The Renoprotective Effects of an Angiotensin-Converting Enzyme Inhibitor, Imidapril

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The Renoprotective Effects of an Angiotensin-Converting Enzyme Inhibitor, Imidapril

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Abbreviations

ACE	angiotensin-converting enzyme
ACE-I	angiotensin-converting enzyme inhibitor
ANOVA	analysis of variance
AT-1 receptor	angiotensin type 1 receptor
Ccr	creatinine clearance
Dahl/R rats	Dahl salt-resistance rats
Dahl/S rats	Dahl salt-sensitive rats
db/db mice	BKS.Cg-+Lepr^{db}/+Lepr^{db} mice
db/+m mice	BKS.Cg-m+^{b}/+Lepr^{db} mice
ELISA	enzyme-linked immunosorbent assay
HS	heparan sulfate
PAS	periodic acid-Schiff
SBP	systolic blood pressure
S.E.M.	standard errors of the mean
STZ	streptozotocin
UalbV	Urinary excretion of albumin
UcreV	Urinary excretion of creatinine

UHSV	Urinary excretion of heparan sulfate
UproV	Urinary excretion of protein

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Declaration

No portion of the work referred to in this thesis has been submitted in support of application for another degree or qualification of this or any other University or other institute of learning.

All animal experiments were reviewed and approved by the Committee of Experimental Animal Care and Use of Mitsubishi Tanabe Pharma Corporation (formerly named Tanabe Seiyaku) and were conducted according to the Guidelines for Animal Experiments of Mitsubishi Tanabe Pharma Corporation.

Preface

Angiotensin converting enzyme (ACE) is one of the key molecule in the renin-angiotensin system, which contribute pathophysiology of the hypertension, stroke, heart failure, and kidney disease. Angiotensin-converting enzyme inhibitors (ACE-Is) reduce the production of angiotensin II, the leading actor of the renin-angiotensin system (Figure).

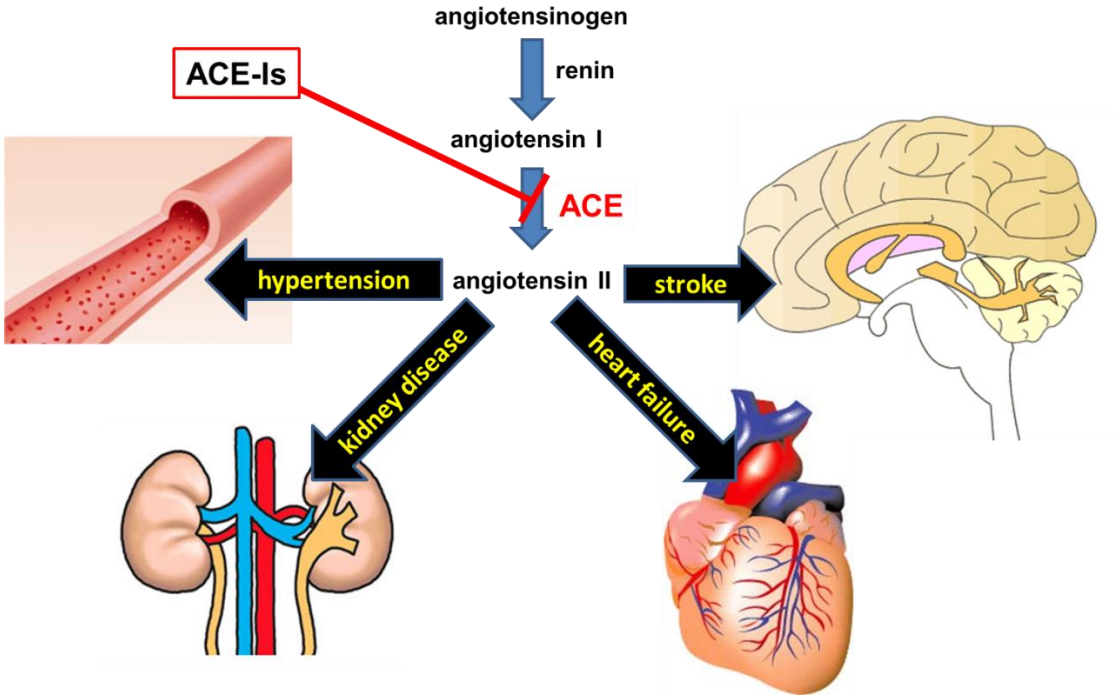

Figure　The role of renin angiotensin system in the organ pathophysiology.

ACE-Is have been primarily developed and approved as the antihypertensive drug. A lot of studies have indicated that ACE-Is have the organ protected effects, and widely used in anticipation of the effects according to the various

guidelines, for example in Japan, hypertension (JSH2009 Hypertension Guidelines), stroke (Japanese Guidelines for the Management of Stroke 2009), heart failure (Guidelines for Treatment of Chronic Heart Failure, JCS 2010), and chronic kidney disease (Evidence-based Clinical Practice Guideline for CKD 2013).

Imidapril hydrochloride (imidapril), one of ACE-Is, has been used by a lot of patients with hypertension in 14 countries, since 1993 of its first approval by Japanese Ministry of Health, Labour and Welfare. Imidapril is an only ACE-I approved for the diabetic nephritis by type 1 diabetes in Japan. The renoprotective effects of imidapril have been reported from clinical (1) and non-clinical studies using diabetes animals (2-4), however, integrated understanding of the renoprotective effects of ACE-Is is still not enough.

In this study, I focused on the studies on the renoprotective effects of imidapril beyond the antihypertensive effects using type 1 and type 2 diabetic mice, and also using non-diabetic nephrosis rats.

Chapter 1　Effects of imidapril in type 1 diabetic mice

1.1 Summary

I investigated whether the prevention of the development of diabetic nephropathy by angiotensin-converting enzyme inhibitors (ACE-Is) is associated with decreases in renal ACE activity and / or blood pressure in diabetic mice. C57Bl/6 mice were injected with streptozotocin (STZ, 200 mg/kg, i.v.) and randomized to receive either imidapril (1 and 5 mg/kg) or captopril (10 and 50 mg/kg) or vehicle by gavage for 28 days. Each assay was performed on 8–10 mice from each treatment. At 28 days after the start of drug treatment, imidapril and captopril significantly reduced blood pressure of the diabetic mice, and this effect of captopril was stronger than that of imidapril. On the other hand, inhibition of renal ACE activity by imidapril was stronger than that by captopril. Imidapril and captopril dose-dependently inhibited urinary albumin excretion to similar extents, but they failed to inhibit the renal hypertrophy and elevation of creatinine clearance. Total renal ACE activity was significantly reduced in diabetic mice, but immunohistochemical localization of ACE was intensive in the vasculature and glomeruli of the diabetic kidney. In conclusion, both effects on

blood pressure and renal ACE activity may be involved in the prevention of development of diabetic nephropathy by imidapril and captopril in STZ-induced diabetic mice. The data suggest that the degrees of contribution of their effects on blood pressure and renal ACE activity to the inhibition of urinary albumin excretion may be different between the two ACE-Is.

1.2 Introduction

ACE-Is have been shown to preserve the renal function in diabetic patients with nephropathy (5-8). In experimental rodent models of diabetes mellitus, advanced lesions were reported to mimic some findings of the early-stage clinical diabetic nephropathy (9, 10) and ACE-Is have also been shown to attenuate renal diseases (5, 10). In human (11) and animal (12, 13) studies, diabetic nephropathy is commonly associated with hypertension. It is well established that hypertension aggravates diabetic nephropathy (14). However, as shown in a meta-analysis of clinical trials, ACE-Is reduce proteinuria and attenuate the progression of renal failure in patients with diabetic nephropathy more effectively than treatment with conventional antihypertensive drugs such as Ca^{2+} channel antagonists and β-adrenoceptor antagonists (15, 16). Moreover,

ACE inhibition seems to be effective in slowing down the progression of human diabetic nephropathy through mechanisms not related to blood pressure (6, 17, 18). Up-regulation of the local tissue renin–angiotensin system in response to injury has been shown in a variety of renal diseases (19-21). These findings leave unanswered the question whether the renoprotective effect of an ACE-I was directly related to the inhibition of angiotensin II or to its eventual antihypertensive effect. In the present study, I investigated the effects of ACE-Is, imidapril and captopril on renal disease, ACE activity and blood pressure in mice with diabetes induced by STZ. In addition, I applied an immunohistochemical technique to determine the localization of ACE in the kidney of diabetic mice.

1.3 Materials and Methods

1.3.1 Chemicals

Imidapril (CAS No. 89396-94-1) was synthesized in Tanabe Seiyaku (Saitama, Japan). Captopril (CAS No. 62571-86-2) and STZ (CAS No. 18883-66-4)were purchased from Sigma Chemical (St. Louis, MO, USA).

1.3.2 Animal experiments

Nine-week-old male C57Bl/6 mice (Japan CLEA, Tokyo, Japan) were housed

in a specific pathogen-free facility and were maintained on standard mouse chow and tap water ad libitum. Under light ether anesthesia, the mice were injected into the tail vein with a bolus injection of 200 mg/kg of STZ dissolved in citrate buffer (pH 4.8). After 4 days, induction of diabetes was confirmed by measurement of the tail blood glucose level using the glucose oxidase method (New Blood-Sugar test; Boehringer Mannheim, Mannheim, Germany), and hyperglycemic mice with glucose levels over 300 mg/dL were used. The diabetic mice were randomly divided into the following five groups (10 animals in each group) treated with vehicle (distilled water), imidapril (1 and 5 mg/kg), and captopril (10 and 50 mg/kg). Ten non-diabetic mice, which had not been injected with STZ, were treated with vehicle. Vehicle or each drug in a volume of 10 mL/kg was given orally to mice by gastric gavage in the morning once a day. Body weight was measured weekly. The systolic blood pressure (SBP) and heart rate of each mouse were measured by the tail-cuff method (UR-5000; Ueda, Tokyo, Japan) before and after the 28-day period of treatment. Blood glucose was measured before treatment and after 14 and 28 days of treatment. On the 29th day, the mice in each group were anesthetized with ether and blood samples were taken from the abdominal aorta. Bilateral kidneys were rapidly

removed and weighed. Half the middle portion of the left kidney was immediately fixed for 60 min with methacarn solution for the estimation of glomerular morphometry, or embedded in the Tissue Tek (Miles, Elkhart, IN, USA), and frozen for immunohistochemical analysis of ACE. The rest of the kidney was frozen in liquid nitrogen and stored at -80℃ until the measurement of ACE activity.

1.3.3 Measurements of urinary albumin level and renal function

On Days 14 and 28, the mice were detained in individual metabolic cages for 24 h for urine collection. The urine volume was measured gravimetrically, and urinary albumin concentrations were determined with an enzyme-linked immunosorbent assay (ELISA) using a murine microalbuminuria kit (Albuwell M; Exocell, Philadelphia, PA, USA). Renal function was evaluated by calculating creatinine clearance (Ccr, mL/min/100 g body weight). The plasma and urinary creatinine levels were measured by an enzymatic method (CRE; Mizuho medy, Saga, Japan) using the autoanalyzer Hitachi 7150 (Hitachi, Tokyo, Japan). The blood (serum) urea nitrogen levels were measured by using the autoanalyzer.

1.3.4 Morphometric analysis of glomerular

Half middle portion of left kidney was fixed with methacarn solution, and

embedded in paraffin. Four-micrometer-thick slices were stained with periodic acid-Schiff (PAS). In PAS stained section of the renal cortex, the glomerular tuft area was determined by the total glomerular area minus the urinary space area and the urinary recesses, as previously described (10). More than 30 glomeruli were counted per kidney and the average was used for analysis.

1.3.5 Measurement of renal ACE activity

Assay of ACE activity was performed on 8–10 mice from each group. Renal ACE activities were measured with tissue samples taken on the 29th day of treatment by using a fluorometric assay described by Cheung and Cushman (22). The ACE activity was calculated as nanomoles of His–Leu generated per mg tissue weight/hour.

1.3.6 Immunohistochemistry for ACE

The frozen renal tissues of five randomly selected mice in the non-diabetic and diabetic groups were cut into 5-mm-thick slices, and mounted on slides. To detect ACE, the slices were fixed in acetone, and incubated with 1 mg/mL of the anti-human ACE monoclonal antibody (9B9; Chemicon, Temecula, CA, USA) for 60 min at room temperature. The samples were subsequently incubated with rabbit anti-mouse immunoglobulin (IgG), and then with a mouse alkaline

phosphatase anti-alkaline phosphatase immune complex. The slide was washed in Tris (pH 7.6) -buffered saline after each step. Bound alkaline phosphatase was visualized by new fuchsin and levamisole to yield a red reaction product. All sections were counterstained with hematoxylin.

