

Amelioration of Diabetic Microalbuminuria and Lipid Peroxidation by Captopril

Hunjoo Ha and Kyung Hwan Kim

Administration of captopril, a scavenger of oxygen derived radicals as well as an inhibitor of angiotensin converting enzyme, has been an efficient way of treating diabetic proteinuria. In the present study, we evaluate whether captopril can ameliorate diabetic proteinuria as an effect on oxidative stress in streptozotocin-induced diabetic rats (STZR). At four weeks after the injection of streptozotocin (50 mg/kg, i.v.), STZR (n=5) exhibited microalbuminuria. The rate of urinary albumin excretion was 0.5 ± 0.1 and 2.6 ± 0.3 mg/24hr in age-matched control rats (CR; n=5) and STZR, respectively. Compared to CR, STZR also showed an extremely increased rate of urinary lipid peroxides (LPO) excretion, an index of oxygen derived radicals generation. The respective values for CR and STZR were 0.6 ± 0.3 and 6.9 ± 0.6 μ mol/24 hr. Significant amelioration of urinary albumin and LPO excretion rate by the treatment of insulin (2 U/day) suggests that these are associated with the diabetic state induced by streptozotocin rather than a direct effect of streptozotocin. Chronic administration of captopril, which did not cause any discernible effect on CR, significantly reduced the urinary albumin excretion rate and decreased LPO excretion in STZR. The urinary albumin excretion rate was significantly correlated with the LPO excretion rate ($p=0.0004$). These results suggest that oxidative stress can be responsible for diabetic microalbuminuria, and captopril could diminish the lipid peroxidation and ameliorate the microalbuminuria in diabetic rats.

Key Words: Microalbuminuria, lipid peroxidation, captopril, insulin

Renal failure is the main cause of mortality and morbidity in diabetic patients. Compared to other chronic renal failure patients, patients having diabetic nephropathy are induced to hemodialysis

more rapidly. Thus, it is especially important to detect diabetic nephropathy in its earlier stage and to start the treatment before nephropathy becomes overt. In this context, the clinical significance of microalbuminuria as a predictor of overt nephropathy is now well accepted (Mogensen 1990).

Many studies were conducted to understand the mechanisms of diabetic microalbuminuria and to elucidate rational treatment. For the functional point of view, either the increased glomerular albumin filtration (Deckert et al. 1988; Myers et al. 1982) or the decreased proximal tubular protein reabsorption (Ha et al. 1991) was suggested as the cause of diabetic microalbuminuria. As a biochemical abnormality, oxidative stress along with a synergistic interaction with protein glycation has recently been considered a common pathway linking diverse diabetic complications (Baynes 1991; Wolff et al. 1991). In terms of diabetic nephropathy, diabetic rats exhibiting microalbuminuria produced extremely increased urinary excretion of lipid perox-

Received March 4, 1992

Accepted July 3, 1992

Department of Pharmacology, Yonsei University College of Medicine, Seoul, Korea

This work was supported in part by a grant from the CMB-YUHAN Research Fund (1990) of the Yonsei University College of Medicine and the Serim Welfare Foundation, Korea.

Address reprint requests to Dr. H Ha, Department of Pharmacology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul, Korea, 120-752

Abbreviations

Captopril-CR; captopril treated control rats

Captopril-STZR; captopril treated streptozotocin-induced diabetic rats

CR; control rats

LPO; lipid peroxides

STZR; streptozotocin-induced diabetic rats

TBA; thiobarbituric acid

ides (LPO), which is an index of oxygen derived free radicals generation (Ha *et al.* 1991).

On the other hand, the inhibition of angiotensin converting enzyme is one of the most efficient ways to reduce proteinuria, albeit the exact mechanisms are not clear, in diverse diseases including diabetes mellitus (Dunn 1990). Captopril, a well known angiotensin converting enzyme inhibitor, has an additional action scavenging of the oxygen derived radicals and preventing reperfusion cardiac injury (Westlin and Mullane 1988; Pi and Chen 1989). We therefore evaluated whether captopril could ameliorate microalbuminuria as an effect on oxidative stress, estimated by lipid peroxidation, in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Male Sprague-Dawley rats from the animal facility, Yonsei University College of Medicine were utilized for this study. Four experimental groups were formed: streptozotocin-induced diabetic rats (STZR), age matched control rats (CR), captopril treated STZR (captopril-STZR) and captopril treated CR (captopril-CR). Plasma glucose, rates of urinary albumin and lipid peroxides (LPO) excretion, and plasma LPO were measured over the course of the early stage of diabetes mellitus, i.e. 1, 2 and 4 weeks after the induction of diabetes. Data obtained at 4 weeks after the induction of diabetes are presented, since at this stage STZR are characterized by increased renal vascular resistance (Ha and Dunham 1987), microalbuminuria and minimal structural changes in the proximal tubules (Woo 1991).

