 0.7**

Anionic sites of glomeruli are significantly decreased in OLETF rats compared with LETO rats. A high dose of CS-866 significantly restored the loss of anionic sites. **$p < 0.01$ compared with nontreated OLETF rats (F-*t* test). [##]$p < 0.01$ compared with nontreated OLETF rats (Dunnett's *t*-test).

increased with time, and remained elevated until the end of the experiment. CS-866 or temocapril treatment did not affect the levels of blood glucose, food consumption, or water intake. However, body weight in low-dose CS-866 groups are significantly higher than that in OLETF rats. Systolic and diastolic BP were significantly higher in OLETF rats. High-dose CS-866 (0.01%) and temocapril treatments were equally potent in reducing systolic and diastolic BP during the experiments. Serum levels of total cholesterol and triglyceride in OLETF rats gradually increased from 36 weeks, but the elevations were significantly prevented by a high dose of CS-866 or temocapril.

Effects on Proteinuria

As shown in Figure 1, urinary protein excretion levels in OLETF rats were not different from those of LETO rats until 12 weeks. However, proteinuria markedly increased from 20 weeks in OLETF rats and remained elevated until the end of the experiment. A high dose of CS-866 or temocapril significantly reduced the increase in urinary protein excretions in OLETF rats. The proteinuria-reducing effect
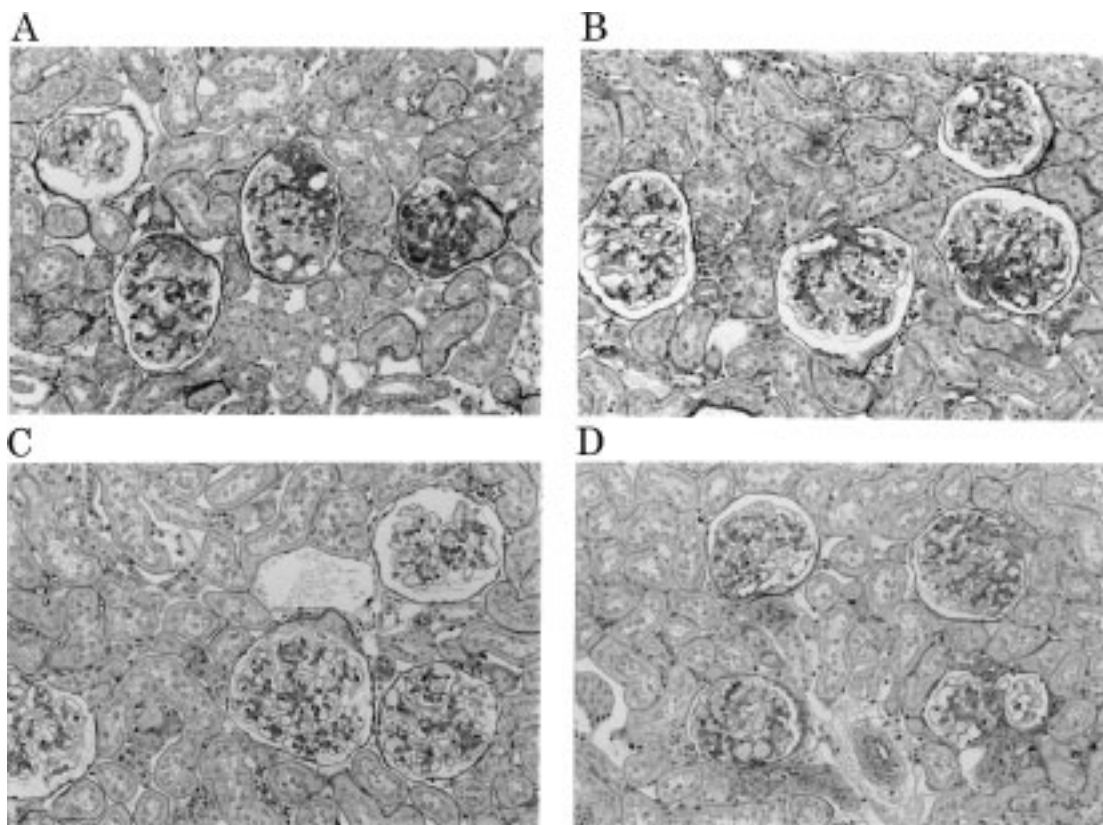

Fig. 3.   Periodic acid-Schiff staining of glomeruli in OLETF rats. (A) No treatment. **(B)** Low-dose (0.001%) CS-866 treatment. **(C)** High-dose (0.01%) CS-866 treatment. **(D)** Temocapril treatment.

