Effects of AST-120 (Kremezin®) on Oxidative Stress and Fibronectin Expression in Experimental Diabetic Nephropathy

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Directed by Professor Shin-Wook Kang

Doctoral Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Written by Ea Wha Kang

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Background: Numerous experimental and clinical evidences indicate that oxidative stress plays an important role in the development and progression of diabetic nephropathy. AST-120 (Kremezin®, Kureha-Chemical Co., Tokyo, Japan) is a well-known oral adsorbent which binds and prevents absorption of several biologically active substances such as indole from the gastrointestinal tract. Indole is a precursor of indoxyl sulfate, which is known as a uremic substance, and serum

indoxyl sulfate concentrations are reported to be increased in patients with decreased renal function. In animal and human studies on renal failure, a close relationship was found between indoxyl sulfate and oxidative stress. Moreover, AST-120 treatment inhibited the progression of diabetic and non-diabetic chronic kidney disease along with a decrease in oxidative stress. To date, however, the effect of AST-120 on non-uremic kidney disease has never been explored.

Purpose: This study was undertaken to investigate whether the administration of oral adsorbent, AST-120, could reduce oxidative stress and ameliorate the development of nephropathy in experimental diabetic rats with normal renal function in terms of extracellular matrix (ECM) accumulation.

Methods: Thirty-two Sprague-Dawley rats were injected with diluent (C, N=16) or 65 mg/kg streptozotocin intraperitoneally (DM, N=16). Eight rats from each group were treated with standard laboratory chow containing 5% AST-120 (Kremezin®) (C+AST-120, DM+AST-120) for 3 months. At the time of sacrifice, 24-hour urinary albumin excretion and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) excretion were determined by ELISA, and serum indoxyl sulfate concentrations by high performance liquid chromatography (HPLC). Glomerular

endothelial nitric oxide synthase (eNOS), subunits of NADPH oxidase (gp91phox, p47phox, and p22phox), and fibronectin mRNA and protein expressions were determined by real-time PCR and Western blot, respectively, with sieved glomeruli, and these protein expressions in renal tissue were also determined by immunohistochemical staining. In addition, Dichlordihydrofluorescin diacetate (DCF-DA) staining was performed to detect glomerular reactive oxygen species production.

Results: Compared to the C group (0.40±0.06 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (1.99±0.17 mg/day, $p<0.01$), and AST-120 treatment significantly reduced albuminuria in DM rats (1.04±0.19 mg/day, $p<0.05$). On the other hand, serum creatinine and indoxyl sulfate levels were comparable among the four groups. Glomerular eNOS, gp91phox, and p47phox mRNA expressions were significantly increased in DM rats by 2.1-, 3.3-, and 2.7-folds, respectively ($p<0.05$), compared to C rats, and these increases in DM glomeruli were significantly abrogated by AST-120 treatment ($p<0.05$). In contrast, there was no difference in glomerular p22phox mRNA expression among the four groups. Fibronectin mRNA expression was also increased in DM compared to C glomeruli, and AST-120 treatment significantly ameliorated this

increase in DM rats ($p<0.05$). The protein expression of these molecules assessed by Western blot showed a similar pattern to the mRNA expression. In addition, immunohistochemical staining for glomerular eNOS, subunits of NADPH oxidase, and fibronectin confirmed the Western blot findings. The intensity of DCF fluorescence was 2.6-fold higher in DM relative to C glomeruli ($p<0.05$), and this increase in DM glomeruli was significantly attenuated by AST-120 treatment ($p<0.05$). Urinary 8-OHdG excretion rates were significantly higher in DM compared to C rats (486.95±182.25 vs. 37.27±13.78 ng/kg/hr, $p<0.001$), and this increase in 8-OHdG excretion rates in DM rats was significantly inhibited by AST-120 treatment ($p<0.01$).

Conclusion: AST-120 treatment alleviates oxidative stress and inhibits the increase in fibronectin expression in diabetic nephropathy. These effects of AST-120 seem to be associated with the amelioration of enhanced NAPDH oxidase and eNOS expression under diabetic conditions. These findings provide a new perspective on the renoprotective effects of AST-120 in diabetic nephropathy.

Key words: AST-120, diabetic nephropathy, oxidative stress, fibronectin

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I. INTRODUCTION

Diabetic nephropathy, the leading cause of end-stage renal disease (ESRD) in the US and many other countries[1], is characterized pathologically by cellular hypertrophy and increased extracellular matrix (ECM) accumulation and clinically by proteinuria[2]. The molecular and cellular mechanisms responsible for these disease characteristics remain incompletely resolved. While the diabetic milieu *per se*, hemodynamic changes, and local growth factors such as transforming growth factor (TGF)-β1 and angiotensin II (AII) are considered to be mediators in the pathogenesis of diabetic nephropathy[3], the underlying pathways mediating these processes are not well understood.

Numerous experimental and clinical evidences indicate that oxidative stress

plays an important role in the development and progression of diabetic nephropathy[4-7]. High glucose *per se*, TGF-β1, and AII are all reported to increase reactive oxygen species (ROS) in cultured mesangial cells, which in turn activates protein kinase C (PKC) and nuclear factor (NF)-κB[8, 9]. In addition, the pathologic changes of glomeruli as well as the increase in urinary albumin excretion in diabetic rats were ameliorated by dietary antioxidant supplementation with vitamin E, taurine, or lipoic acid, supporting the role of oxidative stress in the pathogenesis of diabetic nephropathy[10-13].

The degree of oxidative stress is determined by the balance between the production of ROS and the antioxidant defense system[14]. In experimental and human diabetes, ROS generation is known to be increased via multiple pathways, including glucose auto-oxidation, increased mitochondrial superoxide production, PKC-dependent activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, uncoupled endothelial nitric oxide synthase (eNOS) activity, formation of advanced glycation products (AGEs), and stimulation of cellular ROS production by extracellular AGEs through their receptors[4, 15-18]. Among these, the activation of NADPH oxidase and uncoupling of eNOS mainly contribute to the increase in glomerular superoxide production in experimental diabetic rats[17].

AST-120 (Kremezin®, Kureha-Chemical Co., Tokyo, Japan) is a well-

known oral adsorbent which binds and prevents absorption of several biologically active substances such as indole from the gastrointestinal tract[19]. Indole is a precursor of indoxyl sulfate, which is known as a uremic substance, and serum indoxyl sulfate concentrations are reported to be increased in patients with decreased renal function[20]. In animal and human studies on renal failure, a close relationship was found between indoxyl sulfate and oxidative stress[21, 22]. Moreover, AST-120 treatment inhibited the progression of diabetic[23-25] and non-diabetic chronic kidney disease[26, 27] along with a decrease in oxidative stress[21, 28, 29]. To date, however, the effect of AST-120 on non-uremic kidney disease has never been explored. The present study was undertaken to investigate whether the administration of oral adsorbent, AST-120, could reduce oxidative stress and ameliorate the development of nephropathy in experimental diabetic rats with normal renal function in terms of ECM accumulation.