1.3.7 Statistical analysis

Data are expressed as means ± standard errors of the mean (S.E.M.). Statistical analysis was done by SuperANOVA (Abacus Concepts, Berkeley, CA, USA) on a Mackintosh computer. Comparison between non-diabetic and diabetic mice was performed by Student's *t*-test. Differences among the diabetic groups were analyzed by using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparison. A *p* value less than 0.05 was considered statistically significant.

1.4 Results

In the diabetic mice, two mice in imidapril (5 mg/kg)-treated group and one mouse in captopril (50 mg/kg)-treated group died spontaneously between the 21st and 29th day of treatment. No mice in the other experimental groups died during the period of treatment.

1.4.1 Body weight, blood glucose and blood pressure

Diabetes was associated with reduced weight gains and increased plasma glucose levels which were not influenced by the imidapril and captopril treatments (Table 1.1). Figure 1.1 shows SBP of each group at 28 days after the start of drug treatment. The SBP of the diabetic group was slightly, but not significantly higher than that of the non-diabetic group. The SBP was significantly reduced by treatment with either imidapril or captopril. The blood pressure lowing by captopril was greater than that by imidapril.

1.4.2 Renal hypertrophy and function

Urine volume and kidney weight were increased in the diabetic group compared with non-diabetic group (Table 1.2). Treatment with imidapril and captopril did not influence these parameters. Glomerular tuft areas in diabetic mice were increased (Table 1.2), but morphometric analysis showed no apparent mesangial sclerosis in diabetic mice. Blood urea nitrogen and serum creatinine were not affected in the diabetic group (31 ± 1 and 0.09 ± 0.01mg/dL, respectively) compared with non-diabetic group (30 ± 1 and 0.10 ± 0.00 mg/dL, respectively).

Ccr increased clearly in the diabetic group, but showed no further change in the

mice treated with either imidapril or captopril (Table 1.2).

1.4.3 Effects on urinary albumin excretion

The urinary albumin excretion level of each group on Day 28 is shown in Figure 1.2. The urinary albumin excretion level of the diabetic group was higher than that of the non-diabetic group. The urinary albumin excretion level was dose-dependently reduced by treatment with either imidapril or captopril. This urinary albumin excretion-reducing effect was not different between the treatments with the ACE-Is. There were no significant differences in urinary albumin excretion levels on Day 14 among the diabetic mice treated with drugs.

1.4.4. Activity and immunolocalization of ACE

Renal ACE activity was decreased significantly in the diabetic group compared with the non-diabetic group, and was further decreased by treatment with imidapril and captopril. The ACE activity of the imidapril-treated groups was much lower than that of the captopril-treated groups (Figure 3).

In the non-diabetic mice, immunoreactivity for ACE was slightly present in the endothelium of the renal vein and glomeruli (Figure 4A and C). On Day 29, both the endothelium of renal arteries and the glomeruli in the diabetic group were intensely immunoreactive to the ACE anti-body (Figure 4B and D), but the

intensity of staining for ACE in the tubules was slightly decreased. No immunoreactivity was noted when the ACE antibody was replaced with the non-immune IgG negative control.

1.5 Discussion

The present study demonstrates two major findings. First, the ACE-Is imidapril and captopril equally prevented the elevation of urinary albumin excretion levels in STZ-induced diabetic mice with high blood glucose, but showed differences in the potency of reducing blood pressure and ACE activity. Second, renal ACE activity was significantly decreased in the diabetic group compared with the non-diabetic group, but on immunohistochemical analysis, both the endothelium and the glomeruli of the diabetic kidney were intensely immunoreactive to the ACE antibody.

Imidapril showed a dose-dependent inhibitory effect on the elevation of urinary albumin excretion levels in a pattern identical to the effect seen with captopril in the diabetic mice. Systemic hypertension is a well-known cause of progressive renal injury in both humans (11) and experimental animals (12, 13). In the present study using the diabetic mice, the antihypertensive efficacy of captopril

was stronger than that of imidapril. Therefore, the hypotension did not only account for the inhibitory effects of these ACE-Is on the urinary albumin excretion level in diabetic mice. On the other hand, inhibition of renal ACE activity by imidapril was greater than that by captopril in the diabetic mice. The reason for this difference was not clear, but some ACE-Is, which equally inhibited circulating ACE, had shown major differences in affinity for tissue ACE (23-25). Thus, our observations suggest that the renoprotective effects of the ACE-Is may be attributable to both hypotension and inhibition of renal ACE activity.

Another explanation for the effects of the ACE-Is on the urinary albumin excretion level is the amelioration of permeability properties of the glomerular basement membrane. The permeability of glomerular basement membrane is dependent on both size-selectivity and charge-selectivity. Morelli et al. (26) reported that ACE inhibition diminished glomerular permeability to proteins by enhancing barrier size-selectivity measuring by the dextran fractional clearance in humans with diabetic glomerulopathy.

Angiotensin II has a stimulatory effect on transforming growth factor-ß production (27, 28) and there is evidence that angiotensin II increases

extracellular matrix production in the diabetic kidney (29, 30). These results suggest that one of these effects may be involved in the therapeutic effect of ACE-Is in diabetic nephropathy. ACE was shown to be the rate-limiting step for the biological action of the renin–angiotensin system (31, 32). In addition, tissue ACE activity correlates positively with the tissue angiotensin II levels (33). Therefore, to explore the role of ACE, I compared the activity and immunolocalization of renal ACE in normal and diabetic mice. As a result, renal ACE activity in the diabetic mice was significantly lower than that in normal mice. However, ACE immunostaining intensity was enhanced in the glomeruli and renal vasculature of the diabetic kidney. Thus, I speculate that in these sites in diabetes, the increase in local tissue ACE leads to increased local angiotensin II. These results, which coincide with the results of a study on STZ-induced nephropathy in rats (19), suggest that the findings of reduced whole kidney ACE activity do not necessarily imply a uniform reduction of tissue ACE activity in those sites e.g. glomeruli and vasculature., and that the intrarenal ACE may be involved in the development of nephropathy in STZ-induced diabetic mice.

I showed in this study that imidapril and captopril reduced the increase in the urinary albumin excretion level, but did not affect Ccr and renal hypertrophy.

These results are consistent with previous reports (13, 34), which investigated the effects of ACE-Is administered for 4 or 8 weeks in STZ-induced diabetic models. However, some researchers (10, 29), who treated the animals with ACE-Is for 12 and 24 weeks, found that the ACE-Is inhibited glomerular hyperfiltration and/or renal hypertrophy. Especially, Sassy-Prigent et al. (10) showed that the ACE-Is inhibited the increase in urinary albumin excretion but not the renal hypertrophy at 4 weeks in STZ-induced diabetic rats. Thus, I speculate that the lack of effects of the ACE-Is on increased creatinine clearance and renal hypertrophy may be due to the duration of treatment in our study. Furthermore, using morphometric techniques, I found that the glomerular tuft areas were increased, but mesangial sclerosis did not occur in diabetic mice. I showed that the blood urea nitrogen and serum creatinine levels were not increased in diabetic mice. These results suggest that mice, 4 weeks after the STZ injection, may be in early stage of diabetic nephropathy. Further studies are needed to clarify the influence of other variables known to modulate the ACE.

In conclusion, I found that the ACE-Is in doses equipotent for reduction of the increase in urinary albumin excretion level showed different effects on blood pressure and renal ACE activity. Our results in the present study support the

concept that the prevention by ACE-Is of the development of nephropathy in experimental diabetes may involve both the decreased blood pressure and inhibition of renal ACE activity. In addition, the data suggest that the ACE-Is imidapril and captopril may exhibit major differences in their mechanisms of urinary albumin excretion inhibition in diabetic mice.

1.6 Publication

The study in this chapter was published as “Effects of imidapril and captopril on streptozotocin-induced diabetic nephropathy in mice” in the *European Journal of Pharmacology*, 2000, 398: 381-7.

1.7 Figures and tables

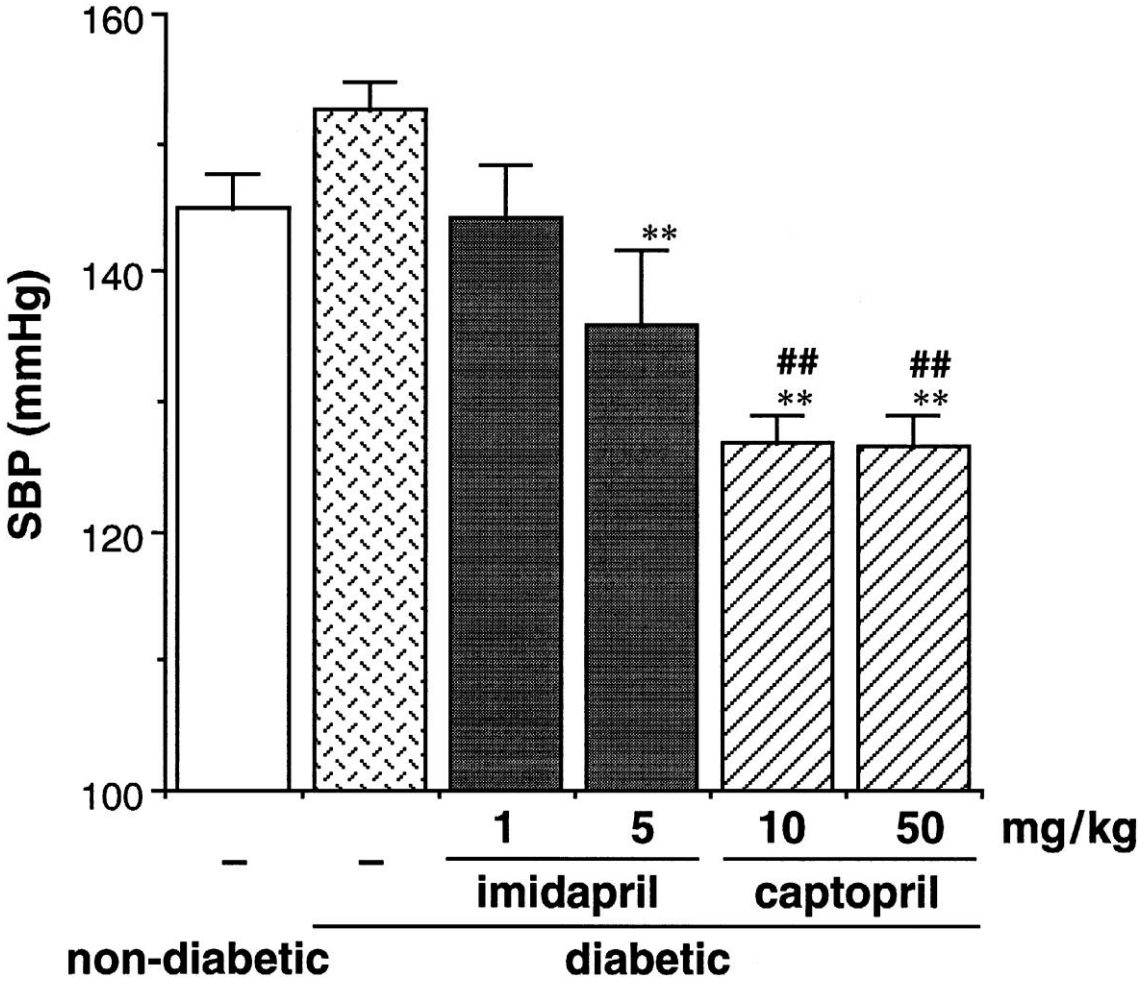

Figure 1.1 Systolic blood pressure (SBP) in the non-diabetic, diabetic, diabetic + imidapril (1 and 5 mg/kg) and diabetic + captopril (10 and 50 mg/kg) groups after the 28-day treatment in streptozotocin-induced diabetic mice. Values are means + S.E.M. *n*=8–10. ***P*<0.01 compared with the diabetic group. ##*P*<0.01 compared with the diabetic imidapril (1 mg/kg) group.