Induction of Experimental Diabetes Mellitus

Diabetes was induced by intravenous injection of streptozotocin (50 mg/kg; Sigma Chemical Co., St. Louis, MO) dissolved in pH 4.5 citrate buffer immediately before injection. Age-matched 7-week-old CR received the same volume of citrate buffer (2.5 ml/kg). Captopril (a gift from Squibb & Sons, Seoul, Korea) dissolved to the amount of either 100 mg or 400 mg per liter of drinking water was provided to the STZR or CR, respectively (captopril-STZR, captopril-CR). A lower dose of captopril to diabetic rats was based on polydipsia associated with diabetic state. The efficacy of the above two doses of captopril was verified by the inhibition of exogenous angiotensin I response in our preliminary

study. Induction of diabetic state was confirmed by estimating urine glucose with commercial enzymatic test strips on the following day.

To exclude the possibility of direct nephrotoxicity of streptozotocin, a separate set of experiments consisted of CR, STZR and insulin treated STZR were conducted. Two days after streptozotocin injection, NPH insulin (Dong Shin Pharm. Co., Ltd., Pyungtag, Korea) was subcutaneously administered at a dose of 2 U/day at 4 P.M.

All rats were maintained on standard rat chow (Samyang rat chow, Seoul, Korea) and tap water with or without captopril.

Measurements of Plasma Glucose and Urinary Albumin Excretion Rate

Blood samples were obtained from the subjects' tail veins and urine was collected for 24 hours. Plasma glucose was measured by the glucose oxidase method using a commercial diagnostic kit (510-DA; Sigma Chemical Co., St. Louis, MO). Urinary albumin was measured by means of quantitative reaction with bromocresol green using a Sigma diagnostic kit (631-2).

Measurement of Lipid Peroxides in Plasma and Urine

The thiobarbituric acid (TBA) method of Ohkawa *et al.* (1979) was used to measure the level of lipid peroxides. Each 100 μ l of the appropriately diluted sample of plasma or urine was mixed with 100 μ l of 4% sodium dodecyl sulfate, and then a reaction mixture consisting 400 μ l of 0.8% 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid) and 400 μ l of 20% acetic acid was added. This solution was placed in a water bath and kept at 95°C for 60 minutes. After stopping the reaction by cooling with tap water, the mixture was centrifuged at 15,000 g for 15 min to precipitate any interfering particulate materials. Our preliminary study revealed that the extraction procedure could be skipped due to enough sensitivity. The amount of lipid peroxides formed was measured by spectrofluorometry (SPF-500 C; SLM instruments, Inc., Urbana, IL) at emission wavelength 553 nm with excitation wavelength 515 nm.

To exclude the possibility of false positive being caused by glucose in TBA method, the effects of glucose up to 448 mM (the urinary concentration of glucose in STZR was about 250 mM) were tested.

Analysis of Data

All results are expressed as means \pm SE. Analysis

of variance (ANOVA) was employed to analyze the values for general characteristics, rates of urinary albumin and LPO excretion, and plasma LPO among experimental groups. If the F statistic was significant, the mean values obtained from each group of rats were compared by the Fisher's least significant difference method. The correlation coefficient was calculated to analyze the relationship between any of two variables among plasma glucose, rates of urinary albumin and LPO excretion, and plasma LPO. The p value less than 0.05 was used as the criterion

for statistically significant differences.