II. MATERIALS AND METHODS

1. Animals

All animal studies were conducted under an approved protocol. Thirty-two male Sprague-Dawley rats (Orientbio, Inc., Seongnam-si, Korea) weighing 220-250 g were studied. Sixteen were injected with diluent (Control, C) and 16 with 65 mg/kg streptozotocin (SigmaChemical Co., St.louis, MO, USA) (Diabetes, DM) intraperitoneally. Blood glucose levels were measured 3 days after the streptozotocin injection to confirm the development of diabetes. Control and diabetic rats were then randomly assigned to two groups. One group, comprised of 8 control and 8 diabetic rats, were feed with standard laboratory chow containing 5% AST-120 (Kremezin®), and the remaining 8 control and 8 diabetic rats with standard laboratory chow. All rats were housed in a temperature-controlled room and were given free access to water and chow during the 3-month study period.

Body weights were checked monthly, and kidney weights were measured at the time of sacrifice. Serum glucose was measured monthly and blood urea nitrogen, creatinine, and 24-hour urinary albumin by ELISA (Nephrat II, Exocell, Inc., Philadelphia, PA, USA) at the time of sacrifice.

2. Measurement of serum indoxyl sulfate concentrations

Serum indoxyl sulfate concentrations were measured by high performance liquid chromatography (HPLC) using a system (Shimadzu, Kyoto, Japan) equipped with an autoinjector at 4°C (SIL-10AD), a degasser (DGU-12A), a pump (LC-10AD), a column oven at 35°C (CTO-10AC), a column (Shiseido capsule pack type SG80, 4.6 x 150 mm, 5 μm), a fluorescence detector (RF-10A XL), a system controller (SCL-10AP), and software (CLASS-VP). Serum samples (10 μl) were analyzed in 10 minutes with a mobile phase, 5% tetrahydrofuran/0.1 M KH_2PO_4 (pH 6.5), at a flow rate of 1 ml/min and with fluorescence detection with excitation at 295 nm and emission at 390 nm.

3. Isolation of glomeruli

Glomeruli were isolated by sieving. Purity of the glomerular preparation was greater than 98% as determined by light microscopy.

4. Isolation of total RNA

Total glomerular RNA was extracted as previously described[30]. Briefly, addition of 100 μl of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA) to the glomeruli was followed by glomerular lysis by freezing and thawing three times. Another 700 μl of RNA STAT-60 reagent was added, the

mixture was vortexed and stored for 5 minutes at room temperature, 160 μl of chloroform was added, and the mixture was shaken vigorously for 30 seconds. After 3 minutes, the mixture was centrifuged at 12,000 g for 15 minutes at 4°C and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 μl isopropanol and pelleted with centrifugation at 12,000 g for 30 minutes at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, was dried using Speed Vac, and was dissolved in DEPC-treated distilled water. Glomerular RNA yield and quality were assessed based on spectrophotometric measures at the wavelength of 260 and 280 nm.

5. Reverse transcription

First strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two μg of total RNA extracted from sieved glomeruli was reverse transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreithol, 25 U RNAse inhibitor, and 40 U AMV reverse transcriptase. The mixture was incubated at 30°C for 10 minutes and 42°C for 1 hour, followed by inactivation of enzyme at 99°C for 5 minutes.

6. Real-time PCR

Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), the PCR was performed in a total volume of 20 μl in each well, containing 10 μl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μl of cDNA, and 5 pmol sense and antisense primers for endothelial nitric oxide synthase (eNOS), NADPH oxidase (gp91phox, p47phox, and p22phox), fibronectin, and 18s, which was determined after the analysis of the optimal concentrations of each primer. The primer sequences for eNOS, gp91phox, p47phox, p22phox, fibronectin, and 18s are shown in Table 1. Each sample was run in triplicates in separate tubes to permit quantification of the gene normalized to 18s.

After real-time PCR, the temperature was increased from 60 to 95°C to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using comparative CT method with $2^{-\triangle\triangle CT}$. The results were given as relative expression of specific gene normalized to the 18s housekeeping gene. Signals from control glomeruli were considered a relative value of 1.0.

Table 1. Sequences of primers used in this study

Name	Forward	Reverse	Size
eNOS	GGCTGCTGCCCGAGATATC	GGCAGTAATTGCAGGCTCTCA	93
gp91phox	CCTGCAGCCTGCCTGAA	AAGGAGAGGAGATTCCGACACA	63
p47phox	CCGGTGAGATCCACACAGAA	TGCACGCTGCCCATCAT	207
p22phox	GGTGAGCAGTGGACTCCCATT	TGGTAGGTGGCTGCTTGATG	79
fibronectin	TGACAACTGCCGTAGACCTG	TACTGGTTGTAGGTGTGGCCG	72
18s	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	67

7. Western blot analysis

Sieved glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol). Aliquots of 50 μg protein were treated with Laemmli sample buffer, were heated at 100°C for 5 minutes, and were electrophoresed at 50 μg/lane in a 8-12% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA, USA), the membrane was incubated in blocking buffer A (1xPBS, 0.1% Tween-20, and 8% nonfat milk) for 1 hour at room temperature, and was incubated overnight at 4°C in a 1:500 dilution of polyclonal antibody detecting eNOS, gp91phox, p47phox, p22phox (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), fibronectin

(Chemicon International, Inc., Temecula, CA, USA), or a dilution of 1:2000 β-actin (Santa Cruz Biotechnology, Inc.). The membrane was then washed once for 15 minutes and twice for 5 minutes in 1xPBS with 0.1% Tween-20, and was incubated in buffer A with horseradish peroxidase-linked goat anti-mouse or anti-rabbit IgG (Amersham Life Science, Inc., Arlington Heights, IL, USA) at 1:1000 dilution. The washes were repeated, and the membrane was developed with chemiluminescent agent (ECL; Amersham Life Science, Inc.). Band densities were measured using TINA image software (Raytest, Straubenhardt, Germany) and were used for analysis.

8. Immunohistochemistry

Slices of kidney for immunohistochemical staining, which were fixed in 10% neutral buffered formalin, were processed in the standard manner, and 4 micron sections were utilized. Slides were deparaffinized and hydrated in ethyl alcohol and were washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 minutes using a vegetable steamer. For staining, polyclonal antibodies recognizing eNOS, gp91phox, p47phox, p22phox (Santa Cruz Biotechnology, Inc.), and fibronectin (Chemicon International, Inc.) were diluted in 1:100-1:200 with 2% casein in BSA and were applied for overnight incubation at room temperature. After washing, a

secondary goat anti-rabbit antibody was added for 20 minutes, the slides were washed and were incubated with a tertiary rabbit-PAP complex for 20 minutes. DAB was added for 2 minutes and the slides were counterstained with hematoxylin. A semi-quantitative score for measuring intensity of staining within glomeruli was developed by examining 30 glomeruli in each section using digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA). Briefly, the degree of staining was quantitated on a scale of 0-4. The score was obtained by multiplying the intensity of staining by the percentage of glomeruli showing the same intensity of staining; these numbers were then summed for each experimental animal to give the final staining score:

$\sum$ (intensity of staining) x (% of glomeruli with that intensity) = staining score

