FR167653, a p38 Mitogen-Activated Protein Kinase (MAPK) Inhibitor, Ameliorates Fibronectin and Type IV Collagen Expression in Diabetic Glomeruli

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Directed by Professor Shin-Wook Kang

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<ABSTRACT>

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Background. Diabetic nephropathy is characterized by glomerular hypertrophy and ECM accumulation, and the p38 MAPK pathway is known to be activated in diabetic glomeruli, leading to an increase in fibronectin and type IV collagen expression. This study was undertaken to investigate the effect of a p38 MAPK inhibitor, FR167653, on urinary albumin excretion and on glomerular fibronectin and type IV collagen expression in diabetic rats.

Methods. Thirty-two Sprague-Dawley rats were injected with diluent (C, N=16) or streptozotocin intraperitoneally (DM, N=16). Eight rats from each group were treated with 10 mg/kg/day FR167653 (C+FR, DM+FR) for 3 months. At the time of sacrifice, 24-hour urinary albumin excretion was determined by ELISA. Glomerular p38 MAPK and c-AMP-responsive element binding protein (CREB) activities were determined by Western blot with phospho-specific antibodies, and glomerular fibronectin and type IV collagen mRNA and protein expression were determined by real-time PCR and Western blot, respectively, with sieved glomeruli.

Results. The ratio of kidney weight to body weight (KW/BW) in DM (1.54±0.13%) was significantly higher than that in C rats (0.53±0.04%; $p<0.01$), and the increase in KW/BW was ameliorated by FR167653 administration (0.84±0.07%; $p<0.01$). FR167653 also significantly inhibited the increase in albuminuria in DM rats (C, 0.40±0.06 mg/day; C+FR, 0.41±0.07; DM, 1.99±0.22 mg/day; DM+FR, 1.04±0.19 mg/day; $p<0.05$). Glomerular p38 MAPK and CREB activities were significantly increased in 3-month DM rats compared to C rats, and FR167653 significantly abrogated the increase in p38 MAPK and CREB activities in DM glomeruli ($p<0.05$). Fibronectin and type IV collagen mRNA expression were significantly increased in DM glomeruli and these increases were inhibited by 86.8% and 79.9%, respectively, with FR167653 treatment ($p<0.05$). FR167653 also ameliorated the increases in fibronectin and type IV collagen protein expression in DM glomeruli ($p<0.05$).

Conclusions. These findings suggest that p38 MAPK could be a potential target for preventing nephropathy in diabetes.

Key words: diabetic nephropathy, p38 MAPK, albuminuria, fibronectin, type IV collagen

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I. INTRODUCTION

The molecular and cellular mechanisms responsible for diabetic nephropathy remain incompletely resolved. While studies indicate involvement of hyperglycemia via the stimulation of growth factor-induced cellular hypertrophy[1, 2], increased production of extracellular matrix protein (ECM) [3, 4], and decreased production of matrix-degrading proteinases[5, 6], the underlying signal transduction mechanisms mediating these processes have been less well explored.

Numerous studies reveal protein kinase C (PKC) activation in diabetic glomeruli[7] and in mesangial cells cultured under high glucose conditions[8, 9]. PKC propagates the physiologic responses of receptor-ligand interactions via an array of downstream signals, such as mitogen-activated protein kinases (MAPKs). p38 MAPK, one of the MAPK family, is known to be activated in

response to stress signals such as proinflammatory cytokines[10-14], ultraviolet irradiation[11], osmolality changes[12], and oxidants[15], leading to alterations in cell growth, prostanoid production, and other cellular dysfunctions[16, 17]. Increased p38 MAPK activity has been observed in the aorta of diabetic rats and in vascular smooth muscle cells and mesangial cells cultured under high glucose conditions[18-20]. Recently, Kang et al[21] have demonstrated that the glomerular p38 MAPK mRNA expression and activity were increased in diabetic rats along with increased MAPK kinase (MKK) 3/6 activity, suggesting the involvement of the p38 MAPK pathway in the pathogenesis of early glomerular hypertrophy and extracellular matrix accumulation.