Table 3.   Glomerular sclerosis index in OLETF rats

	No treatment (n = 12)	CS-866 (0.001%) (n = 11)	CS-866 (0.01%) (n = 12)	Temocapril (n = 11)
Sclerosis index	1.82 ± 0.05	1.73 ± 0.03	1.17 ± 0.05##	1.47 ± 0.05##

High doses of CS-866 or temocapril significantly decreased the glomerular sclerosis index in OLETF rats.
##$p < 0.01$ (Dunnett's t-test).

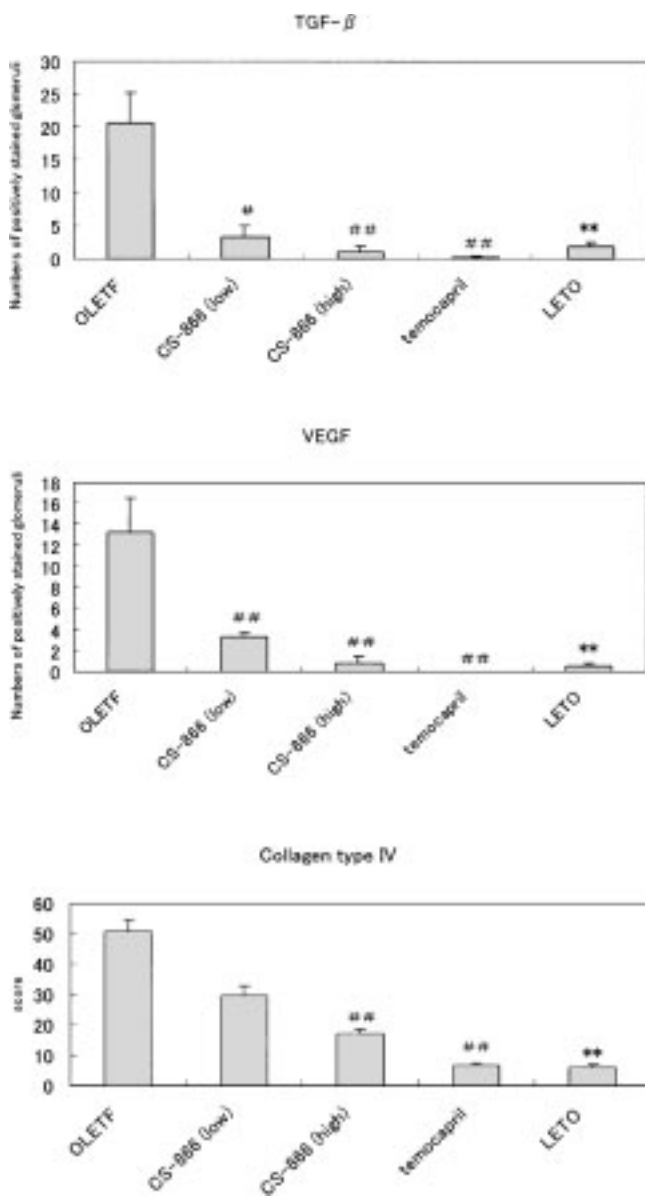**Fig. 4.   Quantitative representation of immunostainings for TGF-β, VEGF, and collagen type IV in glomeruli.** **$p < 0.01$ compared with nontreated OLETF rats (F-t test). #$p < 0.05$ and ##$p < 0.01$ compared with nontreated OLETF rats (Dunnett's t-test).

of temocapril was a little greater than that of a high dose of CS-866; however, there were no significant differences in the extent of proteinuria between the two groups during the experiment.