9. Dichlordihydrofluorescin diacetate (DCF-DA) staining

The fluorescent dye, 2',7'-DCF-DA (Invitrogen, Carlsbad, CA, USA), was used to detect the presence of ROS in sieved glomeruli. 2',7'-DCF-DA diffuses across cell membranes and is hydrolyzed by nonspecific cellular esterases to the nonfluorescent compound dichlorofluorescin (DCFH), which is predominantly trapped within the cell. In the presence of ROS, DCFH

rapidly undergoes one-electron oxidation to the highly fluorescent compound dichlorofluorescein (DCF). Sieved glomeruli were plated into 2-well Lab-Tek®II Chamber Slide (Nalge Nunc International Corp., Naperville, IL, USA), were rinsed with PBS, and then replaced with Dulbecco's Modified Eagle's Medium (DMEM) without phenol red. H_2-DCF-DA was dissolved in DMSO to a final 50 mM stock solution, which was further diluted in DMEM to a final concentration of 50 μM. The glomeruli were then incubated at 37°C for 5 minutes and subsequently rinsed two additional times with DMEM and were imaged immediately. The exposure time was kept to <1 secend to avoid photo-oxidation of the ROS sensitive dyes, and for all treatments, the exposure time was kept constant. At least three independent fields were chosen for each condition, and 5 to 10 glomeruli in a given field were used for quantification of the fluorescence signals. Upon oxidation via interaction with ROS, the dye is cleaved forming the fluorescent compound DCF, and the formation of DCF was monitored in a fluorescent microplate reader with emission at 520 nm and excitation at 500 nm. Results were expressed as fold of control.

10. Measurement of urinary 8-hydroxydeoxyguanosine (8-OHdG) levels

Urinary specimens were centrifuged at 1500 rpm for 10 minutes to remove

particulates. The supernatants were used for the measurement of the 8-OHdG levels using a competitive in vitro enzyme-linked immunosorbent assay (ELISA) kit (Bioxytech, OXIS Health Products, Inc., Portland, OR, USA). A 50 µl urine sample and 50 µl of reconstituted primary antibody were added into each well of 8-OHdG coated microtiter plate and incubated at 37°C for 1 hour. The antibodies in the sample bound to the coated 8-OHdG were washed three times with phosphate-buffered saline. The horseradish peroxidase-conjugated secondary antibody was added to the plate followed by incubation at 37°C for 1 hour, the unbound enzyme-labeled secondary antibody was removed, and the plate was washed again three times. The amount of antibody bound to the plate was determined by the development of color intensity after the addition of a substrate containing 3,3',5,5'-tetra-methyl-benzidine. The reaction was terminated by the addition of phosphoric acid, and the absorbance was measured using a computer-controlled spectrophotometric plate reader at a wavelength of 450 nm. The concentration of 8-OHdG of the urine samples was interpolated from a standard curve drawn with the assistance of logarithmic transformation. The detection range of the ELISA assay was 0.5 to 200 ng/ml. The intra-assay coefficient of variance (CV) was 9.8%, and the inter-assay CV was 6.7%.

11. Statistical analysis

All values are expressed as the mean±standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were confirmed by the Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Animal data

All animals gained weight over the 3-month experimental period, but weight gain was highest in C rats ($p<0.01$). The ratios of kidney weight to body weight in DM (1.34±0.09%) and DM+AST-120 rats (1.28±0.10%) were significantly higher than those in C (0.62±0.04%) and C+AST-120 rats (0.64±0.06%) ($p<0.01$). The mean blood glucose levels of C, C+AST-120, DM, and DM+AST-120 rats were 106.4±5.7 mg/dl, 110.5±4.2 mg/dl, 495.0±8.9 mg/dl, and 482.6±6.3 mg/dl, respectively ($p<0.01$). Compared to the C group (0.40±0.06 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (1.99±0.17 mg/day, $p<0.01$), and AST-120 treatment significantly reduced albuminuria in DM rats (1.04±0.19 mg/day, $p<0.05$). On the other hand, serum creatinine and indoxyl sulfate levels were comparable among the four groups (Table 2).

Table 2. Animal data

	C	C+AST-120	DM	DM+AST-120
Body weight (g)	557.8 ± 18.8	580 ± 15.1	292.1 ± 9.6†	281.3 ± 9.5†
Kwt/Bwt ($X10^{-2}$)	0.62 ± 0.04	0.64 ± 0.06	1.34 ± 0.09†	1.28 ± 0.10†
Glucose (mg/dl)	106.4 ± 5.7	110.5 ± 4.2	495.0 ± 8.9†	482.6 ± 6.3†
S-Cr (mg/dl)	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
UAE (mg/day)	0.40 ± 0.06	0.36 ± 0.08	1.99 ± 0.17†	1.04 ± 0.19*
IS level (mg/dl)	0.06 ± 0.07	0.06 ± 0.03	0.07 ± 0.05	0.05 ± 0.01

Kwt, kidney weight; Bwt, body weight; S-Cr, serum creatinine; UAE, urinary albumin excretion; IS, indoxyl sulfate. †; $p<0.01$ vs. C, *; $p<0.05$ vs. DM

2. Effects of AST-120 on glomerular eNOS, subunits of NADPH oxidase, and fibronectin mRNA expression

Compared to C rats, glomerular eNOS, gp91phox, and p47phox mRNA expressions were significantly increased in DM rats by 2.1-, 3.3-, and 2.7-folds, respectively ($p<0.05$), and these increases in mRNA expressions in DM glomeruli were significantly abrogated by AST-120 treatment ($p<0.05$). In contrast, there was no difference in glomerular p22phox mRNA expression among the four groups (Fig. 1A). Fibronectin mRNA expression was also

increased in DM compared to C glomeruli, and AST-120 treatment significantly ameliorated this increase in glomerular fibronectin mRNA expression in DM rats ($p<0.05$) (Fig. 1B).

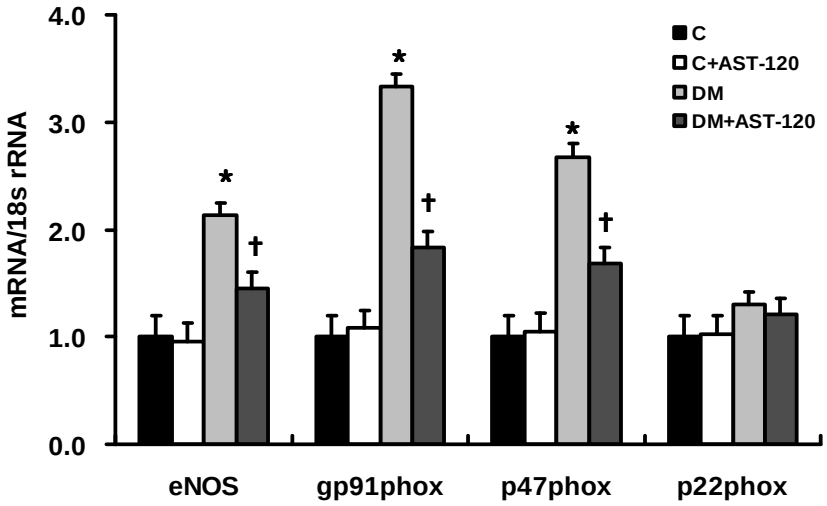

Figure 1A. Glomerular eNOS and subunits of NADPH oxidase mRNA expression assessed by real-time PCR in C, C+AST-120, DM, and DM+AST-120 groups. Compared to C rats, glomerular eNOS, gp91phox, and p47phox mRNA expressions were significantly increased in DM rats by 2.1-, 3.3-, and 2.7-folds, respectively, and these increases in mRNA expressions in DM glomeruli were significantly abrogated by AST-120 treatment. In contrast, there was no difference in glomerular p22phox mRNA expression among the four groups. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