FR167653{1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8(4-pyridyl)pyrazolo(5,1-c)(1,2,4) triazin-2-yl]-2-phenylethanedion sulfate monohydrate} was first discovered to be a dual inhibitor of interleukin (IL)-1 and tumor necrosis factor (TNF)-α production in lipopolysaccharide (LPS)-stimulated human monocytes and phytohemagglutinin-stimulated human lymphocytes[22]. Furthermore, FR 167653 effectively ameliorated ischemia/reperfusion injury in various organs, including kidney[23-25]. Recent studies suggest that FR167653 reduces IL-1β and TNF-α production via specific inhibition of p38 MAPK activity[26-28]. With the kidney, chronic allograft nephropathy, crescentic glomerulonephritis, and autoimmune injury were attenuated with administration of FR167653 by its anti-inflammatory action[29-32]. Besides an anti-inflammatory effect, FR167653 completely suppressed the development of proteinuria in experimental nephrotic syndrome and effectively prevented glomerulosclerosis and renal dysfunction in chronic adriamycin (ADR) nephropathy[33]. Despite the previously reported beneficial effects on various kidney diseases, little is currently known of its effect on diabetic nephropathy.

In this study, we examined the effect of FR167653 on the p38 MAPK pathway in isolated glomeruli from diabetic rats. Besides p38 MAPK, we also

studied: (1) c-AMP-responsive element binding protein (CREB), a transcription factor which is known to be under control of p38 MAPK; (2) fibronectin, a key ECM produced by mesangial cells; and (3) type IV collagen, a main ECM produced by podocytes.

II. MATERIALS AND METHODS

1. Animals

All animal studies were conducted under an approved protocol. Rats weighing 250-280 g were injected either with diluent [n=16, Control (C)] or with 65 mg/kg streptozotocin (STZ) intraperitoneally [n=16, Diabetes (DM)]. Eight rats from each group were treated with 10 mg/kg/day of FR167653 by gavage (C+FR, DM+FR) for 3 months. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 3-months study period.

Body weights were checked monthly, and kidney weights were measured at the time of sacrifice. Systolic blood pressure (SBP) was measured by tail-cuff phlethysmography at 3-months. Blood glucose was measured by glucometer and 24-hour urinary albumin excretion was determined by ELISA (Nephrat II, Exocell, Inc., Philadelphia, PA).

2. Total RNA extraction

Glomeruli were isolated by sieving. Purity of the glomerular preparation was greater than 98% as determined by light microscopy. Total RNA was extracted as previously described[21]. Briefly, 100 μl of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX) was added to the glomeruli, which were lysed by freezing and thawing three times. Another 700 μl of RNA STAT-60 reagent was then added and the mixture was vortexed and stored for 5 minutes at room temperature. Next, 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 X 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 adding 400 μl of isopropanol and then pelleted by

centrifugation at 12,000 X g for 30 minutes at 4°C. The RNA precipitate was washed with 70% ice-cold ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. Glomerular RNA yield and quality were assessed based on spectrophotometric measurements at the wavelength of 260 and 280 nm.

3. 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 were 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 the enzyme at 99°C for 5 minutes.

4. Real-time polymerase chain reaction (PCR)

The primers used for GAPDH, fibronectin, and type IV collagen amplification were shown in Table 1. cDNAs from 25 ng RNA of glomeruli per reaction tube were used for amplification.

Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), PCR was performed with 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. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in

separate tubes to permit quantification of the gene normalized to the GAPDH. The PCR conditions used were as follows: for GAPDH, 35 cycles of denaturation at 94.5°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 minute; and for fibronectin and type IV collagen, 38 cycles of denaturation at 94.5°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec. Initial heating at 95°C for 9 minutes and final extension at 72°C for 7 minutes were performed for all PCRs.

After PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min 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 a comparative C_T method with $2^{-\Delta\Delta CT}$. The results were given as relative expression of fibronectin and type IV collagen normalized to the GAPDH housekeeping gene and expressed in arbitrary units. Signals from C glomeruli were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels revealed a single band.

Table 1. Primers sequences and PCR conditions

	Sequence (5’ 3’)	Annealing Temperature (°C)	No. of cycles
GAPDH			
Sense	GACAAGATGGTGAAGGTCGG	58	35
Antisense	CATGGACTGTGGTCATGAGC		
Fibronectin			
Sense	GCAAGCCTGAACCTGAAGAGACC	62	38
Antisense	CCTGGTGTCCTGATCATTGCATC		
Type IV Collagen			
Sense	GGCTGGCCACTGTTGATATGT	62	38
Antisense	TCGGCTAATACGTGTCCTCAAG		

5. Western blot analysis

Sieved glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol), treated with Laemmli sample buffer, heated at 100°C for 5 minutes, and electrophoresed in an 8% 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), and the membrane was then incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 8% nonfat milk) for 1 hour at room temperature, followed by an overnight incubation at 4°C in a 1:100 dilution of polyclonal antibodies to p38 MAPK, phospho-specific p38 MAPK, CREB, phospho-specific CREB (New England Biolabs, Inc., Beverly, MA, USA), extracellular domain of fibronectin, type IV collagen, or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then washed once for 15 minutes and twice for 5 minutes in 1 x PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science, Inc., Arlington Heights, IL). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.).