Effects on Anionic Sites

Loss of anionic sites on glomerular basement membrane is initially responsible for the development of albuminuria in early phase of diabetic nephropathy (3,4). Therefore, we next investigated whether these treatments could restore the decrease of the charge barrier of glomeruli in OLETF rats. As shown in Figure 2 and Table 2, a decrease in numbers of anionic sites in the glomerular basement membrane in OLETF rats was significantly prevented by a high dose of CS-866.

Effects on Glomerular Morphology

As shown in Figure 3, a high dose of CS-866 or temocapril significantly improved glomerulosclerosis in OLETF rats. Although both treatments improved the index of sclerosis of glomeruli, the protective effect of a high dose of CS-866 was significantly higher than that of temocapril (Table 3).

Effects on Immunohistochemical Staining

As shown in Figures 4 and 5, increased TGF-β, VEGF and collagen type IV stainings were observed in the glomeruli of OLETF rats compared with LETO rats. High-dose CS-866 or temocapril was found to significantly reduce the positive stainings for TGF-β, VEGF, and collagen type IV in OLETF rats.

Effects on Tubular Function and Morphology

Urinary β_2-microglobulin excretion levels are known to be one of the representative markers for tubular injury in diabetes (14); therefore we studied whether tubular injury was ameliorated by either CS-866 or temocapril treatment. As shown in Figure 6, urinary β_2-microglobulin excretions in nontreated OLETF rats dramatically increased from 20 weeks and remained higher compared with those of LETO rats during the experiment. High-dose CS-866 or temocapril treatment was found to significantly decrease urinary β_2-microglobulin excretions in OLETF rats (Fig. 6). Both these treatments also

improved the index of tubular atrophy in OLETF rats (Table 4).

Discussion

We found in the present study that CS-866, a new angiotensin II type 1 receptor antagonist, prevented the elevation of urinary protein excretions in a dose-dependent manner in OLETF rats. Because equihy-potensive doses of CS-866 (high dose) and temo-capril equally ameliorated the elevation of proteinuria (Table 1, Fig. 1), angiotensin II type 1 receptor interaction may be mainly involved in the development of proteinuria in diabetic nephropathy. Recently, Ravid et al. (15) demonstrated that use of an ACE inhibitor reduced the extent of proteinuria and slowed the decline of renal function in normotensive type II diabetic patients with normoalbuminuria over a 6-year follow-up period. These observations suggest that intrarenal angiotensin II type 1 receptor activation may initially participate in the development and progression of diabetic nephropathy. Recently, Taniguchi et al. (16) showed that amlodipine failed to improve nephropathy of OLETF rats despite lowering of blood pressure. These studies support the concept that renoprotective effects of CS-866 and temocapril would be related to specific actions on the renin-angiotensin system rather than to undifferentiated lowering of blood pressure.

Microalbuminuria in the early phase of diabetic nephropathy is considered to be caused initially by a disturbance of the charge barrier of the glomerular basement membrane, in other words, the loss of anionic sites in glomeruli (3,4). Heparan sulfate proteoglycan, a major component of anionic sites in the glomerular basement membrane, is depolymeralized and degraded by reactive oxygen species (ROS) (17,18). Because hyperglycemia-induced angiotensin II type 1 receptor activation is found to be a predominant source of ROS in mesangial cells (19,20), CS-866 may improve proteinuria through preservation of anionic sites in glomeruli by decreasing ROS generation. In support of this, angiotensin II was shown to decrease the production of heparan sulfate proteoglycan by mesangial cells through interaction with type 1 receptor (21). Hyperglycemia itself is also reported to suppress the mesangial synthesis and secretion of heparan sulfate proteoglycan (22,23). This may be one reason that CS-866 treatment did not completely inhibit the elevation of proteinuria in OLETF rats. In our present study, temocapril treatment does not improve anionic site loss significantly, although the level of proteinuria in the group of temocapril is significantly decreased compared to that in the untreated group. These results suggest that temocapril treatment could improve proteinuria in OLETF rats through other mechanism, such as degradation of bradykinin.