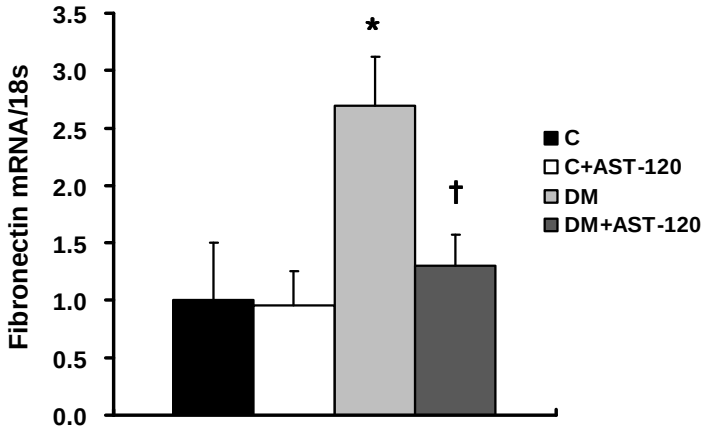

Figure 1B. Glomerular fibronectin mRNA expression assessed by real-time PCR in C, C+AST-120, DM, and DM+AST-120 groups. There was a 2.6-fold increase in fibronectin mRNA expression in DM compared to C glomeruli, and AST-120 treatment significantly ameliorated this increase in glomerular fibronectin mRNA expression in DM rats. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

3. Effects of AST-120 on glomerular eNOS, subunits of NADPH oxidase, and fibronectin protein expression

Similar to the results of mRNA expressions, glomerular eNOS, gp91phox, and p47phox protein expressions assessed by Western blot were significantly increased in DM compared to C rats, and these increases in protein expressions in DM rats were significantly attenuated by AST-120 treatment

($p<0.05$). In contrast, there was no difference in glomerular p22phox protein expression among the four groups. Fibronectin protein expression was also significantly increased in DM glomeruli, and AST treatment significantly inhibited this increase in glomerular fibronectin protein expression in DM rats ($p<0.05$) (Fig. 2).

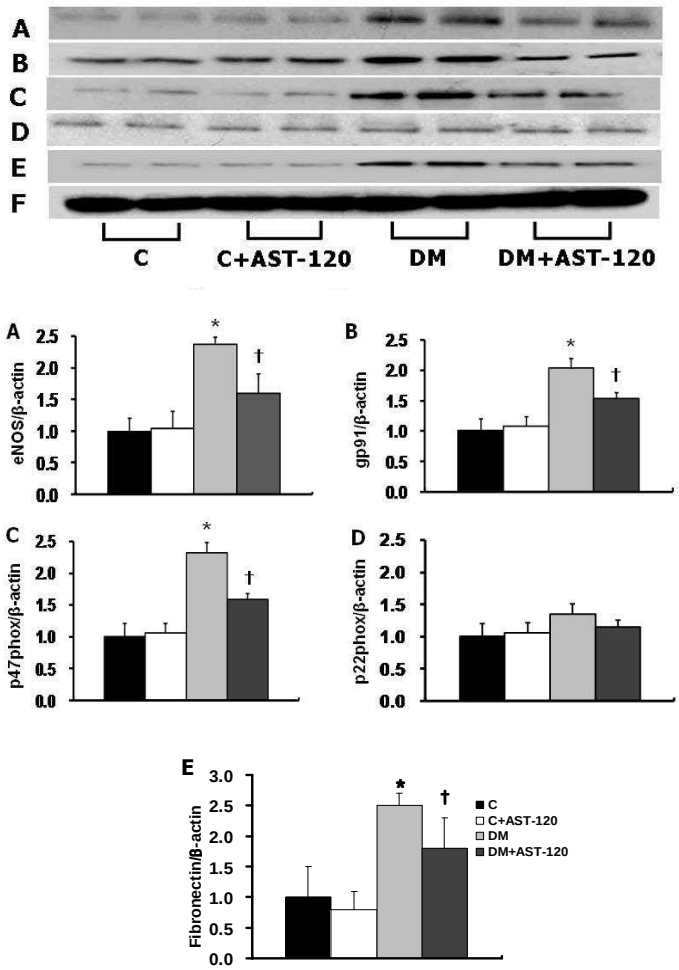

Figure 2. A representative Western blot of glomerular eNOS (A), gp91phox

(B), p47phox (C), p22phox (D), fibronectin (E), and β-actin (F) in C, C+AST-120, DM, and DM+AST-120 groups (representative of four blots). Compared to C rats, glomerular eNOS, gp91phox, p47phox, and fibronectin protein expressions were significantly increased by 2.4-, 2.0-, 2.3-, and 2.5-folds, respectively, in DM rats, and these increases in protein expression were significantly attenuated by AST-120 treatment. In contrast, there was no difference in glomerular p22phox protein expression among the four groups. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

4. Immunohistochemistry

Immunohistochemical staining for glomerular eNOS, subunits of NADPH oxidase, and fibronectin confirmed the Western blot findings. Glomerular eNOS, gp91phox, p47phox, and fibronectin staining were significantly stronger in DM compared to C rats, and AST-120 treatment significantly abrogated these increases in protein expression in DM glomeruli. In contrast, there was no difference in glomerular p22phox staining among the four groups, (Fig. 3A-E). The mean semi-quantitative staining scores for eNOS (50.2±3.8 vs. 28.3±5.8, $p<0.05$), gp91phox (75.4±8.5 vs. 33.1±10.8, $p<0.05$), p47phox (59.1±7.1 vs. 30.7±8.7, $p<0.05$), and fibronectin (78.5±8.8 vs. 36.6±11.1, $p<0.05$) were significantly higher in DM compared to C glomeruli,

and AST-120 treatment in DM rats significantly ameliorated the immunohistochemical staining scores for eNOS (36.7±7.0, $p<0.05$), gp91phox (51.5±8.0, $p<0.05$), p47phox (44.3±6.0, $p<0.05$), and fibronectin (55.1±8.2, $p<0.05$). In contrast, there was no difference in glomerular staining score for p22phox among the four groups.