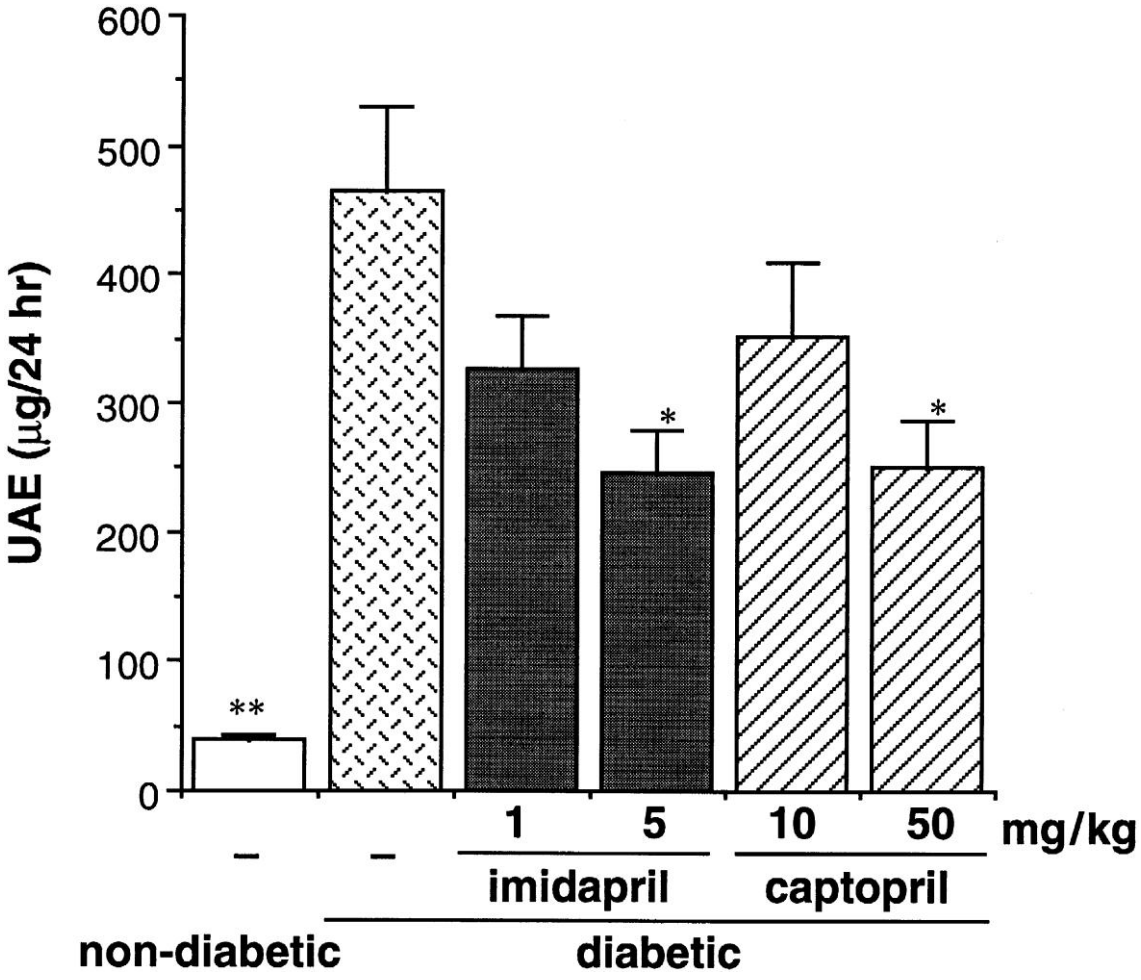

Figure 1.2. Urinary excretion of albumin (UAE) in the non-diabetic, diabetic, diabetic + imidapril (1 and 5 mg/kg) and diabetic + captopril (10 and 50 mg/kg) groups after the 28-day treatment in streptozotocin-induced diabetic mice. Values are means + S.E.M. n=9–10. **P<0.01 and *P<0.05 compared with the diabetic group.

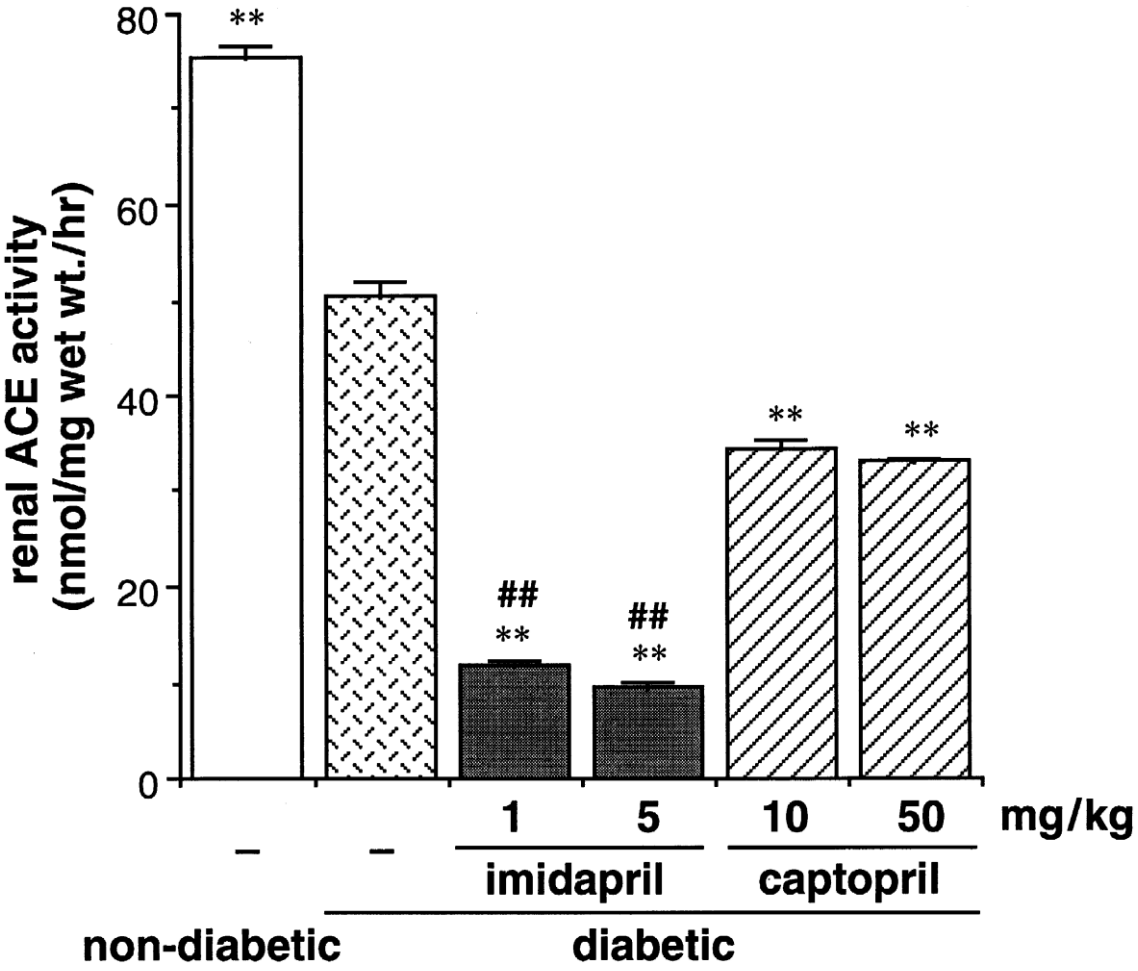

Figure 1.3. Renal angiotensin-converting enzyme (ACE) activity in the non-diabetic, diabetic, diabetic + imidapril (1 and 5 mg/kg) and diabetic + captopril (10 and 50 mg/kg) groups after the 28-day treatment in streptozotocin-induced diabetic mice. Values are means + S.E.M. *n*=8–10. ***P*<0.01 compared with the diabetic group. ##*P*<0.01 compared with the diabetic + captopril (50 mg/kg) group.

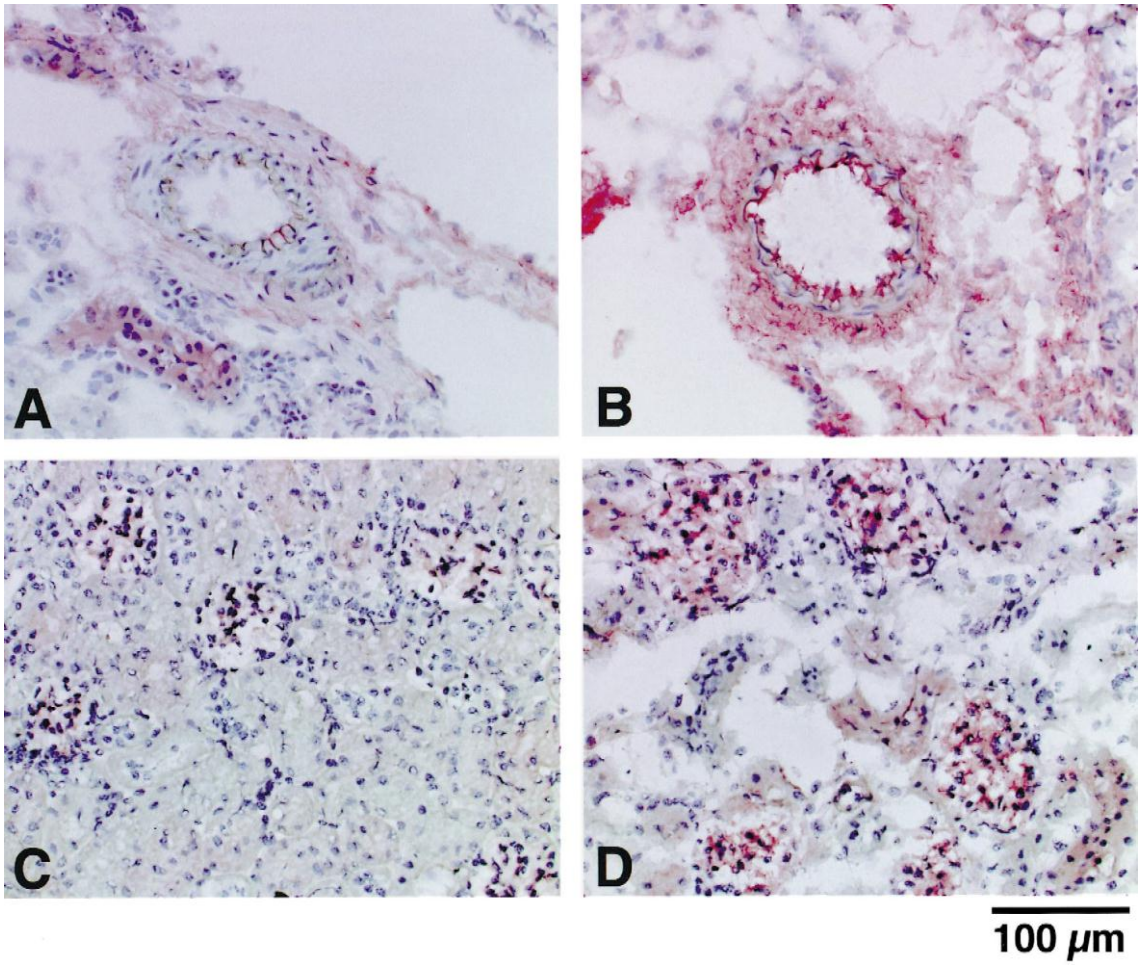

Figure 1.4. Immunohistochemical localization of angiotensin-converting enzyme in the renal arteries (A and B) and glomeruli (C and D) sacrificed on the 33rd day after streptozotocin treatment. A and C: Sections from the kidney of the non-diabetic group. B and D: Sections from the kidney of the diabetic group. Bar indicates 100 μm.

Table 1.1 Body weight and blood glucose concentration of streptozotocin-induced diabetic mice before and after treatment with angiotensin-converting enzyme inhibitors

Group	Dose (mg/kg)	*n*	Body weight (g)	Plasma glucose (mg/dl)
Non-diabetic	–	10		
Day 0			23.2 ± 0.3 *	155.6 ± 4.3 *
Day 28			25.0 ± 0.3 *	148.5 ± 4.9 *
Diabetic	–	10		
Day 0			21.8 ± 0.3	462.2 ± 22.3
Day 28			18.7 ± 0.6	431.2 ± 16.8
Diabetic + imidapril	1	10		
Day 0			21.9 ± 0.3	463.0 ± 17.2
Day 28			18.8 ± 0.7	453.4 ± 18.0
Diabetic + imidapril	5	10		
Day 0			22.0 ± 0.4	463.5 ± 17.2
Day 28			17.6 ± 0.8	413.7 ± 30.1
Diabetic + captopril	10	10		
Day 0			21.9 ± 0.3	462.8 ± 13.8
Day 28			18.6 ± 0.6	449.4 ± 11.5
Diabetic + captopril	50	9		
Day 0			21.9 ± 0.2	464.0 ± 14.2
Day 28			19.0 ± 0.7	493.1 ± 24.2

The non-diabetic mice were treated with vehicle. The diabetic mice were randomly divided into the following five groups treated with vehicle (distilled water), imidapril (1 and 5 mg/kg), and captopril (10 and 50 mg/kg). Values are means ± S.E.M. ***P*<0.01 compared with the diabetic group.