The present study further showed that CS-866 prevented the progression of glomerulosclerosis in OLETF rats. Along with others we have recently
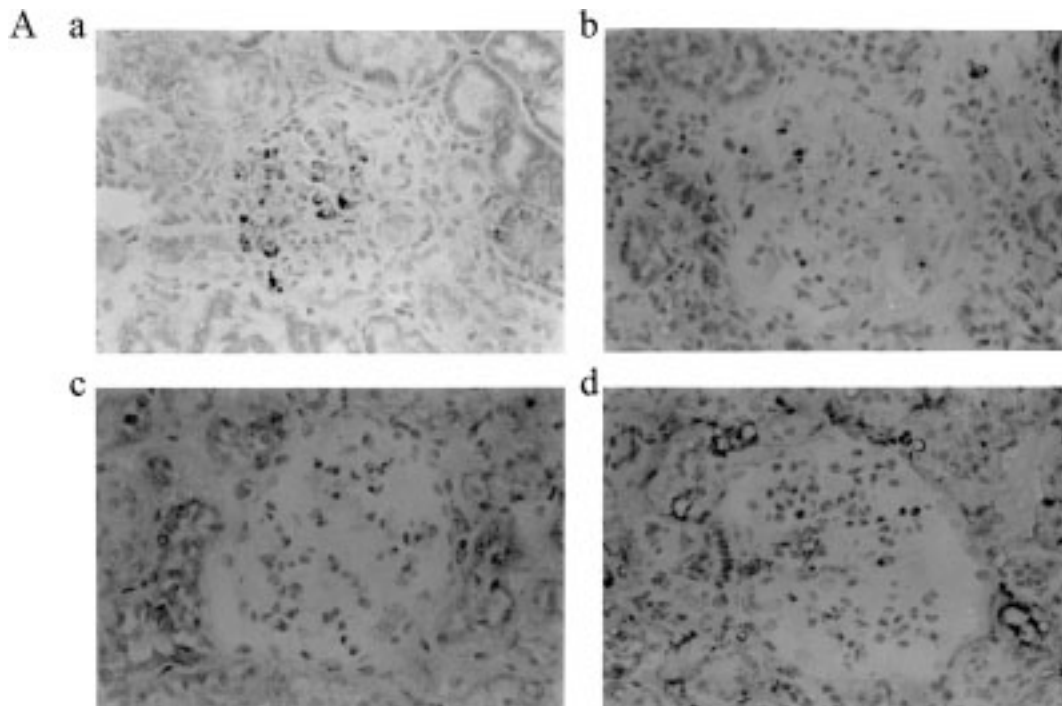