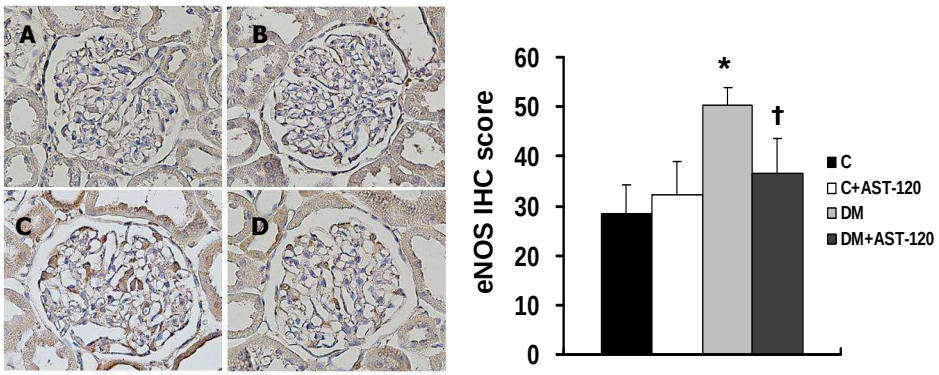

Figure 3A. Immunohistochemical staining for eNOS in C, C+AST-120, DM, and DM+AST-120 groups (x400). The mean semi-quantitative staining scores for glomerular eNOS were significantly higher in DM compared to C and C+AST-120 groups, and AST-120 treatment significantly attenuated this increase in glomerular eNOS staining score in DM rats. A: C, B: C+AST-120, C: DM, D: DM+AST-120. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

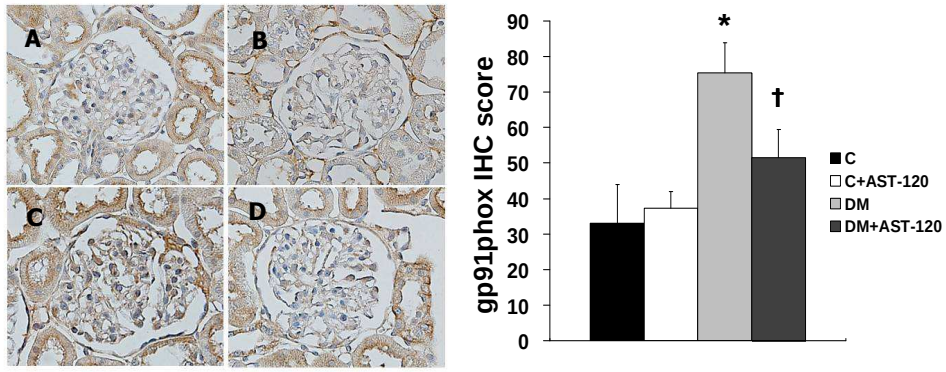

Figure 3B. Immunohistochemical staining for gp91phox in C, C+AST-120, DM, and DM+AST-120 groups (x400). The mean semi-quantitative staining scores for glomerular gp91phox were significantly higher in DM compared to C and C+AST-120 groups, and AST-120 treatment significantly attenuated this increase in glomerular gp91phox staining score in DM rats. A: C, B: C+AST-120, C: DM, D: DM+AST-120. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

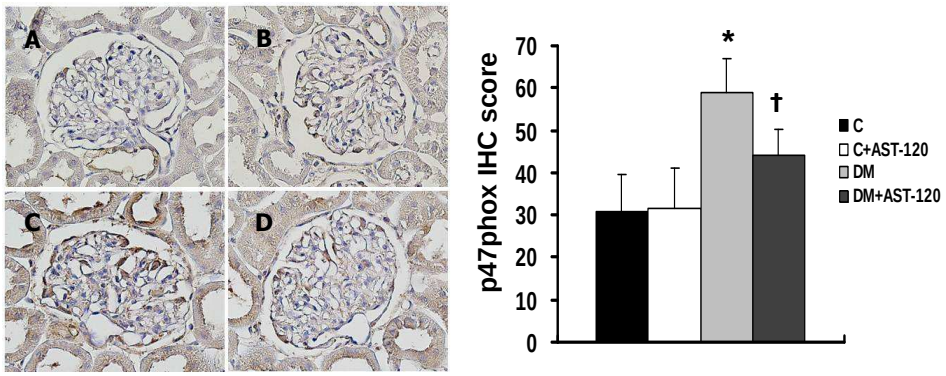

Figure 3C. Immunohistochemical staining for p47phox in C, C+AST-120, DM, and DM+AST-120 groups (x400). The mean semi-quantitative staining scores for glomerular p47phox were significantly higher in DM compared to C and C+AST-120 groups, and AST-120 treatment significantly attenuated this increase in glomerular p47phox staining score in DM rats. A: C, B: C+AST-120, C: DM, D: DM+AST-120. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

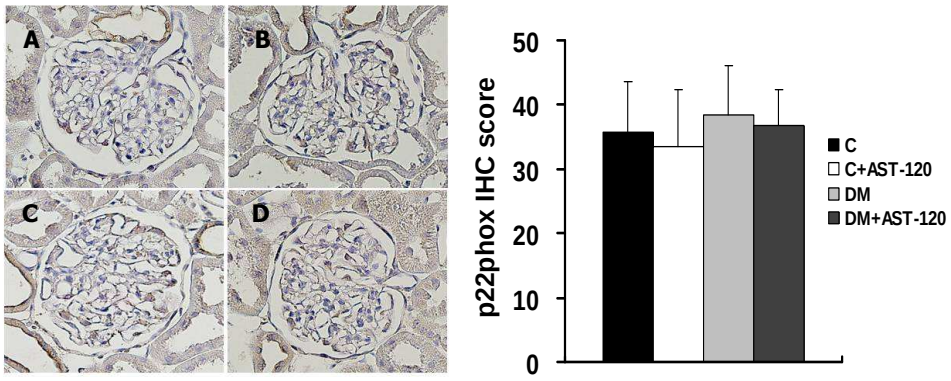

Figure 3D. Immunohistochemical staining for p22phox in C, C+AST-120, DM, and DM+AST-120 groups (x400). The mean semi-quantitative staining scores for glomerular p22phox were comparable among the four groups. A: C, B: C+AST-120, C: DM, D: DM+AST-120.

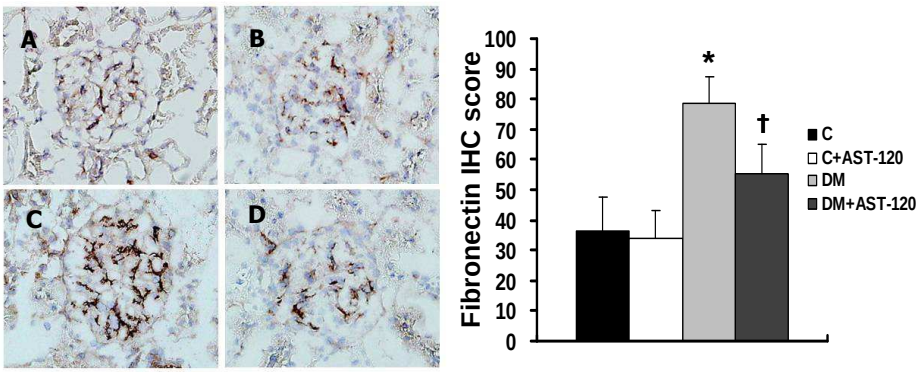

Figure 3E. Immunohistochemical staining for fibronectin in C, C+AST-120, DM, and DM+AST-120 groups (x400). The mean semi-quantitative staining scores for glomerular fibronectin were significantly higher in DM compared to C and C+AST-120 groups, and AST-120 treatment significantly attenuated this increase in glomerular fibronectin staining score in DM rats. A: C, B: C+AST-120, C: DM, D: DM+AST-120. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

5. Effect of AST-120 on glomerular ROS production

Glomerular ROS production was evaluated by determining the fluorescent intensity of DCF. The intensity of DCF fluorescence was 2.6-fold higher in DM compared to C glomeruli ($p<0.05$), and this increase in the intensity of DCF fluorescence in DM glomeruli was significantly inhibited by AST-120 treatment ($p<0.05$).