RESULTS

General Characteristics, Plasma Glucose, Rates of Urinary Albumin and LPO Excretion, and Plasma LPO (Table 1)

Rats whose urine glucose concentration was higher than 2,000 mg/dl at two days after strep-

Table 1. General characteristics, urinary albumin and LPO excretion rates, and plasma LPO in streptozotocin induced diabetic rats and effects of captopril on them

	CR	Captopril-CR	STZR	Captopril-STZR
Plasma Glucose (mg/dl)	103 ± 11	119 ± 2	469 ± 17*	431 ± 36*
Urine Volume (ml/24 hr)	12 ± 6	19 ± 2	141 ± 16*	96 ± 15*, †
Body Weight (g)	300 ± 8	304 ± 4	232 ± 10*	238 ± 15*
Kidney Weight (g)	1.12 ± 0.05	1.16 ± 0.03	1.31 ± 0.06*	1.32 ± 0.06*
Urinary Albumin Excretion rate (mg/24 hr)	0.5 ± 0.1	0.8 ± 0.1	2.6 ± 0.3*	1.6 ± 0.3*, †
Urinary Lipid Peroxides Excretion rate (μmol/24 hr)	0.6 ± 0.3	0.6 ± 0.1	6.9 ± 0.6*	4.6 ± 0.7*, †
Plasma Lipid Peroxides (nmol/ml)	4.0 ± 0.9	1.3 ± 0.4	16.4 ± 5.0*	4.7 ± 1.1†

Values are means ± SE obtained from 5 rats/group at four weeks after treatment (11 to 12 week-old-rats).

LPO; lipid peroxides, CR; Citrate buffer injected age-matched control rats, Captopril-CR; Control rats provided with captopril 400 mg/liter of drinking water, STZR; Streptozotocin (50 mg/kg i.v.) induced diabetic rats, Captopril-STZR; Streptozotocin-induced diabetic rats provided with captopril 100 mg/liter of drinking water

Fisher's least significant difference method was used, when F statistic of ANOVA was significant.

*p < 0.05 compared to CR

†p < 0.05 compared to STZR

Table 2. General characteristics, urinary albumin and LPO excretion rates, and plasma LPO in streptozotocin induced diabetic rats and effects of insulin

	CR	STZR	Insulin-STZR
Number of Animal Used	5	6	5
Plasma Glucose (mg/dl)	101 ± 6	469 ± 13*	196 ± 18*, †
Urine Volume (ml/24 hr)	7 ± 2	169 ± 17*	84 ± 27*, †
Body Weight (g)	328 ± 10	230 ± 15*	328 ± 20†
Urinary Albumin Excretion rate (mg/24 hr)	0.5 ± 0.1	2.0 ± 0.2*	0.8 ± 0.1†
Urinary Lipid Peroxides Excretion rate (μmol/24 hr)	0.5 ± 0.1	7.7 ± 1.4*	4.0 ± 0.7*, †
Plasma Lipid Peroxides (nmol/ml)	4.1 ± 0.3	20.2 ± 2.9*	8.4 ± 1.3†

Values are means ± SE obtained at four weeks after treatment (11 to 12 week-old-rats).

LPO; lipid peroxides, CR; Citrate buffer injected age-matched control rats, Captopril-CR; Control rats provided with captopril 400 mg/liter of drinking water, STZR; Streptozotocin (50 mg/kg i.v.) induced diabetic rats, Captopril-STZR; Streptozotocin-induced diabetic rats provided with captopril 100 mg/liter of drinking water

Fisher's least significant difference method was used, when F statistic of ANOVA was significant.

*p < 0.05 compared to CR

†p < 0.05 compared to STZR

tozotocin injection were used for the study. These rats failed to gain body weight as compared to control and exhibited polyuria. STZR, whose plasma glucose level was significantly higher than control, had significantly lower body weight but higher kidney weight than CR at four weeks after treatment. Captopril-STZR exhibited statistically less urine volume than STZR. However, captopril did not cause any discernible effects on plasma glucose or body and kidney weights of STZR or CR.

The mean values of albumin excretion rate in diabetic STZR were significantly higher than CR. Urinary LPO was much higher (12 times) in STZR compared to CR. This increase in urinary LPO was partly due to increase in plasma LPO (4 times) in STZR. Captopril ameliorated all these changes in STZR without causing any discernible effects on any of these values in CR.