6. Pathology

Slices of kidney for immunohistochemical and immunofluorescence staining were fixed in 10% neutral buffered formalin, processed in the standard manner, and 5 μm sections of paraffin embedded tissues were utilized. Slides were deparaffinized, hydrated in ethyl alcohol and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 minutes using a Black and Decker vegetable steamer. For fibronectin staining, the primary

polyclonal antibody to extracellular domain of fibronectin (Santa Cruz Biotechnology, Inc.) was diluted in 1:100 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, the secondary goat anti-rabbit antibody for fibronectin was added for 20 minutes, and the slides were washed and incubated with a tertiary rabbit-PAP complex for 20 min. DAB was added for 2 minutes and the slides were counterstained with hematoxylin.

For type IV collagen staining, the polyclonal antibody to type IV collagen (Santa Cruz Biotechnology, Inc.) was diluted in 1:100 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary Cy3-conjugated AffiniPure donkey anti-goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 60 minutes and then washed for 10 minutes.

A semi-quantitative score for measuring intensity of fibronectin and type IV collagen staining within glomeruli was determined by examining thirty glomeruli in each section and by digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA).

7. Statistical analyses

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). Results were analyzed using the Kruskal-Wallis non-parametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Animal data (Table 2)

All animals gained weight over the 3-months experimental period, but weight gain was highest in C rats ($p<0.01$). The ratio of kidney weight to body weight in DM rats (1.54±0.13%) was significantly higher than those in C (0.53±0.04%), C+FR (0.59±0.06%), and DM+FR rats (0.84±0.07%; $p<0.01$). The mean blood glucose levels of C, C+FR, DM, and DM+FR rats were 110.1±9.2 mg/dl, 106.3±7.9 mg/dl, 473.2±16.3 mg/dl, and 467.2±12.5 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±0.22 mg/day; $p<0.05$), and FR treatment significantly reduced albuminuria in DM rats (1.04±0.19 mg/day; $p<0.05$).

Table 2. Animal data of the four groups

	Control	Control+FR	DM	DM+FR
Kidney weight/Body weight (%)	0.53±0.04	0.59±0.06	1.54±0.13*	0.84±0.07
Blood glucose (mg/dl)	110.0±9.2	106.3±7.9	473.2±16.3†	467.2±12.5†
Urinary albumin excretion (mg/day)	0.40±0.06	0.41±0.07	1.99±0.22‡	1.04±0.19

*; $p<0.01$ vs. C, C+FR, and DM+FR group, †; $p<0.01$ vs. C and C+FR groups, ‡; $P<0.05$ vs. C, C+FR, and DM+FR groups.

2. p38 MAPK activity and protein expression

Figure 1 shows a representative Western blot of equal amounts of protein

from the lysates of sieved glomeruli from the four groups. The blot was first probed with an antibody to phospho-specific p38 MAPK, then stripped and probed with an antibody that recognizes total p38 MAPK. Phospho-specific p38 MAPK levels, which represent p38 MAPK activity, were significantly greater in DM glomeruli compared to C and C+FR glomeruli ($p<0.05$). Densitometric measurements revealed a 1.8-fold increase in p38 MAPK activity in DM relative to C glomeruli. FR167653 treatment nearly normalized the increase in glomerular p38 MAPK activity in DM rats ($p<0.05$). In contrast, there was no significant difference in total p38 MAPK levels among the four groups.