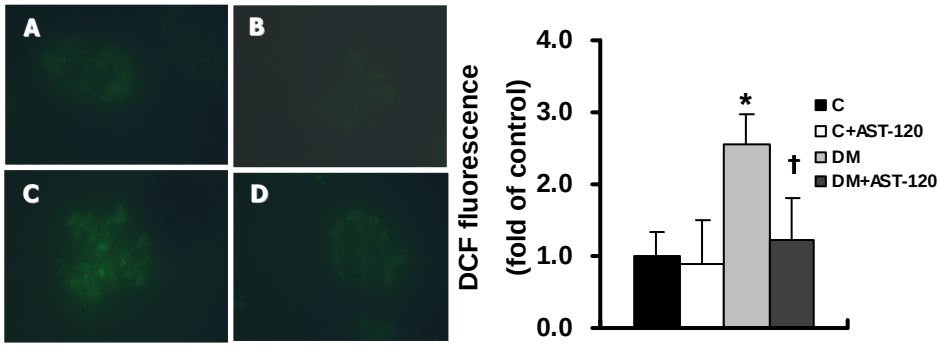

Figure 4. A representative DCF-DA staining for glomerular ROS production. The intensity of DCF fluorescence was 2.6-fold higher in DM compared to C glomeruli, and this increase in the intensity of DCF fluorescence in DM glomeruli was significantly inhibited by AST-120 treatment. A: C, B: C+AST-120, C: DM, D: DM+AST-120. *; $p<0.05$ vs. C and C+AST-120, †; $p<0.05$ vs. DM

6. Effect of AST-120 on urinary 8-OHdG excretion

Urinary 8-OHdG excretion rates were significantly higher in DM compared to C rats (486.95±182.25 vs. 37.27±13.78 ng/kg/hr, $p<0.001$), and this increase in 8-OHdG excretion rates in DM rats was significantly abrogated by AST-120 treatment ($p<0.01$) (Fig. 5).

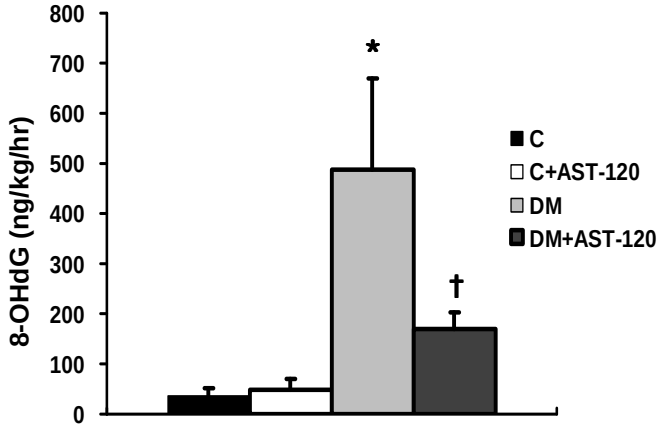

Figure 5. Urinary 8-OHdG excretion rates assessed by ELISA in C, C+AST-120, DM, and DM+AST-120 groups. Urinary 8-OHdG excretion rates were significantly higher in DM compared to C rats, and this increase in 8-OHdG excretion rates in DM rats was significantly abrogated by AST-120 treatment. *; $p<0.001$ vs. C and C+AST-120, †; $p<0.01$ vs. DM

IV. DISCUSSION

Recent experimental and clinical studies have demonstrated that AST-120, an oral absorbent, inhibited the progression of diabetic[23-25] and non-diabetic chronic kidney disease[26, 27] along with a decrease in oxidative stress[21, 28, 29], but the effect of AST-120 on non-uremic kidney disease has never been explored. In this study, I demonstrate for the first time that AST-120 treatment inhibited the increase in albuminuria and enhanced glomerular fibronectin expression in diabetic rats. In addition, the results of this study suggest that reduced oxidative stress may contribute to these effects of AST-120.

AST-120 is an oral adsorbent that consists of fine spherical carbonic particles of approximately 0.2-0.4 mm in diameter and can adsorb low molecular weight substances such as indole[19]. Indole is a precursor of indoxyl sulfate, which is known as one of the circulating uremic toxins, to be accumulated in renal failure[20], to stimulate glomerular sclerosis and interstitial fibrosis[31, 32], and to be effectively removed by AST-120[33, 34]. Since Sanaka et al[23] demonstrated that AST-120 attenuated the rates of decline of reciprocal creatinine in 27 undialyzed uremic patients, numerous investigations have been taken to elucidate not only the effects of AST-120 on the progression of renal failure but also the underlying mechanism of the beneficial effects of

AST-120. The progression of renal failure has been retarded or improved in both diabetic and non-diabetic patients treated by AST-120 irrespective of the degree of renal failure[24, 25, 35, 36]. In addition, AST-120 has been shown to improve renal function, glomerulosclerosis, and tubulointerstitial fibrosis in various animal models of acute and chronic renal failure[33, 34, 37].

These beneficial effects of AST-120 were considered to be attributed to the removal of indole, leading to a decrease in serum indoxyl sulfate levels[38]. Aoyama et al[39] demonstrated that the administration of indoxyl sulfate stimulated the expression of TGF-β1, tissue inhibitor of metalloproteinase (TIMP)-1, and pro-α1(I) collagen in the kidneys and these changes in uremic Otsuka Long-Evans Tokushima Fatty rats were reversed by AST-120 treatment. In addition, AST-120 significantly inhibited the increases in renal cortical nuclear factor (NF)-κB DNA-binding activity in 3/4 nephrectomized rats, resulting in less monocyte chemoattractant protein-1 (MCP-1) mRNA expression and macrophage infiltration[40]. Recently, there have been some studies suggesting a close association between that indoxyl sulfate and oxidative stress. Gelasco et al[41] and Dou et al[42] found that indoxyl sulfate induced a significant production of ROS in cultured rat mesangial cells and cultured human umbilical vein endothelial cells, respectively. In addition, AST-120 suppressed the progression of renal failure in 5/6 nephrectomized

rats, at least in part, by attenuation of oxidative stress assessed by urinary excretion of acrolein and 8-OHdG[28]. Furthermore, a recent study by Kato et al[43] revealed that carboxymethyllysine (CML) and pentosidine levels were significantly correlated with serum indoxyl sulfate concentrations in hemodialysis patients. In the present study, oxidative stress, assessed by DCF-DA staining and urinary 8-OHdG excretion, was significantly higher in diabetic compared to control kidney and this increase was abrogated by AST-120 treatment in accordance with aforementioned studies, suggesting that AST-120 abrogated enhanced oxidative stress levels under diabetic conditions. In contrast to the previous studies, however, there was no difference in serum indoxyl sulfate concentrations among the study groups. Since the diabetic rats I used in this study do not develop renal failure at 3 months after the induction of diabetes, it seems that similar levels of serum indoxyl sulfate is due to comparable renal function between control and diabetic rats. On the other hand, besides indoxyl sulfate AST-120 is known to adsorb hippuric acid, p-cresol, and AGEs, including methylglyoxal and CML, all of which are known to be associated with oxidative stress[44-46]. Therefore, further study is needed to clarify the molecules which levels are increased in diabetic rats and are decreased by AST-120 treatment, that is, the molecules which are responsible for the antioxidant effect of AST-120 in diabetic rats.