Table 1.2 Urine volume, kidney weight and creatinine clearance after the 28-day treatment with angiotensin-converting enzyme inhibitors in streptozotocin-induced diabetic mice

Group	Dose (mg/kg)	Urine volume (ml/24 h)	Kidney weight/ body weight (mg/g)	Glomerular tuft area (μm^2)	Creatinine clearance (ml/min/100 g body weight)
Non-diabetic	–	$1.7 \pm 0.1^{*}$	$11.86 \pm 0.11^{*}$	$2257 \pm 31^{*}$	$1.53 \pm 0.06^{*}$
Diabetic	–	26.7 ± 1.7	19.84 ± 0.72	3055 ± 69	2.57 ± 0.09
Diabetic + imidapril	1	26.1 ± 2.8	18.37 ± 0.53	3034 ± 55	2.63 ± 0.25
Diabetic + imidapril	5	22.9 ± 2.8	19.62 ± 0.64	3040 ± 58	3.08 ± 0.22
Diabetic + captopril	10	25.3 ± 1.2	20.12 ± 0.55	2985 ± 45	2.46 ± 0.16
Diabetic + captopril	50	25.7 ± 2.5	18.58 ± 0.61	2922 ± 31	2.22 ± 0.16

The non-diabetic mice were treated with vehicle. The diabetic mice were randomly divided into the following five groups treated with vehicle (distilled water), imidapril (1 and 5 mg/kg), and captopril (10 and 50 mg/kg). Values are means ± S.E.M. n=8–10. Kidney weight indicates total weight of left and right kidneys. **P<0.01 compared with the diabetic group.

Chapter 2　Effects of imidapril in type 2 diabetic mice

2.1 Summary

I investigated the renoprotective effects of an ACE-I, imidapril in a diabetic animal model. I used BKS.Cg-+Lepr^{db}/+Lepr^{db} (db/db) mice, a genetic animal model of obese type 2 diabetes. Diabetic db/db mice suffered from glomerular hyperfiltration, albuminuria and hypoalbuminemia. Oral administration of 5 mg/kg/day of imidapril for 3 weeks suppressed renal hyperfiltration, reduced albuminuria and normalized hypoalbuminemia. Imidapril did not influence body weights, blood pressure or blood glucose concentrations in db/db mice. Urinary excretion of heparan sulfate (HS) in non-treated 11-week-old db/db mice was significantly lower than that in age-matched non-diabetic db/+m mice. HS is a component of HS proteoglycans, which are present in glomerular basement membranes and glycocalyx of cell surfaces. Reduced urinary HS excretion indicated glomerular HS loss in db/db mice. Imidapril increased urinary excretion of HS to concentrations observed in non-diabetic BKS.Cg-m+^{b}/+Lepr^{db} (db/+m) mice, indicating that imidapril prevented the loss of renal HS. These results suggest that imidapril ameliorates renal hyperfiltration and loss of renal contents

of HS. Improvement of filtration function and maintenance of HS, which is an important structural component of glomeruli, may contribute to renoprotective effects of imidapril.

2.2 Introduction

ACE-Is have been shown to preserve renal function in diabetic patients with nephropathy (5-8). As shown in meta-analyses of clinical trials, ACE-Is reduce proteinuria and attenuate the progression of renal failure in patients with diabetic nephropathy more effectively than treatment with conventional antihypertensive drugs, such as Ca^{2+} channel blockers or β-adrenoceptor antagonists(15, 16) (5,6). Moreover, ACE inhibition appears to be effective in slowing the progression of human diabetic nephropathy through mechanisms that are not related to blood pressure (6, 17, 18) . Up-regulation of local tissue renin–angiotensin system has been shown in a variety of renal diseases (19-21). Imidapril, an ACE-I, has been used as an antihypertensive drug, and is indicated for diabetic nephropathy caused by type 1 diabetes. Clinical usage of imidapril has been well reviewed by Robinson et al. (1) Preclinical studies have suggested that imidapril reduces proteinuria and renal lesions in type 1 (Chapter

1) and type 2 (2-4) diabetic nephropathy models. However, the renal protective mechanisms of ACE-Is, including imidapril, are not fully understood. In the present study, I investigated the effects of imidapril on renal function and urinary excretion of heparan sulfate (HS), which is a glomerular structural component of glomeruli, in a type 2 diabetic model, db/db mice.

2.3 Materials and Methods

2.3.1 Chemicals

Imidapil (CAS No. 89396-94-1) was synthesized at Mitsubishi Tanabe Pharma (Osaka, Japan).

2.3.2 Animals

Male db/db mice and their non-diabetic controls, db/+m mice, were obtained from CLEA Japan (Tokyo, Japan). They were individually housed in polycarbonate cages (3600 M, Tecniplast, Milan, Italy) throughout the experiments. They were allowed free access to standard laboratory chow, CRF-1 (Oriental Yeast, Tokyo, Japan), and tap water. The animal room was controlled for temperature (23 ± 2 ℃), humidity (55 ±15%) and light (12-h light: dark cycle). All animals used for the experiments were at 8 weeks of age after 1

week of acclimation.

2.3.3 Experimental procedures

Sixteen diabetic db/db mice divided into two experimental groups that were matched for SBP and blood glucose concentrations. Diabetic db/db mice received imidapril (5 mg/kg/day p.o.) or purified water once daily for 3 weeks. Eight non-diabetic db/+m mice also received purified water once daily for 3 weeks. SBP was determined using the tail-cuff method (UR-5000; Ueda, Tokyo, Japan) before and 20 days after treatment with imidapril. Body weight and blood glucose were monitored weekly. Urine was collected before and 22 days after treatment with imidapril. At the end of the experimental period, the mice were sacrificed by exsanguination from the abdominal aorta under ether anesthesia. The blood was centrifuged, and the obtained plasma was stored at 30℃ until further analyses. Both kidneys were removed and weighed.

2.3.4 Measurements

Plasma glucose, urinary albumin and HS concentrations were determined using commercially available kits (New Blood Sugar Test, Boehringer Mannheim, Mannheim, Germany; Mouse Albumin ELISA Kit, Shibayagi, Gunma, Japan; and Heparan Sulfate ELISA Kit, Seikagaku Corporation, Tokyo, Japan, respectively).

Plasma and urinary creatinine concentrations were determined using an autoanalyzer (Hitachi 7070, Hitachi, Tokyo, Japan). Urinary excretions of albumin and HS were calculated according to the equations: urinary excretion at an initial period = urine volume × urinary concentration / body weight, and urinary excretion at the end of the experiments = urine volume × urinary concentration / kidney weight. Compared with db/+m mice, urinary excretion was lower and body weight was significantly higher in diabetic db/db mice at the end of the experiment. Therefore, kidney weight was used as a denominator instead of body weight to avoid overestimation of their excretory functions.

2.3.5 Statistical analysis

Data are expressed as means ± S.E.M. Statistical analysis was conducted by the closed testing procedure. Comparisons of the difference between non-diabetic db/+m control and diabetic db/db mice were made by Student's t-test first. After that, effects of imidapril in db/db mice were compared between with or without treatment db/db mice by Student's t-test. A *p* value less than 0.05 was considered statistically significant.

2.4 Results

Time course of body weight, blood glucose concentrations and SBP is shown in Figure 2.1. Compared with db/+m control mice, body weight was significantly higher, blood glucose concentrations were greatly higher, and SBP was slightly but significantly lower in db/db mice. These significant differences were maintained throughout the experiments and were not affected by treatment with imidapril (Figure 2.1). Urinary excretions of creatinine and HS per body weight in db/db mice were not different from those in db/+m control mice at 8 weeks of age (Table 2.1). However, urinary excretion of albumin per body weight in db/db mice was greater than that in db/+m control mice (Table 2.1).

After 22 days of treatment with 5 mg/kg of imidapril, kidney weights and plasma creatinine concentrations were almost similar in all experimental groups (Figure 2.2). Urine volume and creatinine clearance (Ccr) in db/db mice were greater than those in db/+m control mice. Imidapril significantly corrected hyperfiltration as indicated by the increased Ccr observed in db/db mice, but it did not affect urine volumes (Figure 2.2).

Urinary excretion of albumin in db/db mice was much higher than that in db/+m mice, and imidapril tended to decrease urinary excretion of albumin in db/db mice ($p=0.07$, Figure 2.3). Plasma albumin concentrations in db/ db mice was

similar to those in db/+m mice, and imidapril increased these concentrations significantly. Urinary excretion of HS in db/db mice was significantly lower than that in db/+m mice, and the imidapril-treated group showed significantly higher HS excretion than nontreated db/db mice (Figure 2.3). Plasma HS concentrations in db/db mice were slightly but significantly higher than those in db/+m mice, and imidapril did not affect these concentrations (Figure 2.2).

2.5 Discussion

Previous studies have indicated that imidapril inhibits the progression of renal diseases independently of its antihypertensive effects (3). They demonstrated a reduction in proteinuria, leading to diminished renal histological changes following treatment with imidapril. Furthermore, Nawano et al. reported that imidapril enhances the expression of insulin receptor substrate proteins (2). However, few studies indicate which molecules in renal structures are influenced by imidapril.

In the present study, imidapril ameliorated hyperfiltraion with a tendency of reduction in albuminuria accompanied by an increase in plasma albumin concentration, confirming the renoprotective effects of imidapril in db/db mice

during the hyperfiltration period. It is well known that clinical use of ACE-Is, including imidapril, maintains glomerular filtration function for long period of time in patients with renal diseases. Slight decrease in glomerular filtration has also been demonstrated during the early phase of treatment with ACE-Is (35). Taken together, these data suggest that ACE-Is may reduce the excessive glomerular filtration load of effective glomeruli.

Plasma HS concentrations in db/db mice were slightly but significantly higher than those in db/+m control mice at 11 weeks of age. Although the underlying mechanisms are not clear, the higher HS concentrations may be related to the larger body mass of db/db mice, because HS-containing proteins are expressed ubiquitously (36). The higher turnover rate of systemic HS may be another contributing factor. On the other hand, urinary excretion of HS in 11-week-old db/db mice was significantly lower than that in age-matched db/+m mice, surely, reflecting glomerular HS contents rather than circulating HS concentrations. Moreover, these results suggest that HS contents in the kidneys of db/db mice may be markedly decreased. Decreased urinary excretion of HS in 11-week-old db/db mice may reflect HS loss in glomeruli as a result of hyperglycemia and/or hyperfiltration. Glomerular structures may be injured during the early stages of

diabetes in db/db mice. Imidapril prevented the reduction in urinary excretion of HS without affecting plasma HS concentrations. These results suggest that imidapril maintains glomerular HS contents, but the precise mechanisms remain unclear.

Although the exact reasons for decrease of HS in glomeruli remain unclear, reduction of the expression of HS-containing proteins and accelerated enzymatic deglycosylation of HS proteoglycans are some of the possibilities. Recently, van den Hoven et al. (37) reported that glomerular heparanase activity was increased in adriamycin nephropathy in rats.

Furthermore, a clinical report showed a decline of HS in glomerular basement membranes without changes in the contents of core protein, agrin, in patients with diabetes (38).

In the present study, I did not determine glomerular heparanase activities or contents of HS-containing proteins. However, aforementioned reports suggest that accelerated enzymatic deglycosylations of HS-proteoglycans could be involved. Further studies are required to determine the precise protective mechanisms of imidapril in terms of renal HS containing proteins. Nevertheless, urinary HS may be a convenient marker for the condition of glomerular HS

containing proteins. Imidapril corrected glomerular filtration function and may protect renal structures at least in part by maintaining contents of HS containing proteins.

Since Brenner et al. (39) proposed the hypothesis of the charge barrier several decades ago, many researchers have believed that renal glomerular molecular selectivity was comprised two factors: the size barrier and the charge barrier. HS has been considered as an important component of the renal charge barrier, and early studies have established the importance of HS proteoglycans and their glycosaminoglycan chains as components of charge barrier (40). However, some studies have reported no proteinuria following the deletion of HS containing proteins in glomeruli (41, 42). Moreover, clinical findings also deny a relationship between severity of proteinuria and HS proteoglycans (43). Recently, HS proteoglycans have been recognized as the molecules involved in cellular signals in podocytes and other cell types (44). Hence, the putative roles of HS-containing proteins may have shifted from charge barrier molecules to the signal transducers of glomerular epithelial cells. Imidapril has functional renoprotective effects involving correction of glomerular filtration function, and it may also have structural effects that protect filtration surface structures by

maintaining HS containing proteins.

The relationship of angiotensin II signals and HS contents in glomeruli is still unclear. There is a report that angiotensin II type receptor-mediated HS-proteoglycans production in SV40 transformed podocyte (45), but the above-mentioned in vivo observations indicate that the most important factor is not whole protein contents but HS glyco-chain loss. However, further investigations are required to determine the relationship between angiotensin II signals and HS contents.

In this study, imidapril improved hyperfiltration and protected against HS loss, as indicated by decreased urinary excretion of HS, in diabetic db/db mice. These data suggest that both functional and structural mechanisms play a significant role in the renoprotective effect of imidapril.

2.6 Publication

The study in this chapter was published as “Involvement of heparan sulfate in the renoprotective effects of imidapril, an angiotensin-converting enzyme inhibitor, in diabetic db/db mice” in the *Journal of Receptors and Signal Transduction*, 2014, 34: 21-5.