Effects of Insulin Treatment on Plasma Glucose, Rates of Urinary Albumin and LPO Excretion, and Plasma LPO in STZR (Table 2)

Injection of insulin 2 U/day significantly ameliorated hyperglycemia and polyuria exhibited in STZR. These insulin-treated STZR developed a similar body weight to the CR. The increased lipid peroxidation and rate of albumin excretion were also attenuated by insulin. Thus, the albumin excre-

tion rate and plasma LPO in insulin-treated STZR were similar to those of the CR.

Relationship among Plasma Glucose, Rates of Urinary Albumin and LPO Excretion, and Plasma LPO (Table 3)

When the data obtained from all the groups of present study were pooled and analyzed, the correlation coefficient values always exhibited statistical significance between any of two variables. Table 3

Table 3. Correlation coefficient among plasma glucose, urinary albumin and LPO excretion rates, and plasma LPO

	r	p
Albumin Excretion vs Plasma Glucose	0.758	0.0001
Albumin Excretion vs LPO Excretion	0.704	0.0004
Albumin Excretion vs Plasma LPO	0.415	0.0615
LPO Excretion vs Plasma Glucose	0.542	0.011
LPO Excretion vs Plasma LPO	0.489	0.0247
Plasma LPO vs Plasma Glucose	0.452	0.04

Data from 11 streptozotocin-induced diabetic rats (STZR), 5 captopril treated STZR, and 5 insulin treated STZR were analyzed.

LPO; lipid peroxides

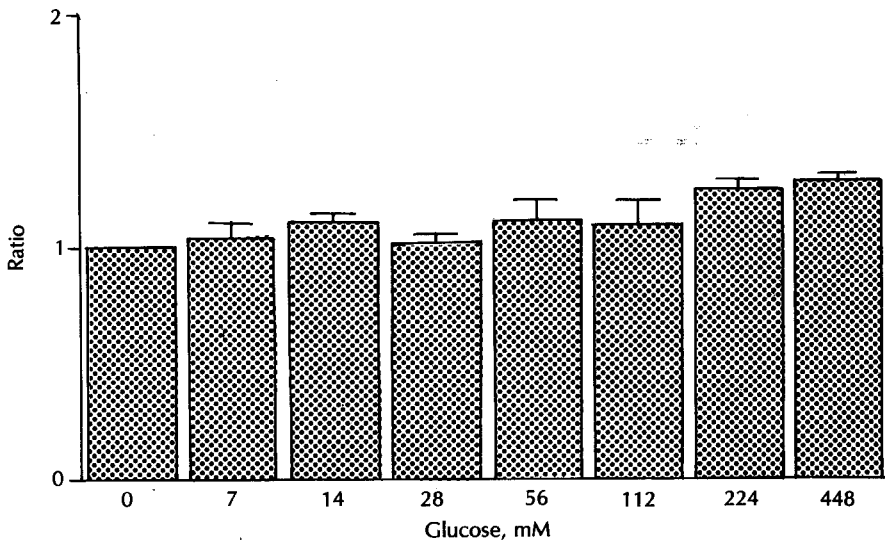


Fig. 1. Effects of glucose on lipid peroxides measured by thiobarbituric acid method. Values are means \pm SE obtained from five different samples of urine. Detailed procedures are described in Methods.

summarizes the correlation coefficient values after excluding the data from the CR. The rate of albumin excretion had strong correlations with plasma glucose and LPO excretion rate.

Analysis of the values from STZR and captopril-STZR revealed that the relationship between urinary excretion rates of albumin and LPO was the most significant ($r=0.754$; $p<0.01$) followed by the relationship between urinary LPO and plasma glucose ($r=0.739$, $p<0.01$).

Effects of Glucose on Thiobarbituric Acid Method (Figure 1)

Glucose up to the concentration which was equivalent to 448 mM in collected urine did not cause any significant effect on LPO measured in the present study ($p=0.092$).