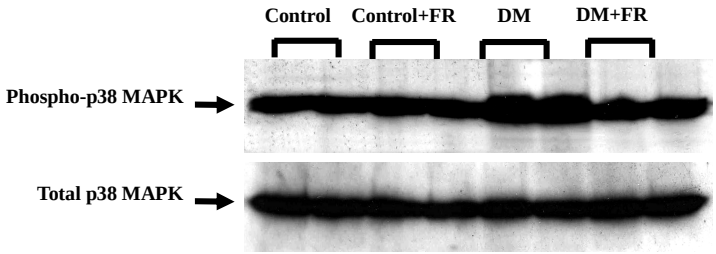


Figure 1. A representative Western blot of glomerular phospho-specific and total p38 MAPK in C, C+FR, DM, and DM+FR groups. Phospho-specific p38 MAPK levels were significantly greater in DM glomeruli compared to C and C+FR glomeruli ($p<0.05$), and FR167653 treatment nearly normalized the increase in glomerular p38 MAPK activity in the DM group ($p<0.05$). In contrast, there was no difference in total p38 MAPK levels among the four groups.

3. CREB activity and protein expression

In order to determine whether activation of the p38 MAPK pathway could induce parallel increases in the activity of a p38 MAPK target transcription factor, I examined the activity and protein expression of CREB. I subjected glomerular protein lysates to immunoblotting using antibodies to

phospho-specific (activated) CREB and to total CREB. The representative blot in Figure 2 shows that CREB activity was significantly increased in DM glomeruli relative to C and C+FR glomeruli ($p<0.01$). Densitometric measurements revealed a 2.3-fold increase in CREB activity in DM compared to C glomeruli. Similar to p38 MAPK activity, FR167653 treatment ameliorated the increase in glomerular CREB in DM rats ($p<0.01$). In contrast, there was no significant difference in total CREB levels among the four groups.

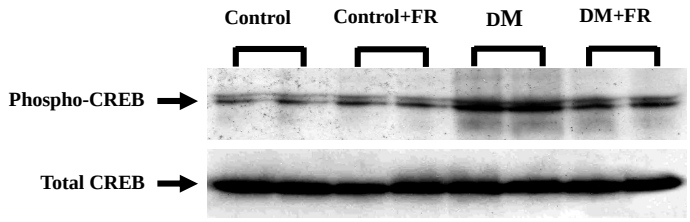


Figure 2. A representative Western blot of glomerular phospho-specific and total CREB in C, C+FR, DM, and DM+FR groups. CREB activity was significantly increased in DM glomeruli relative to C and C+FR glomeruli ($p<0.01$), and FR167653 treatment ameliorated the increase in glomerular phospho-specific CREB in the DM group ($p<0.01$). In contrast, there was no difference in total CREB levels among the four groups.

4. Fibronectin and type IV collagen mRNA and protein expression

In order to correlate my observations to evolving diabetic nephropathy, I examined the mRNA and protein expression of fibronectin and type IV collagen, key ECM proteins of mesangial cells and podocytes, respectively. The glomerular fibronectin/GAPDH and type IV collagen/GAPDH mRNA ratios were 2.1-fold and 1.9-fold higher, respectively, in DM compared to C rats ($p<0.05$), and these increments in fibronectin and type IV collagen mRNA

expression in DM glomeruli were inhibited by 86.8% and 79.9%, respectively, with FR167653 treatment ($p<0.05$; Fig. 3). The protein expression of fibronectin and type IV collagen protein showed similar patterns to their mRNA expression (Fig. 4). On the other hand, FR 167653 treatment had no effects on fibronectin and type IV collagen mRNA and protein expression in C rats.

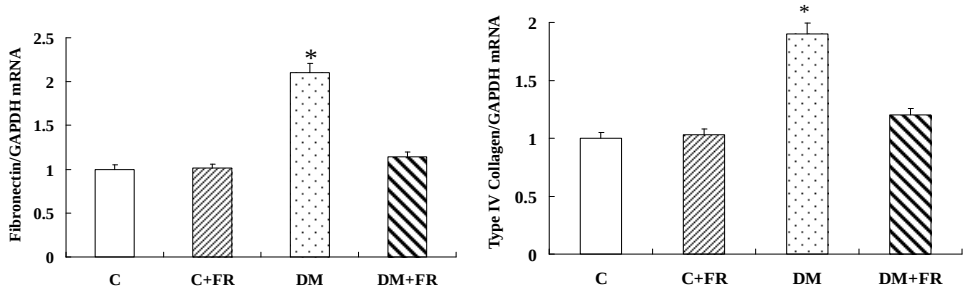


Figure 3. Glomerular fibronectin/GAPDH and type IV collagen/GAPDH mRNA ratios in C, C+FR, DM, and DM+FR groups. Fibronectin mRNA and type IV collagen mRNA expression were significantly increased in DM compared to C and C+FR glomeruli ($p<0.05$), and these increments in fibronectin and type IV collagen mRNA expression in DM glomeruli were significantly inhibited by FR167653 treatment ($p<0.05$).