2.7 Figures and tables

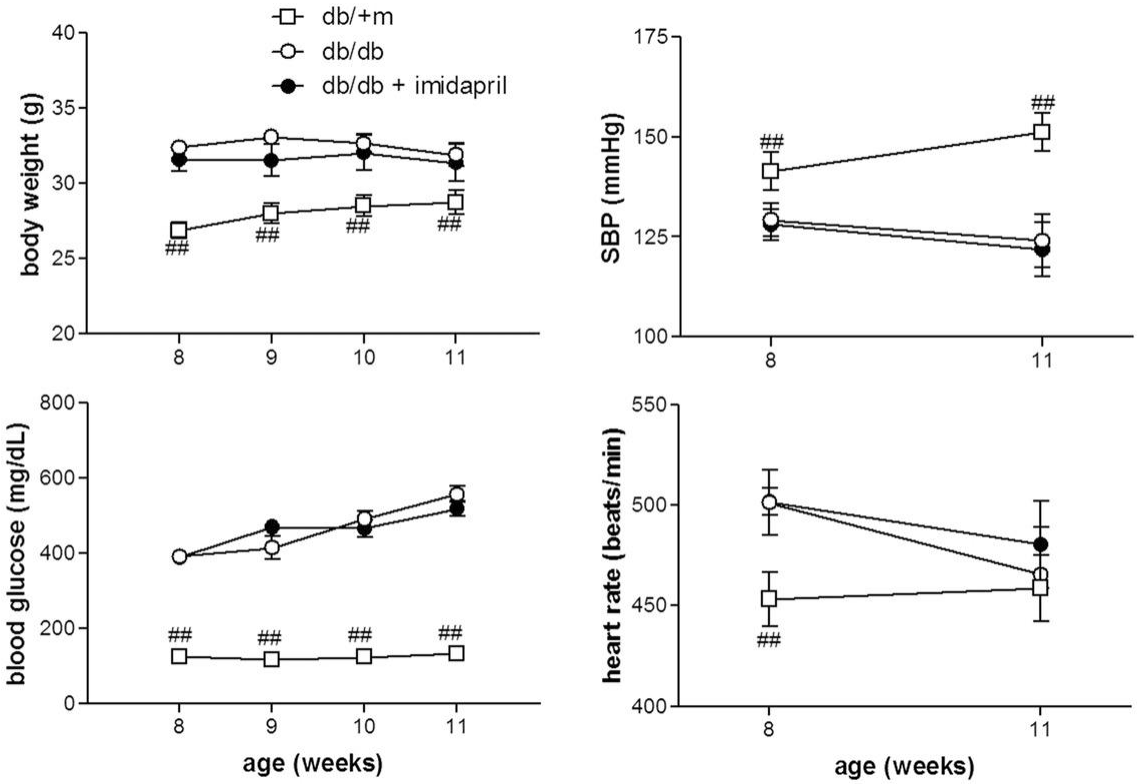

Figure 2.1 Time course of body weight, blood glucose concentrations and systolic blood pressure (SBP). □, db/tm; ○, db/db; ●, db/db treated with imidapril; Means ± S.E.M. *n*=8. ##*p*<0.01 compared with db/db control using Student's t-test.

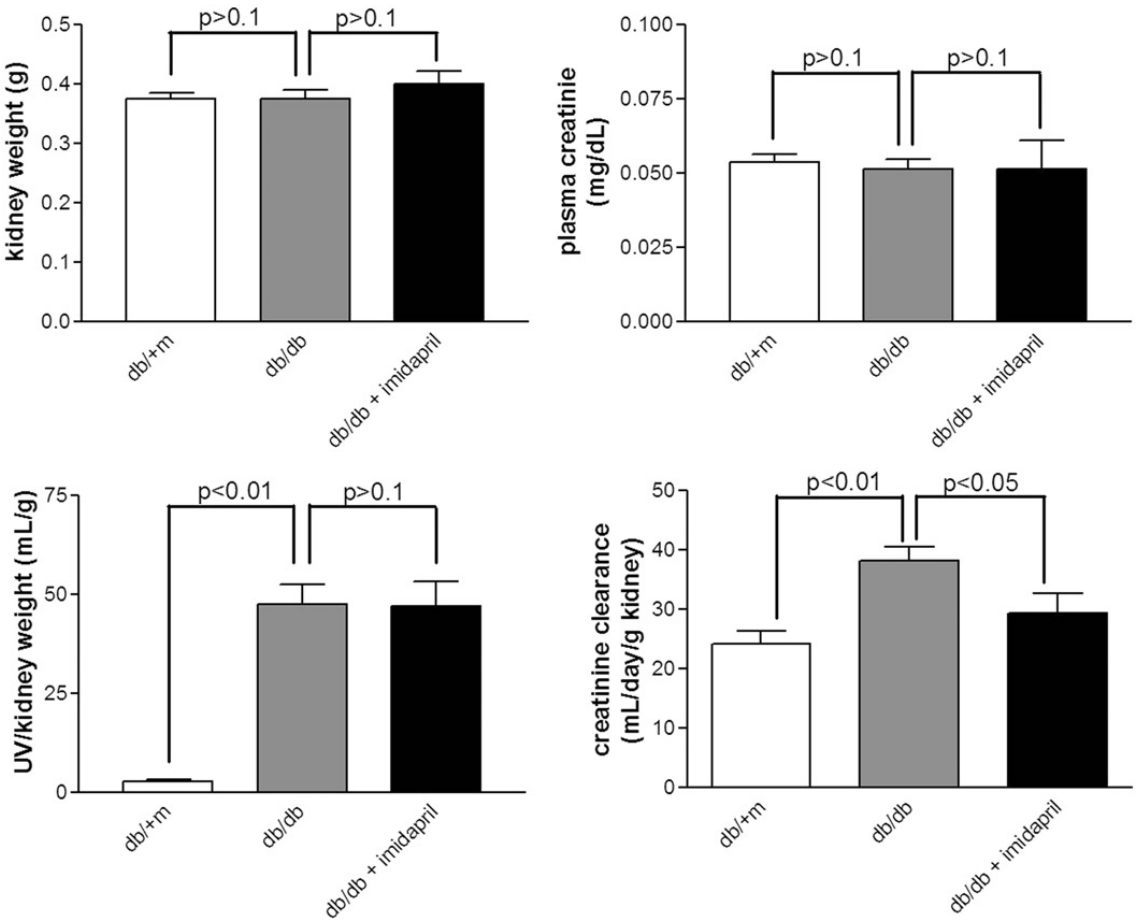

Figure 2.2 Effects of 3-week administration of imidapril on kidney weight, plasma creatinine concentration, urine volumes per kidney weight (UV/kidney weight), and Ccr per kidney weight in 11-week-old mice. Mean + S.E.M. of eight animals. Each p Value was calculated using Student's t-test.

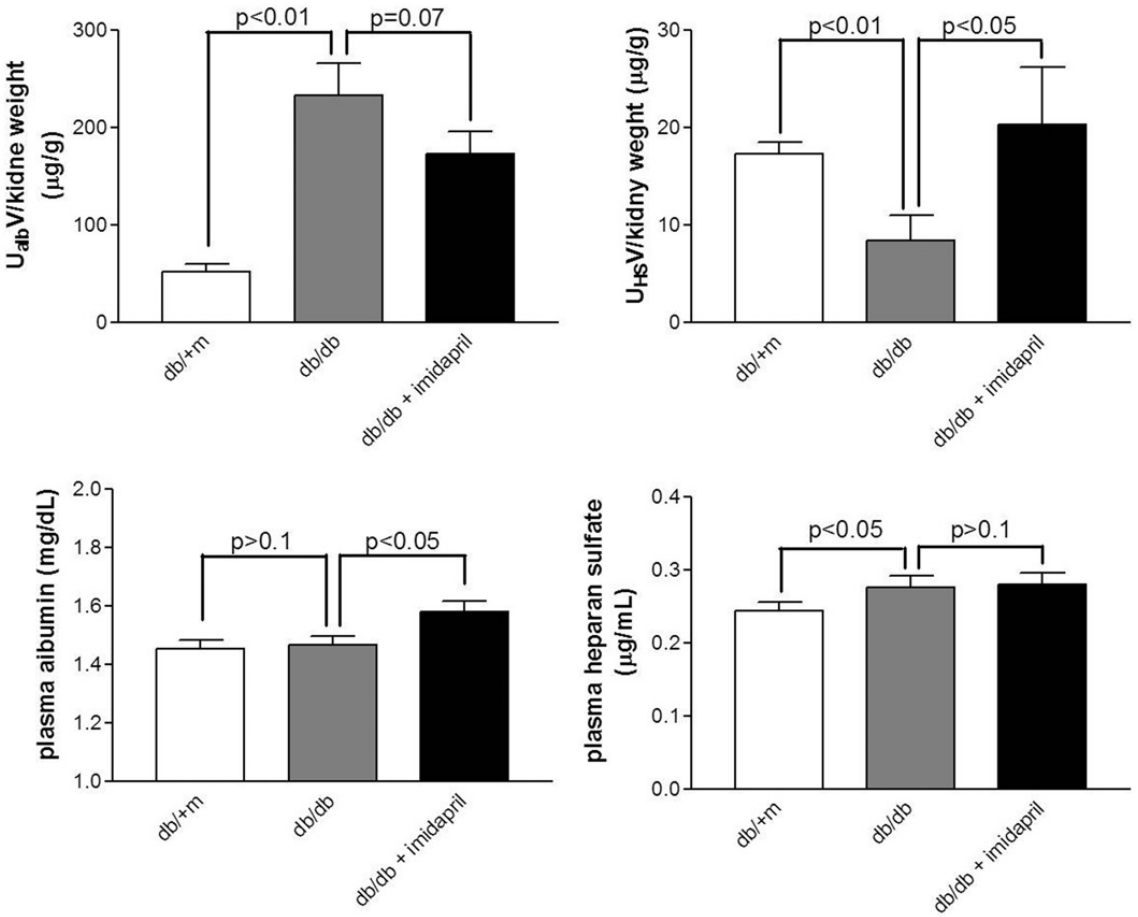

Figure 2.3 Effects of 3-week administration of imidapril on urinary excretion of albumin ($U_{alb}V$) and heparan sulfate ($U_{HS}V$) per kidney weight and plasma concentration of albumin and HS in 11-week-old mice. Mean + S.E.M. *n*=8. Each p Value was calculated using Student's t-test.

Table 2.1 Urinary parameters of 8-week-old mice before treatment with imidapril.

	db/+m	db/db	db/db + imidapril
UcreV/bwt (mg/g)	0.24 ± 0.01	0.28 ± 0.02	0.28 ± 0.02
UalbV/bwt (μg/g)	1.0 ± 0.1##	2.9 ± 0.2	2.9 ± 0.5
UHSV/bwt (μg/g)	0.27 ± 0.01	0.36 ± 0.06	0.30 ± 0.04

UcreV/bwt, UalbV/bwt, and UHSV/bwt indicate urinary excretion of creatinine, albumin, and heparan sulfate per body weight, respectively. Means ± S.E.M. *n*=8. ##p<0.01, as compared with db/db using Student's t -test.

Chapter 3　Effects of imidapril in nephrosis rats without diabetes

3.1 Summary

I investigated the effect of angiotensin-converting enzyme inhibition on spontaneous nephrosis in Dahl salt-sensitive (Dahl/S) rats. Dahl/S rats fed on a normal sodium diet spontaneously developed nephrosis and mild hypertension from a young age. In young Dahl/S rats, an ACE-I, imidapril, attenuated the development of proteinuria accompanied by a decrease in blood pressure. Methylprednisolone, a potent therapeutic agent for proteinuria, did not affect the development of nephrosis. An angiotensin type 1 (AT-1) receptor antagonist, losartan, but not a Ca^{2+} channel blocker, verapamil, inhibited the development of nephrosis while both agents decreased blood pressure to a similar extent as imidapril. In mature Dahl/S rats, imidapril suppressed not only the development of proteinuria but also the glomerular lesions. It is concluded that the development of spontaneous nephrosis in Dahl/S rats is mediated by angiotensin II.

3.2 Introduction

Dahl salt-sensitive Dahl/S. rats are genetically predisposed to develop hypertension when fed on a high salt diet (46). Dahl/S rats have been used as a model of salt-sensitive hypertension. Salt-induced hypertension in Dahl/S rats leads to renal damage (47), and there are slight morphological changes in Dahl/S rats fed on a normal sodium diet. Other investigators have demonstrated that the kidneys of Dahl/S rats are abnormal. Sterzel et al.(48) demonstrated that young Dahl/S rats had proteinuria which was not prevented by a low sodium diet. O'Donnell et al. (49) demonstrated that Dahl/S rats fed on standard laboratory chow had proteinuria and hyperlipidemia, and developed focal and segmental glomerulosclerosis. Abnormalities of lipid metabolism in Dahl/S rats also have been reported (50-53). Although Dahl/S rats develop nephrosis spontaneously, there are few studies about nephrosis in Dahl/S rats fed on a normal sodium diet. The present study was undertaken to clarify the role of angiotensin II in the development of spontaneous nephrosis in Dahl/S rats.