Even though the term ‘oxidative stress’ has been of wide use, no clear definition exists yet. A number of researchers, however, define oxidative stress as a status where production of the short-lived, highly-reactive molecules is increased. Sies H[47] referred oxidative stress to a pro-oxidant status following the disturbance in the pro-oxidant/antioxidant balance. Oxidative stress involves various molecules called ROS of which superoxide anion, hydrogen peroxide, nitric oxide (NO), and peroxynitrite are known to be important, and plays a pivotal role in the pathogenesis of diabetic nephropathy[48, 49]. An extensive pool of researches suggests that oxidative stress is increased in both diabetic animals and humans. High glucose increased production of DCF-sensitive ROS in cultured mesangial cells in a time-dependent manner[8], and this increase in ROS is known to be dependent on PKC, NADPH oxidase, and mitochondrial electron gradient[9, 50]. TGF-β1 and AII also increased ROS production in cultured mesangial cells in a NADPH oxidase-dependent manner[9, 51]. In addition, ROS is known to mediate both *in vivo* and *in vitro* activation of PKC, NF-κB, TGF-β1, fibronectin, and plasminogen activator inhibitor (PAI)-1 under diabetic conditions[8, 50, 52]. In high glucose-stimulated mesangial cells, antioxidant treatment normalized PKC and NF-κB activation as well as PAI-1 expression, suggesting that ROS might mediate the high-glucose induced changes in cultured mesangial cells[9,

[53, 54]. In diabetic animals, increased activities of glomerular PKCδ and PKC-ε were also inhibited by treatment with taurine, an antioxidant[55]. Similarly, antioxidant treatment suppressed the increase in glomerular TGF-β1 and fibronectin mRNA expression and ameliorated glomerular thickening, mesangial expansion, and proteinuria in diabetic rats[50, 56]. Not only urinary albumin excretion but also glomerular basement membrane thickening and TGF-β1 and type IV collagen protein expressions were significantly attenuated in diabetic rats whose copper/zinc superoxide dismutase, a catalytic antioxidant, was over-expressed[57]. As inflammation was recently found to be involved in the pathogenesis of diabetic nephropathy, the interest has been drawn to MCP-1, the most important mediator of inflammatory cell infiltration[58, 59]. ROS was reported to contribute to the high glucose-induced increase in MCP-1 expression in cultured mesangial cells[8]. It was also found to mediate TGF-β1-induced phsphorylation of mitogen-activated protein kinase and Smad 2 as well as epithelial-mesenchymal transition in the tubular epithelium of normal rats[60]. The results of this study show that fibronectin mRNA and protein expression are increased in diabetic glomeruli and this increase in fibronectin expression are attenuated by AST-120 treatment along with reduced oxidative stress, suggesting that these changes in fibronectin expression are in part mediated by alleviation of enhanced oxidative stress by

AST-120 in diabetic rats.

NADPH oxidase is a major source of oxidants in renal cells such as tubular epithelial cells and glomerular mesangial cells[61]. NADPH oxidase was originally found in neutrophils and is composed of 5 subunits: 2 membrane-associated subunits; gp91phox and p22phox, and 3 major cytosolic subunits; p67phox, p47phox, and p40phox[61]. Under diabetic conditions, the expression of some subunits of NADPH oxidase is known to be increased *in vitro* and *in vivo*, resulting in enhanced oxidative stress levels. Kitada et al[62] demonstrated that oxidative stress in early diabetic glomeruli is mediated by NADPH oxidase activation and that the membranous translocation of p67phox and p47phox via PKC-β activation plays a crucial role. A study by Onozato et al[6] also showed that renal expression of p47phox were increased in early diabetic kidney along with an increase in plasma lipid peroxidation products and renal hydrogen peroxide production and nitrotyrosine deposition and that these changes were inhibited by ACEi or ARB, indicating a pathogenic role for AII in the development of oxidative damage in early diabetic nephropathy. In addition, Nox4, which is a homologue of gp91phox, was reported to be the major source of ROS in the kidney and to mediate renal hypertrophy and fibronectin expression in the early stage of diabetic nephropathy[63]. Similar to the previous studies, the present study shows that the expression of gp91phox

and p47phox mRNA and protein expressions are increased in diabetic glomeruli and these increases are ameliorated by AST-120 treatment, suggesting that AST-120 may reduce oxidative damage in diabetic kidney via the inhibition of NADPH oxidase-mediated ROS production. Among the subunits of NADPH oxidase, there was no difference in p22phox expression among the study groups. Even though Kitada et al[62] also found no difference in p22phox expression between control and diabetic glomeruli, the reasons for the divergence of changes in NAPDH-oxidase subunits are not clear and need to be further investigated.

In addition to NADPH oxidase, increased ROS generation under diabetic conditions is attributed to changes in NOS, xanthine oxidase, cyclooxygenase, and lipoxygenase[14]. Particularly, in diabetic nephropathy, the major sources of ROS are known to be NADPH oxidase and uncoupled NOS, resulting in a reduction of glomerular NO production despite increased expression of eNOS[17]. In addition, eNOS itself is also found to produce O_2^-, which requires Ca^{2+}/calmodulin and is primarily regulated by tetrahydrobiopterin rather than L-arginine[64]. Even though there are conflicting results on the expression of eNOS in diabetic nephropathy, many studies have shown that eNOS expression is increased in animal[6, 17, 65] and human[66] diabetic glomeruli, which is consistent with the findings of this study. Even though the functional

significance of increased eNOS expression was not examined in the present study, I surmise that the increase in eNOS expression is mainly attributed to the increase in uncoupled eNOS, which needs to be confirmed by checking the expression of eNOS dimer form.

In conclusion, AST-120 treatment alleviates oxidative stress and inhibits the increase in fibronectin expression in diabetic nephropathy. These effects of AST-120 seem to be associated with the attenuation of enhanced NAPDH oxidase and eNOS expression under diabetic conditions. These findings provide a new perspective on the renoprotective effects of AST-120 in diabetic nephropathy.

V. CONCLUSION

In this study, I investigated whether the administration of oral absorbent, AST-120, could reduce oxidative stress and ameliorate the development of nephropathy in experimental diabetic rats with normal renal function. The results were as follows;

1. Compared to the C group, 24-hour urinary albumin excretion was significantly higher in the DM group, and AST-120 treatment significantly reduced albuminuria in DM rats. On the other hand, serum creatinine and indoxyl sulfate levels were comparable among the four groups.
2. Glomerular eNOS, gp91phox, p47phox, and fibronectin mRNA and protein expressions were significantly increased in DM compared to C rats, and these increases in DM glomeruli were significantly abrogated by AST-120 treatment.
3. Glomerular eNOS, gp91phox, p47phox, and fibronectin staining by immunohistochemistry were significantly stronger in DM relative to C rats, and AST-120 treatment significantly ameliorated these increases in protein expression in DM glomeruli.
4. There was no difference in glomerular p22phox expression among the four

groups.