DISCUSSION

An understanding of the mechanisms for diabetic microalbuminuria, which has become a well accepted predictor of overt nephropathy (Mogensen 1990), is more important than ever for providing rational treatment. While the exact mechanisms for diabetic microalbuminuria remain to be discovered, the inhibition of angiotensin converting enzyme is a known efficient way to reduce proteinuria in diverse disease including diabetes mellitus (Dunn 1990). Considering diverse effects of angiotensin II on the kidney (Ichikawa and Harris, 1991), the mechanism of this antiproteinuric effect of angiotensin converting enzyme inhibitor could be related to either the specific renal hemodynamic effects due to the inhibition of angiotensin II synthesis (Yoshioka *et al.* 1986; Pelayo *et al.* 1990) or a non-hemodynamic mechanism of action (Remuzzi *et al.* 1990; Heeg *et al.* 1991). Woo (1991) also reported that captopril ameliorated microalbuminuria without any discernible effect on basal renal hemodynamics of STZR. Whatever abnormalities in renal physiology caused microalbuminuria, oxidative stress has been suggested as a biochemical abnormality in diabetic kidney (Baynes 1991; Wolff *et al.* 1991). The present study was conducted to evaluate whether captopril can ameliorate microalbuminuria as a result of reduced oxidative stress in STZ-induced diabetic rats, on the basis that 1) LPO excretion, an index of oxidative stress, is increased in diabetic rats exhibiting microalbuminuria (Ha *et al.* 1991), and 2) captopril is a scavenger for oxygen derived radicals

(Westlin and Mullane 1988, Pi and Chen 1989).

At four weeks after the injection of streptozotocin, the STZR exhibited typical characteristics of diabetes mellitus such as hyperglycemia, polyuria, reduced growth rate and hypertrophied kidneys (Table 1). These rats also produced increased LPO as well as albumin excretion, which agrees with a previous study (Ha *et al.* 1991). Insulin treatment (2 U/day) reduced albumin and LPO excretion along with plasma glucose. This indicates that microalbuminuria and increased urinary excretion of LPO in STZR would be due not to direct nephrotoxic effects of streptozotocin but to drug induced diabetic renal dysfunction. It would have been better if insulin at higher dose normalizing hyperglycemia had been employed in the present study.

In agreement with a previous report (Dunn 1990), captopril ameliorated microalbuminuria in STZR. Captopril also ameliorated lipid peroxidation associated with diabetes without any significant effect on corresponding values of control rats. This suggests that oxidative stress may be involved in diabetic microalbuminuria as we hypothesized. Oxidative stress has already been reported as a possible cause for several different renal disease (Shah 1989 and references therein). Although a direct way to address the involvement of oxidative stress in diabetic microalbuminuria would be the quantification of reactive oxygen species, we employed an indirect method for measuring LPO. Some phases of oxidative stress have been discussed in relation to lipid peroxidation (Haugaard 1968), and the values of LPO are often regarded as an index of reactive oxygen species generation. Despite several cautions raised by Janero (1990), the TBA method employed in the present study has been the leading method for measurement of LPO among several methods (Gutteridge and Halliwell 1990). There is a significant correlation between the TBA method and reduced iodide methods using urine as a sample, and the TBA method is a sensitive and relatively specific determinant for urinary LPO (Ha and Endou 1992). Glucose is one of the known substances which produces false positive values in TBA method. Nevertheless the concentration of glucose equivalent up to 448 mM in collected urine did not influence LPO measured in this study. This excludes the possibility that the increased urinary LPO in STZR is a methodological artifact resulted from high glucose in urine. Moreover, captopril-STZR whose plasma and urinary glucose were similar to STZR exhibited lower LPO excretion than STZR. This provided an additional evidence that LPO measured in

diabetic urine represented increased lipid peroxidation, i.e. increased oxidative stress.

Ceriello *et al.* (1991) suggested that the increased superoxide generation in serum of diabetic patient could be reduced by metabolic control. The increase of plasma LPO, but not urinary LPO excretion, in STZR was virtually normalized by captopril treatment in the present study (Table 1). Together with this, a greater increase in urinary LPO compared to plasma LPO suggests that the diabetic kidney may be involved in LPO production. Ha *et al.* (1991) showed that LPO was increased in proximal tubules of the diabetic kidney. If STZR had exhibited renal hyperfiltration, this could have increased LPO filtered and excretion. However, inulin clearance revealed that diabetic STZR employed in the present study had similar glomerular filtration rate as CR, and captopril did not alter glomerular filtration rate of STZR (Data not shown). Unlike moderate hyperglycemic rats treated with insulin, non-insulin treated diabetic rats have not exhibited renal hyperfiltration (O'Donnell 1988 and reference therein). Therefore, neither increased LPO excretion in STZR nor decreased LPO excretion by captopril can be attributable to an altered glomerular filtration rate. While the exact characteristics of increased urinary LPO in STZR remain to be discovered, the significant correlation between urinary excretion rates of albumin and LPO (Table 3) further suggests that urinary LPO can be an alternative index for diabetic microalbuminuria.