3.3 Materials and Methods

3.3.1 Drugs

Imidapril (CAS No. 89396-94-1) and losartan (CAS No. 124750-99-8) were synthesized by Tanabe Seiyaku (Osaka, Japan). Methylprednisolone (CAS No. 83-43-2) and verapamil (CAS No. 152-11-4) were purchased from Sigma (St. Louis, MO, USA). Each compound was dissolved in purified water and administered p.o. once a day at noon.

3.3.2 Animals

Male Dahl salt-resistance (Dahl/R) rats and Dahl/S rats were purchased from SEAC Yoshitomi (Fukuoka, Japan). These strains of Dahl rats originated in Møllegaards Avlslaboratorium (Ejby, Denmark). The rats were given standard laboratory chow containing 0.39% (w/w) of sodium (CE-2, CLEA, Tokyo, Japan) and tap water was available ad libitum throughout the experiments. Four rats were housed in one cage except during the periods of urine collection.

3.3.3 Experimental procedure

3.3.3.1 Effects of imidapril and methylprednisolone on nephrosis in young Dahl/S rats

A total of 32 Dahl/S rats aged 4 weeks were divided into four groups. Animals in each group were daily given either vehicle, imidapril (0.5 or 2 mg/kg) or methylprednisolone (2 mg/kg) for 15 days from the age of 5 weeks. Eight

age-matched Dahl/R rats were given vehicle and served as a reference. The 24-h urine collection was performed 5 days before and 1, 8 and 15 days after the start of drug administration. Urinary excretion of protein (UproV) was calculated from the urinary protein concentration and urine volume. SBP)was measured by the tail-cuff method (UR-5000, Ueda, Tokyo, Japan) 3 days before and 4 and 11 days after the start of drug administration. Blood pressure was measured in the morning just before drug administration. On the day of last urine collection, animals were anesthetized with sodium pentobarbital (50 mg/kg) by an intraperitoneal injection, and then blood samples were collected via the abdominal aorta. Plasma levels of total cholesterol, triglyceride, albumin, total protein, and plasma and urine levels of creatinine were measured, and the Ccr was calculated.

3.3.3.2 Effects of losartan and Ĺerapamil on nephrosis in young Dahl/S rats

A total of 24 Dahl/S rats aged 4 weeks were divided into three groups and were daily given either vehicle, losartan (10 mg/kg), or verapamil (60 mg/kg) for 15 days from the age of 5 weeks. Urine and blood collection, and measurements of SBP were performed following the same procedure as described above.

3.3.3.3. Effect of imidapril on nephrosis in mature Dahl/S rats

A total of 16 Dahl/S rats aged 12 weeks were divided into two groups and were daily given either vehicle or imidapril (2 mg/kg) for 22 days from the age of 13 weeks. Eight age-matched Dahl/R rats were given vehicle and served as a reference group. The 24-h urine collection was performed 5 days before and 1, 8, 15 and 22 days after the start of drug administration. Systolic blood pressure was measured 3 days before and 4 and 11 days after the start of drug administration. On the day of last urine collection, animals were anesthetized, and then blood samples were collected as described above. Then the left kidney was removed for the histological examination..

3.3.3.4 Measurements

Blood samples were placed in heparin-containing tubes on ice, and plasma samples were obtained by centrifugation (2500 rpm, 15 min, 4℃). The creatinine concentration in plasma and urine was measured by an enzymatic method (Creatinizyme, Eiken, Tokyo, Japan), using an automatic analyzer (Hitachi705, Hitachi, Tokyo, Japan). Urinary protein concentration was determined by the pyrogallol red method (Micro TP-AR, Wako, Osaka, Japan), using an automatic analyzer. Plasma levels of total cholesterol, triglyceride, albumin and total protein were measured by routine methods, using an automatic analyzer.

3.3.3.5 Histological examination

Each kidney specimen was fixed in phosphate-buffered 10% formalin and dehydrated with ethanol. The tissues were then embedded in paraffin and sectioned. The sections were stained with the PAS reagent for light microscopic examination. The percentage of glomeruli with lesions was determined by blind observation.

3.3.3.6 Statistical analysis

Data are expressed as the means ± S.E.M. Analysis of variance followed by Dunnett's test was used for comparisons between data for the control Dahl/S rats in the experiments with young Dahl/S rats. Unpaired *t*-test was used for data obtained for mature Dahl/S rats. A *P*-value less than 0.05 were considered to be statistically significant. The values for Dahl/R rats are shown as a reference, but were not included in the statistical analysis.

3.4 Results

3.4.1 Effects of imidapril and methylprednisolone on nephrosis in young Dahl/S rats

The time courses of changes in body weight, UproV, and SBP are shown in

Figure 3.1. Control Dahl/S rats developed mild hypertension and marked proteinuria during the experimental periods. Imidapril inhibited both the hypertension and the increase in UproV in a dose-dependent manner. Methylprednisolone did not affect the development of proteinuria or hypertension, but significantly inhibited the increase in body weight. Plasma chemistry values and creatinine clearance are shown in Table 3.1. Control Dahl/S rats had high levels of total cholesterol and triglyceride and low levels of albumin and total protein. Treatment with imidapril at a dose of 2 mg/kg significantly lowered the concentration of total cholesterol and triglyceride, and elevated the level of total protein and albumin. Methylprednisolone suppressed the plasma level of total cholesterol but increased the triglyceride level. Ccr was not affected by these drugs.

3.4.2 Effects of losartan and Ĺerapamil on nephrosis in young Dahl/S rats

The time courses of changes in body weight, UproV, and SBP are shown in Figure 3.2. Losartan and verapamil similarly suppressed the increase in SBP. Losartan inhibited the development of proteinuria. In contrast, verapamil inhibited UproV only on the 1st day, but not on the 8th and 15th days after the start of administration. Verapamil caused a slight but significant suppression of

the increase in body weight. Plasma chemistry values and Ccr are shown in Table 3.2. Losartan significantly elevated the plasma albumin level. Verapamil significantly elevated the plasma total cholesterol level and Ccr. Ccr was not affected by losartan.

3.4.3 Effect of imidapril on nephrosis in mature Dahl/S rats

The time courses of changes in body weight, UproV, and SBP are shown in Figure 3.3. Imidapril decreased UproV and SBP. Plasma chemistry values and Ccr were not affected by imidapril (Table 3.3). Hyaline droplets and hypertrophy of podocytes were frequently seen in some glomeruli, and adhesion of the glomerulus to Bowman's capsule was less frequently observed in control Dahl/S rats (Figure 3.4). Imidapril significantly decreased the incidence of these glomerular lesions (Figure 3.5A). The percentage of disordered glomeruli was significantly correlated with the values of UproV in Dahl/S rats (Figure 3.5B).

3.5 Discussion

This is the first report that drug treatment attenuates the development of spontaneous nephrosis in Dahl/S rats fed on a normal salt diet. There was marked proteinuria in 4-week old Dahl/S rats. Other investigators demonstrated

that Dahl/S rats showed proteinuria at the same age (48). The average absolute daily urinary excretion of protein at 4, 7, 12 and 16 weeks of age in control Dahl/S rats was 10, 89, 78 and 125 mg/day, respectively. Proteinuria in Dahl/S rats became steady after 7 weeks of ages. The 7-week old Dahl/S rats had abnormal plasma chemistry values, including high levels of total cholesterol and triglyceride, and low levels of albumin and total protein. Furthermore, I observed glomerular lesions in 16-week old Dahl/S rats. These changes were mild, but similar to those in patients with nephrosis. Thus, Dahl/S rats spontaneously develop nephrosis from a young age.

Imidapril decreased proteinuria and normalized plasma chemistry variables in young Dahl/S rats. An AT-1 receptor antagonist, losartan, also inhibited the development of nephrosis in Dahl/S rats, like imidapril. The data suggest that angiotensin II is one of the causes and/or malignant factors in spontaneous nephrosis in Dahl/S rats. I did not measure plasma angiotensin II levels in this study.

There is a report that serum angiotensin II levels of Dahl/S rats without sodium loading are the same as those of Dahl/R rats, but glomerular angiotensin II receptor density in Dahl/S rats is higher than that in Dahl/R rats Bouhnik and

Sahlgren (54, 55) reported that plasma renin activity and plasma angiotensin II concentration in Dahl/S rats tended to be lower than those in Dahl/R rats. Further studies on glomerular angiotensin II receptors are required to make clear the relationship between spontaneous nephrosis in Dahl/S rats and angiotensin II.

Verapamil, a Ca^{2+} channel blocker, prevented the development of mild systemic hypertension but not proteinuria. These results suggest that imidapril and losartan attenuate the development of nephrosis by mechanisms other than systemic hypotension. In patients with proteinuria, angiotensin-converting enzyme inhibition decreases proteinuria independently of the decrease in systemic blood pressure (56). In remnant kidney rats, a Ca^{2+} channel blocker that decreased blood pressure to the same extent as an angiotensin-converting enzyme inhibitor failed to suppress proteinuria (57). Dahl/S rats fed on a high salt diet develop severe hypertension and renal damage (47). Prevention of the hypertension by nifedipine in salt-loaded Dahl/S rats reduces renal morphological changes (58). However, spontaneous nephrosis in Dahl/S rats is different from hypertension-induced renal damage because I could not observe any morphological changes typical of hypertensive renal impairment, such as

arteriolosclerosis, in this study.

On the day after the start of drug administration, verapamil decreased proteinuria temporarily (Figure 3.2). There is a report that a decrease in blood pressure initiates the antiproteinuric effect of ACE inhibition in patients with renal disease (59). The transient reduction of proteinuria produced by imidapril or verapamil may result in part from the fall in blood pressure. A sustained attenuation of proteinuria requires inhibition of the renin–angiotensin system.

The increase in Ccr elicited by verapamil suggests the possibility that this compound causes hyperfiltration in the glomeruli (Table 3.2). I did not determine single nephron hemodynamics. ACE inhibition, however, may not affect glomerular filtration in Dahl/S rats adversely. It has been reported that an ACE-I reduces glomerular filtration pressure but that a Ca^{2+} channel blocker dose not (60). In mature Dahl/S rats, imidapril suppressed mild hypertension and proteinuria, but failed to normalize plasma chemistry variables. The reason was that Dahl/S rats treated with imidapril still leaked a certain amount of protein into the urine. Imidapril, however, did ameliorate glomerular lesions. To evaluate glomerular damages, scoring methods have often been used (61). In this study, 60–85% of glomeruli in control Dahl/S rats did not have significant histological

changes: typical glomerular lesions were the accumulation of hyaline droplets in podocytes (Figure 3.4). The changes in podocytes indicate that there was an increase in the glomerular filtration of protein. Glomerular damage, such as increase in mesangial matrix material or glomerulosclerosis, which are well-known changes in Dahl/S rat fed on a high-salt diet (61), was not frequently observed in this study. I, therefore, used the incidence of disordered glomeruli to determine glomerular lesions. There was a positive correlation between UproV and the incidence of glomerular lesions in mature Dahl/S rats. Proteinuria and glomerular lesions are closely associated in Dahl/S rats. It has been reported that the prevention of proteinuria by an ACE-I is important to renal protection in the adriamycin nephrosis rat (62).

Abnormalities of lipid metabolism in Dahl/S rats have been demonstrated (50-53). Hirano et al. (51) reported abnormal properties of lipoprotein in Dahl/S rats. Probucol treatment in Dahl/S rats fed on a normal sodium diet decreased high-density lipoprotein cholesterol levels, but it could not correct proteinuria and hypoalbuminemia (63). In this study, imidapril treatment resulted in the suppression of proteinuria and in a reduction of hypercholesterolemia and hypertriglycemia in young Dahl/S rats. The findings indicate the possibility that

the abnormal lipid metabolism inDahl/S rats is primarily caused by spontaneous nephrosis.

The development of nephrosis in Dahl/S rats was resistant to methylprednisolone, which is widely used in patients with proteinuria. I think that the dosage of methylprednisolone was enough because methylprednisolone increased the plasma level of triglyceride and suppressed the gain in body weight, which are common side effects of glucocorticoids (64). Nephrosis in Dahl/S rats is different from immunemediated glomerulonephritis. ACE-Is can be used to treat some aspects of steroid-resistant nephrosis.

In summary, Dahl/S rats developed nephrosis without sodium loading. Imidapril attenuated the development of nephrosis and renal glomerular lesions in Dahl/S rats. Angiotensin II may play an important role in the development of spontaneous nephrosis in Dahl/S rats.

3.6 Publication

The study in this chapter was published as “Involvement of angiotensin II in development of spontaneous nephrosis in Dahl salt-sensitive rats” in the *European Journal of Pharmacology*, 1998, 362: 213-9.