*; $p<0.05$ vs. C, C+FR, and DM+FR groups.

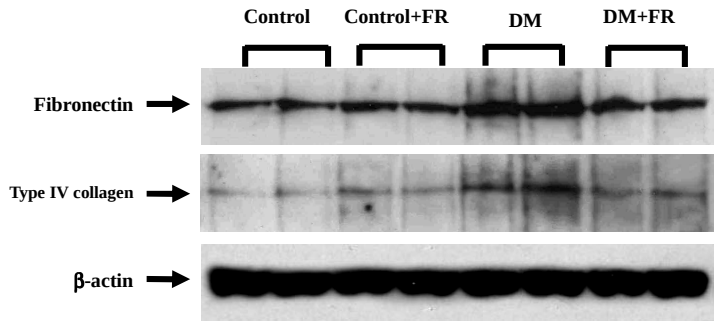


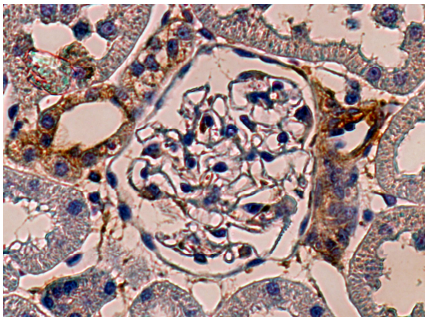
Figure 4. A representative Western blot of glomerular fibronectin and type IV collagen in C, C+FR, DM, and DM+FR groups. Fibronectin and type IV collagen protein expression were

significantly increased in DM relative to C and C+FR glomeruli ($p<0.05$), and these increments in fibronectin and type IV collagen protein expression in DM glomeruli were significantly inhibited by FR167653 treatment ($p<0.05$). In contrast, there was no difference in β-actin protein expression among the four groups.

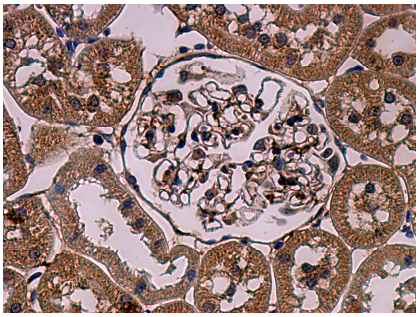
5. Pathology

Immunohistochemical staining for glomerular fibronectin and immunofluorescence for glomerular type IV collagen confirmed the Western blot findings. Glomerular fibronectin staining within mesangial regions and type IV collagen staining along the GBM were significantly stronger in DM compared to C and C+FR rats, and FR167653 treatment inhibited the increases in fibronectin and type IV collagen accumulation in DM rats (Fig. 5, Fig. 6).

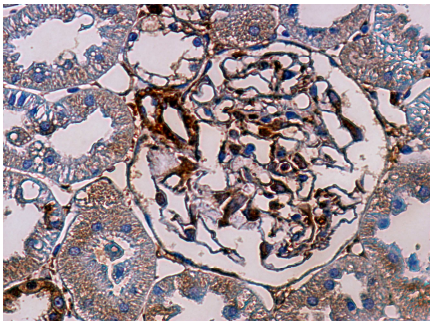
(A)



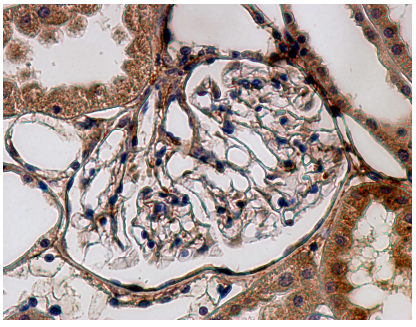
Control



Control+FR167653



DM



DM+FR167653

(B)