Fig. 5.   Typical photomicrographs of immunostainings for TGF-β (A), VEGF (B), and collagen type IV (C) in glomeruli.
(a) No treatment. (b) Low-dose (0.001%) CS-866 treatment. (c) High-dose (0.01%) CS-866 treatment. (d) Temocapril treatment.
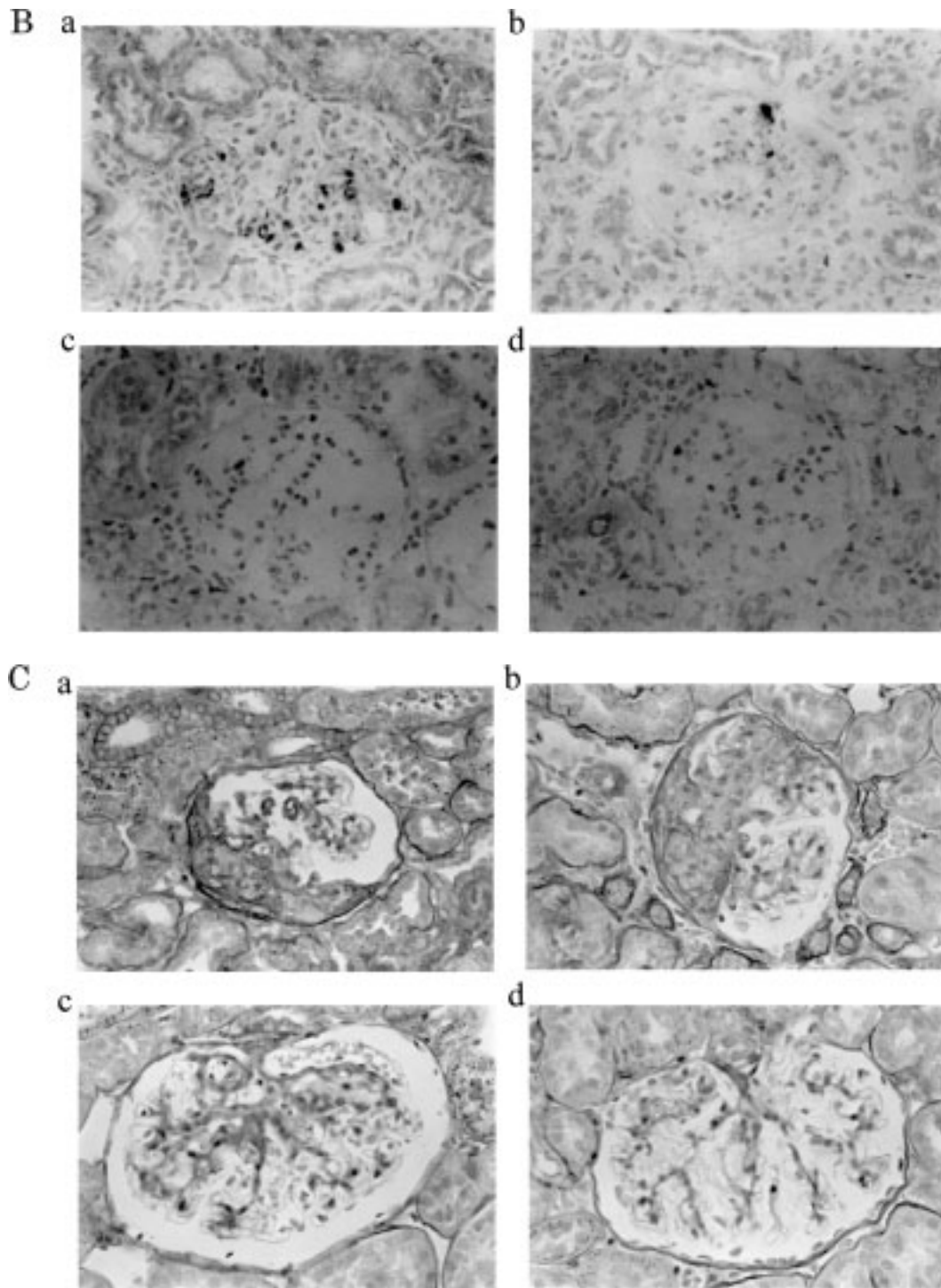
Fig. 5. (Continued)

shown that overproduction of VEGF and TGF-β elicited the functional and structural changes of diabetic nephropathy (13,24,25). In fact, both treatments retarded the progression of glomerulosclerosis by blocking overproduction of these growth factors in glomeruli. The observation that angiotensin II enhances the production of VEGF and