3.7 Figures and tables

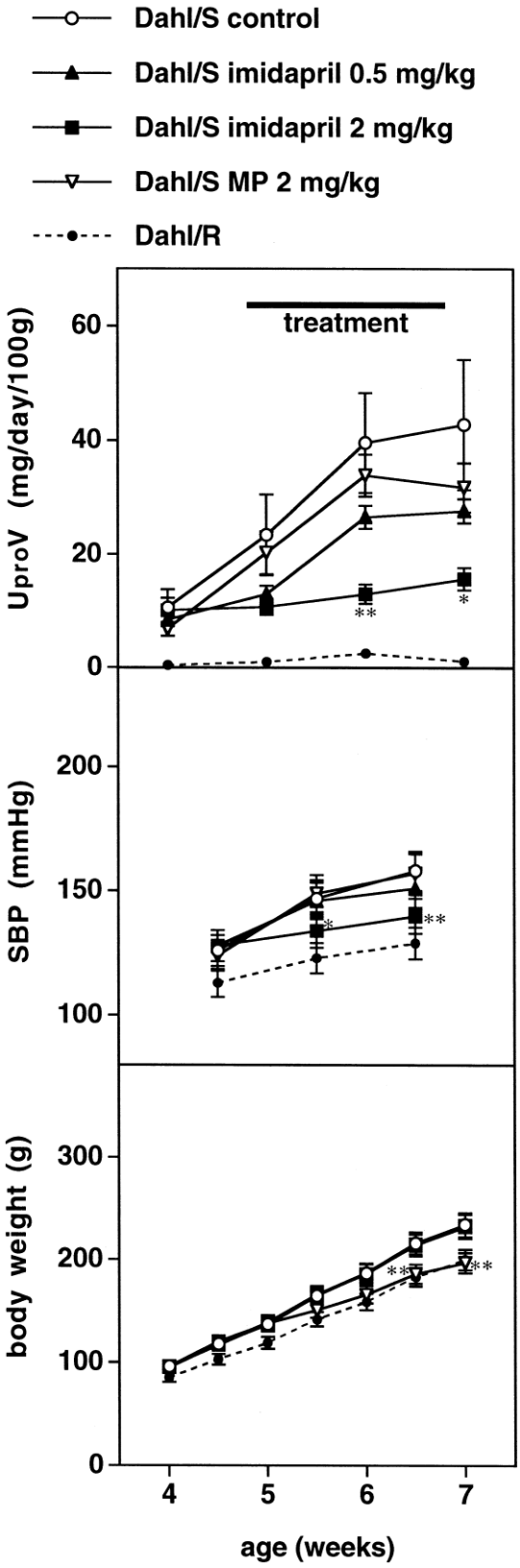

Figure 3.1 Effects of imidapril and methylprednisolone (MP) on urinary protein

excretion (UproV), systolic blood pressure (SBP), and body weight in young Dahl/S rats. Data are expressed as the means ± S.E.M. n=8. *P<0.05 and **P<0.01 as compared with corresponding Dahl/S control values by Dunnett's test. Dahl/R: Dahl salt-resistant rats.

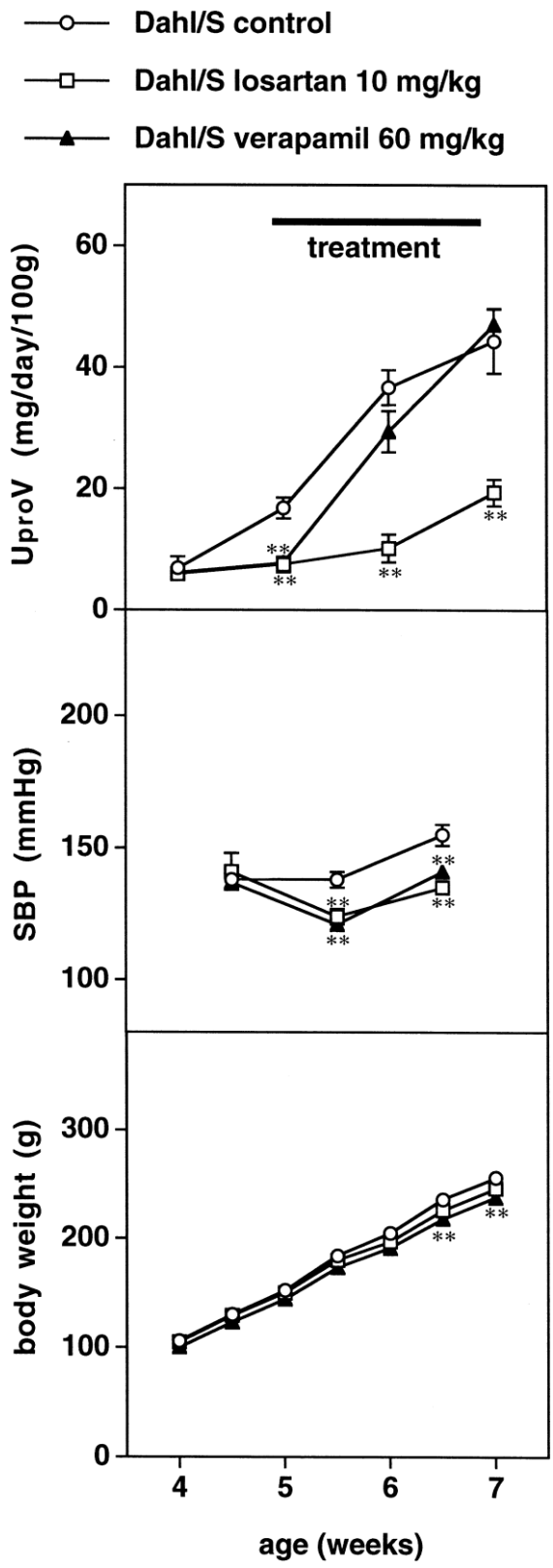
Dahl/S control
Dahl/S losartan 10 mg/kg
Dahl/S verapamil 60 mg/kg
treatment
UproV (mg/day/100g)
SBP (mmHg)
body weight (g)
age (weeks)
**

Figure 3.2 Effects of losartan and verapamil on urinary protein excretion (UproV), systolic blood pressure (SBP), and body weight in young Dahl/S rats. Data are expressed as the means ± S.E.M. *n*=8. ***P*<0.01 as compared with corresponding Dahl/S control values by Dunnett's test.

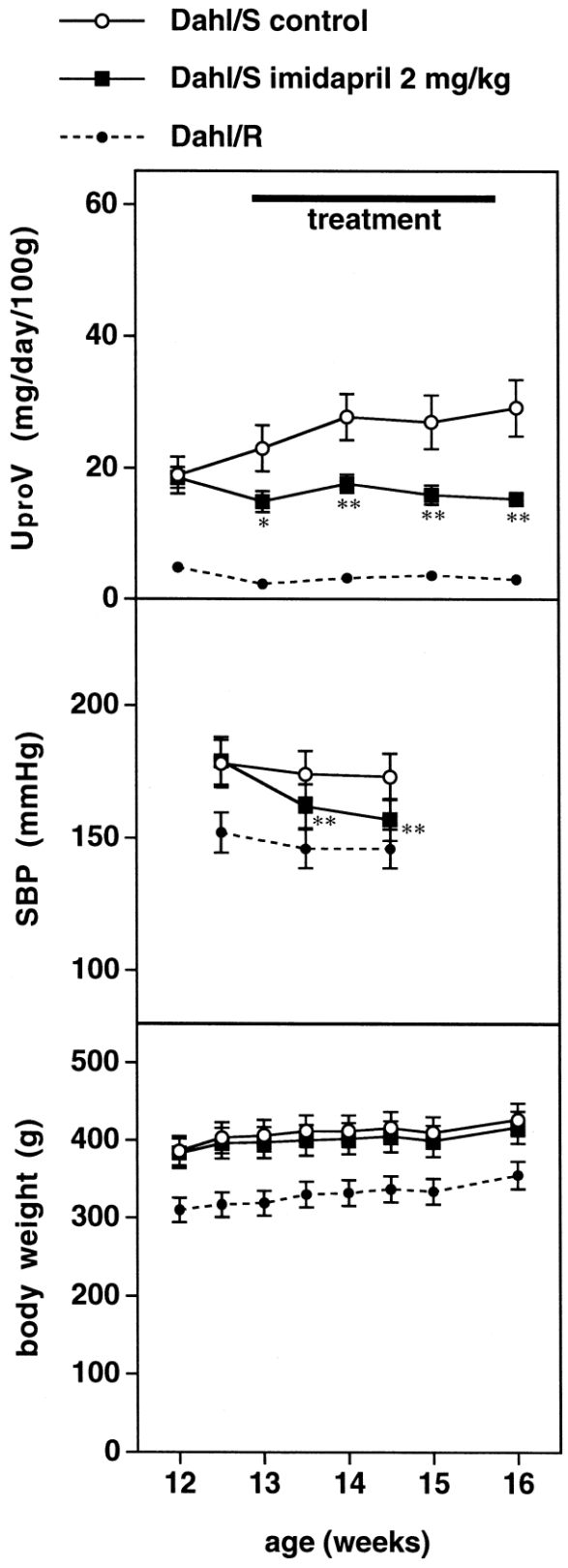
Dahl/S control
Dahl/S imidapril 2 mg/kg
Dahl/R
treatment
UproV (mg/day/100g)
60
40
20
0
*
**
**
**
SBP (mmHg)
200
150
100
**
**
body weight (g)
500
400
300
200
100
0
12
13
14
15
16
age (weeks)

Figure 3.3 Effects of imidapril on urinary protein excretion (UproV), systolic blood pressure (SBP), and body weight in mature Dahl/S rats. Data are expressed as the means ± S.E.M. n=8. *P<0.05 and **P<0.01 as compared with corresponding Dahl/S control values by unpaired t-test. Dahl/R: Dahl salt-resistant rats.

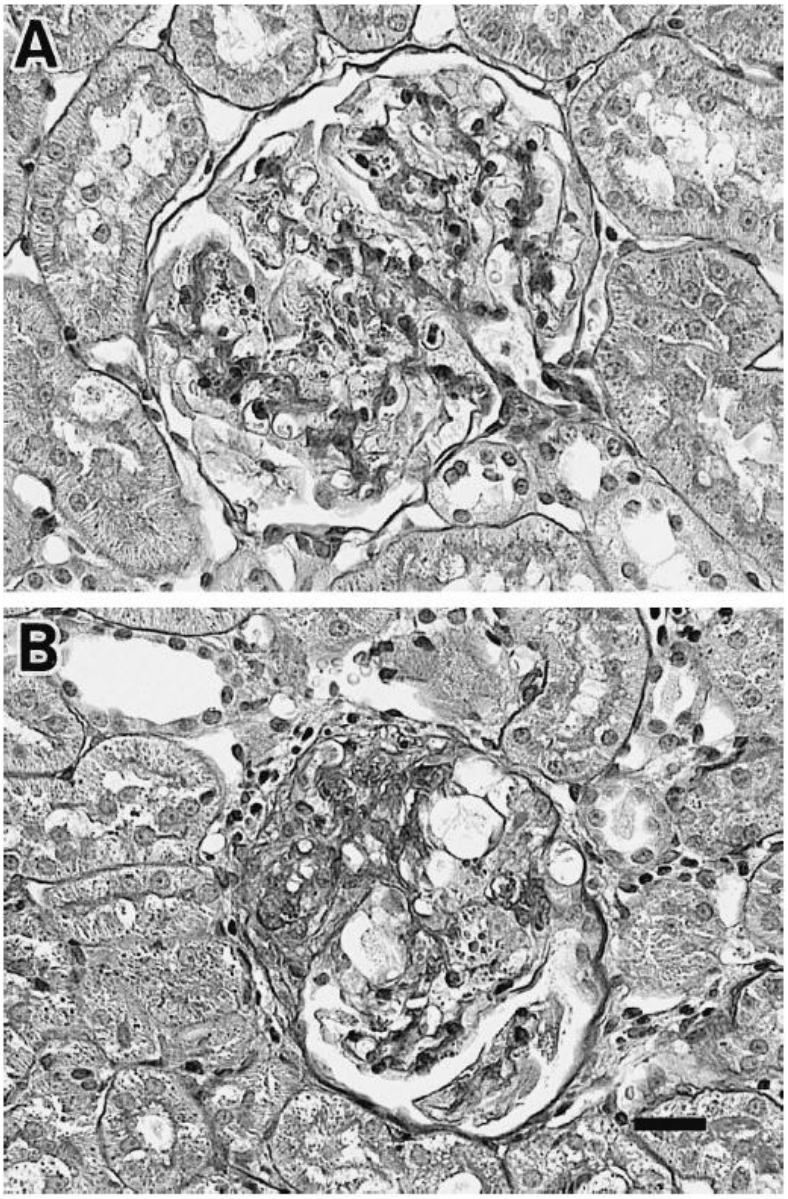

Figure 3.4 Light micrographs of typical glomerular lesions in 16-week old Dahl/S rats (periodic acid–Schiff reagent stain, bar: 25 mm) A: There are numerous hyaline droplets and hypertrophic podocytes in the glomerular tuft. B: The glomerulus is partially adhered to the Bowman's capsule, causing a segmental sclerosis.