5. The intensity of DCF fluorescence for glomerular ROS production was also significantly increased in DM compared to C glomeruli, and this increase in DM glomeruli was significantly attenuated by AST-120 treatment.
6. Urinary 8-OHdG excretion rates were significantly higher in DM compared to C rats, and AST-120 treatment significantly inhibited this increase in 8-OHdG excretion rates in DM rats.

In summary, AST-120 treatment alleviates oxidative stress and inhibits the increase in fibronectin expression in diabetic nephropathy. These effects of AST-120 seem to be associated with the amelioration of enhanced NAPDH oxidase and eNOS expression under diabetic conditions. These findings provide a new perspective on the renoprotective effects of AST-120 in diabetic nephropathy.

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ABSTRACT (In Korean)

당뇨병성 신병증에서 AST-120 (크레메진®)이 산화성 스트레스와 fibronectin의 발현에 미치는 영향

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서론: 당뇨병성 신병증은 전세계적으로 말기 신부전증의 가장 흔한 원인 질환으로, 병리학적으로는 사구체 및 세뇨관의 비후와 세포 외 기질의 축적, 그리고 임상적으로는 단백뇨를 특징으로 한다. 이러한 당뇨병성 신병증의 병인으로 거론되고 있는 여러 가지 요인 중 최근에는 당뇨 조건 하에서 증가된 산화성 스트레스가 당뇨병성 신병증의 발생과 밀접한 관련이 있는 것으로 보고되고 있다. AST-120 (크레메진®)은 잘 알려져 있는 경구 흡착제로, 신기능이 감소되어 있는 경우 체내에 축적되는 요독 물질 중 하나인 indoxyl sulfate의 전구 물질인 indole과 같은 단백 대사 물질을 흡착하여 indoxyl sulfate의 축적을 억제하는 효과가 있는 약제이다. 실험적 요독증 동물 모델에서 AST-120의 투여로 TGF-β1와 TIMP-1의 발현이

감소되었을 뿐만 아니라 사구체경화증이 호전되었다는 연구 결과가 있으며, 당뇨 및 비당뇨성 만성 신장 질환 환자에서도 AST-120이 신장 질환의 진행을 억제하는 효과가 있었다고 보고되고 있다. 또한, 최근에는 요독증 동물 모델과 사람에서의 이러한 AST-120의 효과가 산화성 스트레스의 감소와 연관되어 있다는 연구 보고도 있다. 그러나, 산화성 스트레스와 밀접한 관련이 있는 당뇨병성 신병증에서 AST-120의 효과에 대한 연구는 미미한 실정이며, 특히 신기능이 정상인 당뇨병성 신병증에 대한 AST-120의 효과를 관찰한 연구는 전무한 상태이다. 이에 본 연구에서는 신기능이 정상인 실험적 당뇨 백서에서 AST-120의 투여가 신장 내 산화성 스트레스 및 대표적인 세포 외 기질인 fibronectin의 발현에 미치는 영향을 알아보고자 하였다.

방법: Sprague-Dawley 백서 32마리를 대상으로, 16마리는 streptozotocin (65 mg/kg)으로 당뇨를 유발시켰으며 (당뇨군), 16마리는 위약을 투여하였다 (대조군). 각 군에서 8마리는 5% AST-120 (크레메진®, Kureha-Chemical Co., Tokyo, Japan)이 포함된 사료로 사육하였으며, 3개월 후에 24시간 소변을 채집한 다음 희생시켜 신장을 적출하였다. 24시간 뇨단백 배설량과 뇨 8-OHdG 배설량은 ELISA를 이용하여 측정하였으며, 혈청 indoxyl

sulfate 농도는 HPLC로 측정하였다. 사구체 내 eNOS, NADPH oxidase의 subunits (gp91phox, p47phox, p22phox), 그리고 fibronectin의 mRNA와 단백 발현은 sieve를 사용하여 분리한 사구체를 이용하여 각각 real-time PCR과 Western blot으로 분석하였다. 면역조직염색을 이용한 사구체 내 eNOS, NADPH oxidase의 subunits (gp91phox, p47phox, p22phox), 그리고 fibronectin의 단백 발현도 조사하였다. 사구체 내 활성 산소족의 생성 정도는 DCF-DA 면역형광염색을 시행하여 분석하였다.

결과: 24시간 뇨단백 배설량은 당뇨군 (1.99±0.22 mg/day)에서 대조군 (0.40±0.06 mg/day)에 비하여 의의있게 증가되었으며 ($p<0.01$), AST-120 투여로 당뇨군에서의 단백뇨가 유의하게 감소되었다 (1.04±0.19 mg/day, $p<0.05$). 혈청 크레아티닌 및 indoxyl sulfate 농도는 네 군 사이에 의미있는 차이가 없었다. 사구체 eNOS, gp91phox, p47phox, 그리고 fibronectin의 mRNA 발현은 당뇨군에서 대조군에 비하여 각각 2.1배, 3.3배, 2.7배, 그리고 2.6배 증가되었으며 ($p<0.05$), AST-120의 투여가 당뇨군에서의 eNOS, gp91phox, p47phox, 그리고 fibronectin mRNA의 발현 증가를 의의있게 감소시켰다 ($p<0.05$). 이에 반해, p22phox의 mRNA 발현은 네 군 사이에 유의한 차이가

없었다. Western blot 및 면역조직염색을 이용하여 분석한 각 유전자의 단백 발현도 mRNA의 발현 변화와 유사하였다. DCF-DA 면역형광염색상 활성 산소족 생성을 나타내는 DCF 염색 강도가 당뇨 사구체에서 대조군 사구체에 비하여 2.6배 증가되었으며 ($p<0.05$), AST-120 투여로 당뇨 사구체에서의 DCF 염색 강도가 의미있게 감소되었다 ($p<0.05$). 또한, 당뇨군에서 뇨 8-OHdG 배설률이 대조군에 비하여 의의있게 높았으며 (486.95±182.25 vs. 37.27±13.78 ng/kg/hr, $p<0.001$), AST-120의 투여가 당뇨군에서 증가된 뇨 8-OHdG 배설률을 유의하게 감소시켰다.

결론: 본 연구에서 AST-120은 당뇨병성 신병증에서 산화성 스트레스를 호전시키고 사구체 내 fibronectin의 발현 증가를 억제시켰으며, 이런 효과는 당뇨 사구체 내 NADPH oxidase와 eNOS의 발현 증가가 AST-120에 의하여 억제되는 것과 관련이 있을 것으로 생각된다. 따라서, 항산화 및 항섬유화 효과가 있는 AST-120이 당뇨병성 신병증의 발생 및 진행 억제에 유용할 것으로 생각된다.

핵심 되는 말: AST-120, 당뇨병성 신병증, 산화성 스트레스, 세포 외 기질