TGF-β by mesangial cells further suggests the involvement of angiotensin II type 1 receptor interaction in overproduction of VEGF and TGF-β in diabetic nephropathy (26,27). In our study, low-dose CS-866 failed to improve nephropathy in OLETF rats despite reduction of positive stainings for TGF-β and VEGF. These results suggest that the
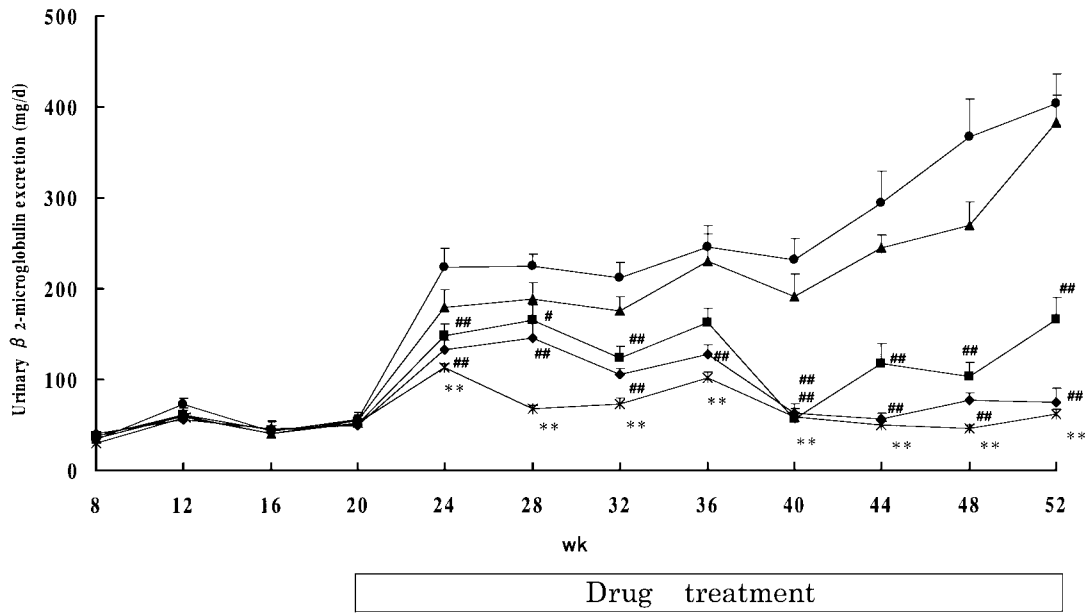
Fig. 6. Effects CS-866 or temocapril treatment on urinary β_2-microglobulin excretions in OLETF rats. (●) Nontreated OLETF rats; (▲)0.001% CS-866–treated OLETF rats; (■) 0.01% CS-866–treated OLETF rats; (◆) temocapril–treated OLETF rats; (*) LETO rats. **$p < 0.01$ compared with nontreated OLETF rats (F-t test). #$p < 0.05$ and ##$p < 0.01$ compared with nontreated OLETF rats (Dunnett's t-test).

concomitant lowering of total cholesterol and triglyceride levels might also be involved in renoprotective effects of CS-866 or temocapril. Many experimental studies have demonstrated a significant deleterious role for hyperlipidemia in both the initiation and progression of renal injuries, supporting our speculation (28).

We showed here that high-dose CS-866 or temocapril significantly lowered both cholesterol and triglyceride levels in OLETF rats. Because CS-866 or temocapril were reported to have no effect on plasma lipid levels in type II diabetic animals or patients (29,30), the effects of CS-866 or temocapril on lipid parameters in OLETF rats might be species-specific actions.

In this study, the renoprotective effects of high-dose CS-866 on glomerular sclerosis was significantly larger than that of an equihypotensive dose of temocapril. Angiotensin II type 1 receptor

antagonists result in the unopposed stimulation of angiotensin II type 2 receptor, which may explain the more beneficial effects of CS-866 on the progression of glomerular sclerosis (31,32).

High-dose CS-866 or temocapril treatment was also found to improve tubular injury in OLETF rats. Tubular atrophy and interstitial fibrosis most closely correlated with declining creatinine clearance, one of the representative prognostic markers for diabetic nephropathy (33). Our present study suggests that the new angiotensin II type 1 receptor antagonist, CS-866, may prove useful as a therapeutic strategy for the treatment of diabetic patients with nephropathy.

Acknowledgments

Table 4. Index of tubular atrophy in OLETF rats

	No treatment ($n = 12$)	CS-866 (0.001%) ($n = 11$)	CS-866 (0.01%) ($n = 12$)	Temocapril ($n = 11$)
Index of tubular atrophy	3.0 ± 0.2	3.0 ± 0.2	1.9 ± 0.2#	1.3 ± 0.1##

High-dose CS-866 or temocapril treatment significantly improved the tubular atrophy index in OLETF rats.
#$p < 0.05$ and ##$p < 0.01$ compared with OLETF rats (Dunnett's t-test).

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