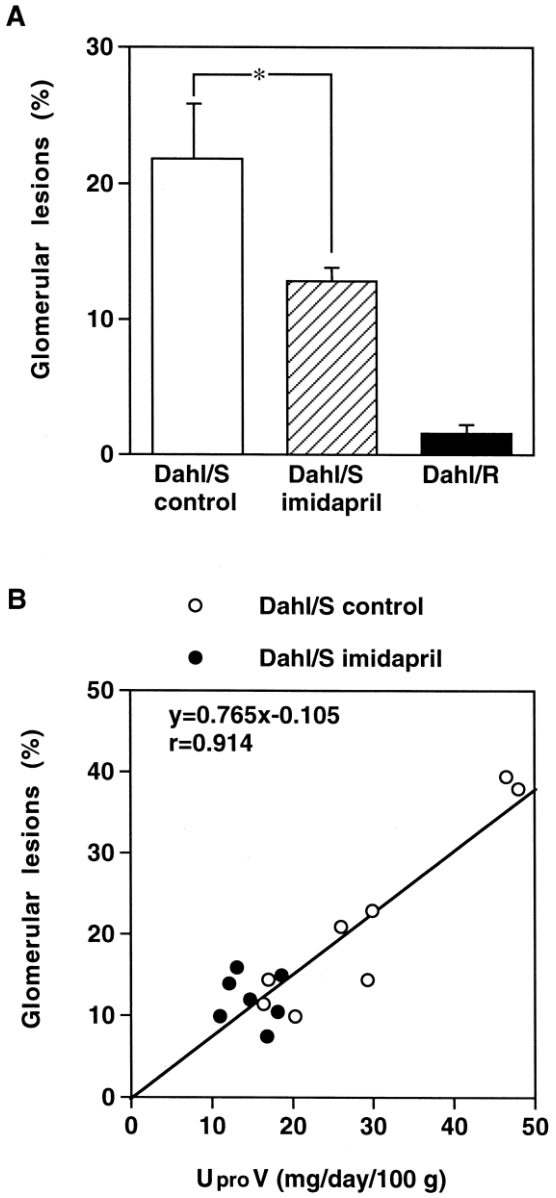

Figure 3.5 Effect of imidapril on glomerular lesions (A) and correlation between urinary protein excretion (UproV) and glomerular lesions (B) in 16-week old Dahl/S rats. Data are expressed as the means ± S.E.M. *n*=8. **P*<0.05 as compared with corresponding Dahl/S control values by unpaired *t*-test. Dahl/R: Dahl salt-resistant rats.

Table 3.1 Effects of imidapril and methylpredonisolone on plasma concentration of total cholesterol (CHO), triglyceride (TG), total protein (TP) and albumin (ALB), and creatinine clearance (Ccr) in 7-week old Dahl/S rats

Strain	Drug	Dose (μg/kg)	CHO (mg/dl)	TG (mg/dl)	TP (g/dl)	ALB (g/dl)	Ccr (ml/day/100 g)
Dahl/S	–	–	96 ± 9	153 ± 2	5.10 ± 0.07	3.07 ± 0.10	1015 ± 44
Dahl/S	imidapril	0.5	85 ± 2	132 ± 8	5.30 ± 0.04	3.26 ± 0.04	1056 ± 44
Dahl/S	imidapril	2.0	79 ± 2[a]	109 ± 8[b]	5.35 ± 0.06[a]	3.41 ± 0.03[b]	1046 ± 60
Dahl/S	MP[d]	2.0	71 ± 2[b]	329 ± 31[b]	4.98 ± 0.08	3.12 ± 0.06	1118 ± 51
Dahl/R[c]	–	–	62 ± 1	118 ± 12	4.87 ± 0.07	3.34 ± 0.04	1047 ± 28

Values are the means ± S.E.M. n=8. [a]P<0.05 and [b]P<0.01 as compared with corresponding Dahl/S control values by Dunnett's test. [c] Dahl salt-resistant rats and [d] methylpredonisolone.

Table 3.2 Effects of losartan and verapamil on plasma concentration of total cholesterol (CHO), triglyceride (TG), total protein (TP) and albumin (ALB), and creatinine clearance (Ccr) in 7-week old Dahl/S rats

Strain	Drug	Dose (μg/kg)	CHO (mg/dl)	TG (mg/dl)	TP (g/dl)	ALB (g/dl)	Ccr (ml/day/100 g)
Dahl/S	–	–	82 ± 2	167 ± 13	5.16 ± 0.07	3.27 ± 0.06	1021 ± 123
Dahl/S	losartan	10	75 ± 2	114 ± 7[b]	5.18 ± 0.01	3.43 ± 0.03	1093 ± 42
Dahl/S	verapamil	60	93 ± 2[b]	162 ± 10	5.16 ± 0.08	3.21 ± 0.04	1373 ± 66[a]

Values are the means ± S.E.M. *n*=8. [a]*P*<0.05 and [b]*P*<0.01 as compared with corresponding Dahl/S control values by Dunnett's test.

Table 3.3 Effects of imidapril on plasma concentration of total cholesterol (CHO), triglyceride (TG), total protein (TP) and albumin (ALB), and creatinine clearance (Ccr) in 16-week old Dahl/S rats

Strain	Drug	Dose (μg/kg)	CHO (mg/dl)	TG (mg/dl)	TP (g/dl)	ALB (g/dl)	Ccr (ml/day/100 g)
Dahl/S	–	–	70 ± 3	127 ± 13	5.47 ± 0.50	2.84 ± 0.30	736 ± 60
Dahl/S	imidapril	2	67 ± 2	147 ± 24	5.46 ± 0.03	2.93 ± 0.05	751 ± 41
Dahl/R[a]	–	–	49 ± 5	111 ± 15	5.20 ± 0.03	3.05 ± 0.07	820 ± 89

Values are the means ± S.E.M. *n*=8. [a]Dahl salt-resistant rats.

Concluding Remarks

There are a lot of studies about the ACE-I since its first clinical appearance in 1981. ACE-I was approved for the systemic hypertension primarily. The first report of its renoprotective effects was brought up using systemic hypertensive rats (65). After continuous studies about renoprotective effects of ACE-I, some ACE-Is included in imidapril approved for diabetic nephropathy in the late 1990's or early 2000's. In 1994, the first AT-1 receptor blocker was approved for the systemic hypertension. Since then, the interest of studies on renin-angiotensin system sifted to using AT-1 receptor blockers instead of ACE-Is in both area of basic and clinical science. It must be caused the simple mechanisms of AT-1 receptor blockers, while the ACE is known to have broad substrate specificity (66). The ACE-I is a very classical drug, however, its pharmacological mechanisms still not clear enough.

This study demonstrated that an ACE-I, imidapril has renoprotective effects in animals with albuminuria / proteinuria both of diabetic and non-diabetic conditions. Its renoprotection may be included following actions of imidapril:

1) inhibition of systemic renin-angiotensin system which produce lowering systemic blood pressure,

2) inhibition of renal renin-angiotensin system which lead protection of renal heparin sulfate, and correction of renal hyperfiltration,

3) the other mechanisms (?)

The chapter 1 and 3 indicated that lowering systemic blood pressure should be important, but the only vasodilation is not enough to produce renoprotective effects. Additionally, imidapril showed renoprotective effect without lowering systemic blood pressure in the chapter 2. These findings suggest that renoprotective effcts of imidapril exist beyond the antihypertensive effects. The antihypertensive effects of ACE-Is based on the inhibition of systemic renin-angiotensin system, and additionally facilitates of vasodilation by bradykinin. Inhibition of the renal local renin-angiotensin system might be important in the renoprotective effects of imidapril.

Major parts of renoprotective effects of imidapril may produce as direct action to the renal renin-angiotensin system. The balance of contribution to renoprotective effects between antihypertensive effects and inhibition the renal local renin-angiotensin system should be different from drugs, even in the ACE-Is. Imidapril showed stronger inhibition of the renal ACE activity than another ACE-I, so that the effects may strongly contribute renoprotective effects of imidapril. The

renal renin-angiotensin system are important to maintain the glomerular structures (67). The chapter 2 demonstrated that imidapril suggested that protected haparan sulfate, which is one of the glomerular basement membrane components. These facts indicated that the over works of renal renin-angiotensin system might injure glomerular structures.

Renal glomerular hyperfiltration is one of the typical symptoms in diabetes. Imidapril showed correction of hyperfiltration in the chapter 2, but did not affect in the chapter 1 and 3. Imidapril increased renal blood flow and Ccr in volume-loaded dogs (68). Glomerular filtration is controlled by a complicated procedure, for example blood pressure, hydration state, renin-angiotensin system, and so on. Therefore the effect of imidapril against renal filtration might be variable. In the clinical use of ACE-Is, acute renal failure is a considerable adverse effect (69). The facts proved that the renal renin-angiotensin system participates the fundamental function of the kidney both of normal and disease states. Therefore the correction of hyperfiltration states accompanied with diabetes would be useful for the renoprotective effects. In the chapter 3, verapamil, which did not reduce proteinuria enough, increased Ccr. Excess glomerular filtration might be wrong even in the non-diabetic condition.

The chapter 3 suggested that proteinuria closely related with glomerular damage. Throughout this study, I could not resolve whole mechanisms of reducing albminuria or proteinuria by imidapril, however, these effects of imidapril should strongly contribute to protect the renal glomerular structures.

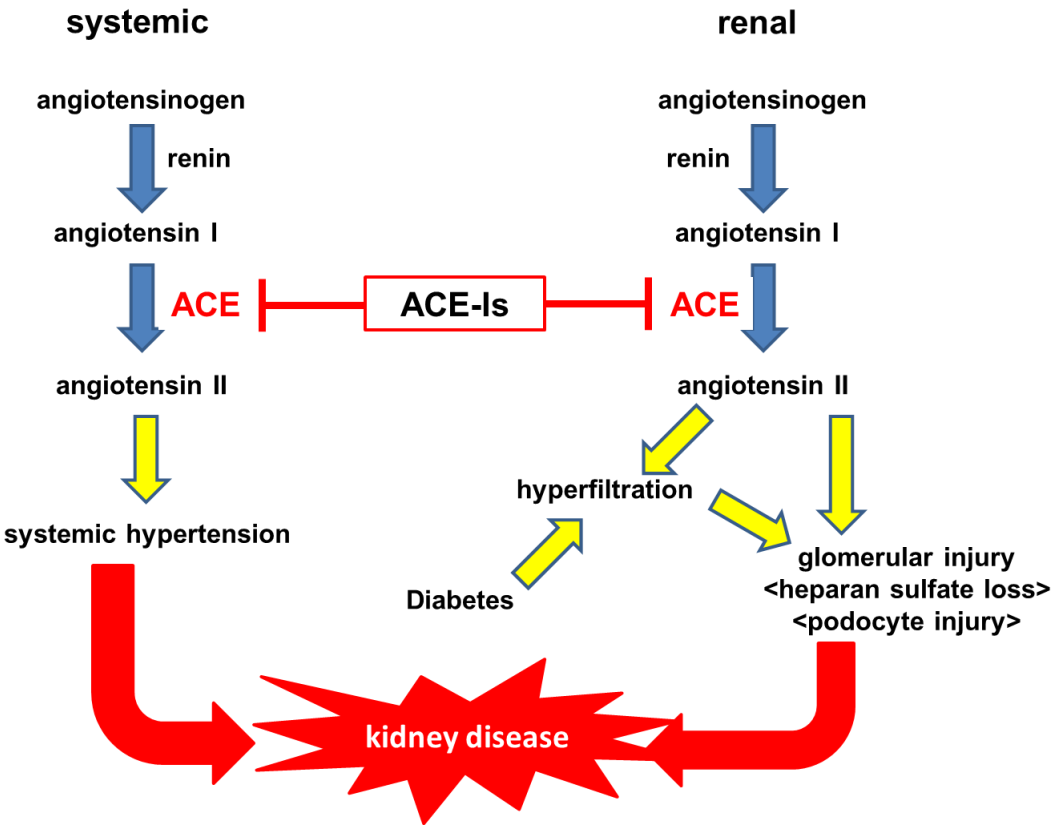

Figure Angiotensin-converting enzyme inhibitors (ACE-Is) prevents kidney disease by inhibiting both of the systemic and renal renin-angiotensin system.

Considering these findings, imidapril produce its renoprotective effects through both functional and structural improvement in the kidney. Furthermore, imidapril

exhibits its renoprotective effects not only diabetic conditions but non-diabetic nephrosis.

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