Collectively, the present study demonstrates that captopril ameliorates microalbuminuria and reduces lipid peroxidation, and, further, the urinary excretion rates of albumin is significantly correlated with LPO excretion rates. It would be interesting to evaluate whether other drugs having the ability to scavenge reactive oxygen metabolites can also ameliorate diabetic microalbuminuria.

REFERENCES

- Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405-412, 1991
- Ceriello A, Guigliano D, Quatraro A, Russo PD, Lefebvre PJ: Metabolic control may influence the increased superoxide generation in diabetic serum. *Diabetic Medicine* 8: 540-542, 1991
- Deckert T, Feldt-Rasmussen B, Djurup R, Deckert M: Glomerular size and charge selectivity in insulin-dependent diabetes mellitus. *Kidney Int* 33: 100-106, 1988
- Dunn MJ: Prostaglandins, angiotensin II, and proteinuria. *Nephron* 55 (Suppl 1): 30-37, 1990
- Gutteridge JMC, Halliwell B: The measurement and mechanism of lipid peroxidation in biological systems. *TIBS* 15: 129-135, 1990
- Ha H, Endou H: Lipid peroxidation in isolated nephron segments. *Am J Physiol* in press, 1992
- Ha H, Tojo A, Endou H: Proximal tubular dysfunction as a cause of diabetic microalbuminuria (Abstract). *Physiologist* 34: 249, 1991
- Haugaard N: Cellular mechanisms of oxygen toxicity. *Physiol Rev* 48: 311-373, 1968
- Heeg JE, De Jong PE, van der Hem GK, Zeeuw D: Angiotensin II does not acutely reverse the reduction of proteinuria by long-term ACE inhibition. *Kidney Int* 40: 734-740, 1991
- Ichikawa I, Harris RC: Angiotensin action in the kidney: Renewed insight into the old hormone. *Kidney Int* 40: 583-596, 1991
- Janero DR: Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biol Med* 9: 515-540, 1990
- Mogensen CE: Prediction of clinical diabetic nephropathy in IDDM patients. Alternatives to microalbuminuria? *Diabetes* 39: 761-767, 1990
- Myers BD, Winetz JA, Chui F, Michaels AS: Mechanisms of proteinuria in diabetic nephropathy: A study of glomerular barrier function. *Kidney Int* 21: 633-641, 1982
- O'Donnell MP, Kasiske BL, Keane WF: Glomerular hemodynamic and structural alteration in experimental diabetes mellitus. *FASEB J* 2: 2339-2347, 1988
- Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351-358, 1979
- Pelayo JC, Quan AH, Shanley PF: Angiotensin II control of the renal microcirculation in rats with reduced mass. *Am J Physiol* 258: F414-F422, 1990
- Pi X-j, Chen X: Captopril and ramiprilat protect against free radical injury in isolated working rat heart. *J Mol Cell Cardiol* 21: 1261-1271, 1989
- Remuzzi A, Puntoriere S, Battaglia C, Bertani T, Remuzzi G: Angiotensin converting enzyme inhibition ameliorates glomerular filtration of macromolecules and water and lessens glomerular injury in the rat. *J Clin Invest* 85: 541-549, 1990
- Shah SV: Role of reactive oxygen metabolites in experimental glomerular disease. *Kidney Int* 35: 1093-1106, 1989
- Westlin W, Mullane K: Dose captopril attenuate reperfusion-induced myocardial dysfunction by scavenging free radicals? *Circulation* 77 (Suppl 1): 130-

139, 1988

Wolff SP, Jiang ZY, Hunt JV: Protein glycation and oxidative stress in diabetes mellitus and aging. *Free Radical Biol Med* 10: 339-352, 1991

Woo HC: The role of renin-angiotensin system in the

pathogenesis of diabetic nephropathy (Ph. D. Thesis). Seoul, Korea, Yonsei University, 1991

Yoshioka T, Mitarai T, Kon V, Deen WM, Rennke HG, Ichikawa I: Role for angiotensin II in an overt functional proteinuria. *Kidney Int* 30: 538, 1986