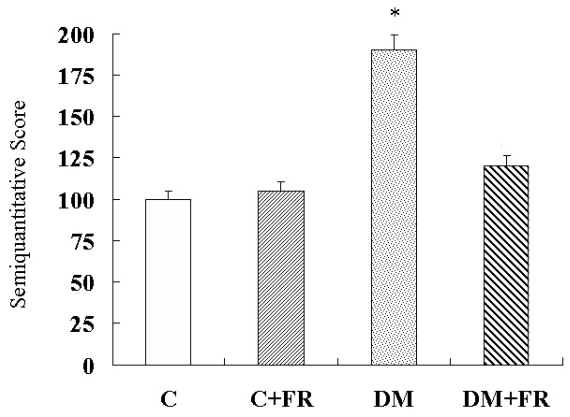
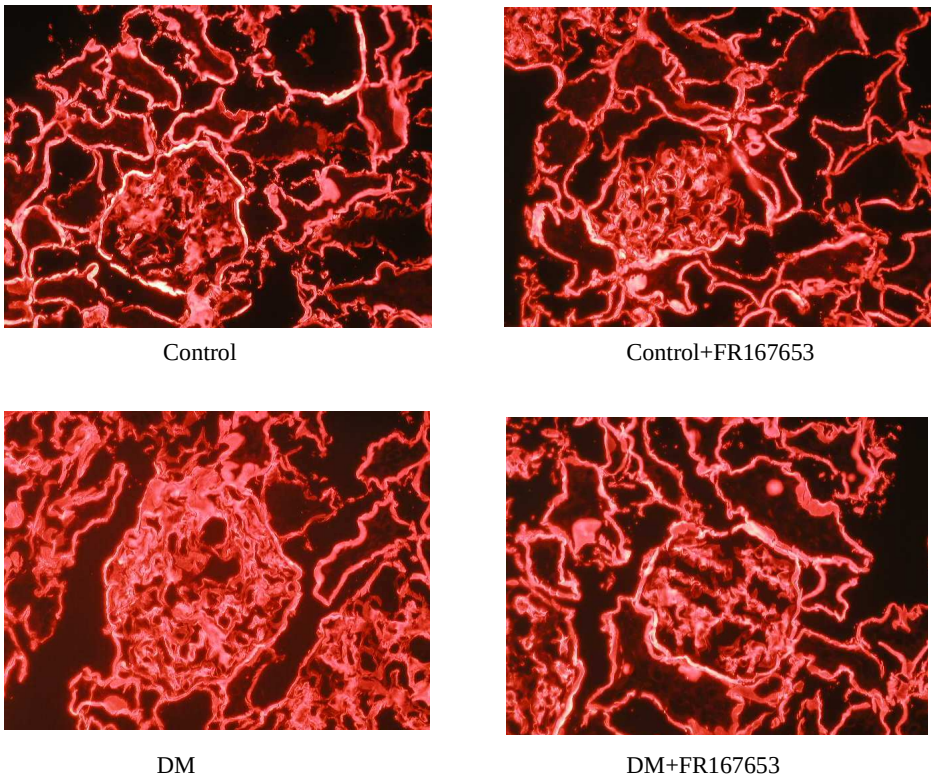


Figure 5. (A) Immunohistochemical staining for fibronectin in C, C+FR, DM, and DM+FR groups. There was a significant increase in glomerular fibronectin protein expression in DM compared to C and C+FR rats, and this increase was ameliorated by FR167653 treatment (x 400). (B) Semiquantitative immunohistochemical score for glomerular fibronectin was significantly higher in DM relative to C and C+FR rats ($p<0.05$), and this increment was inhibited by the administration of FR167653.

*; $p<0.05$ vs. C, C+FR, and DM+FR groups.

(A)



(B)

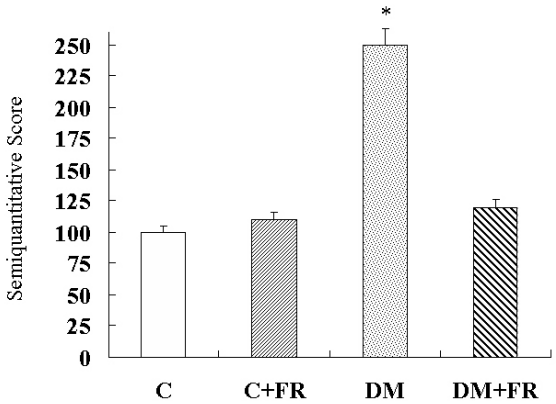


Figure 6. (A) Immunofluorescence staining for type IV collagen in C, C+FR, DM, and DM+FR

groups. There was a significant increase in glomerular type IV collagen protein expression in DM compared to C and C+FR rats, and this increase was ameliorated by FR167653 treatment (x 400). (B) Semiquantitative immunofluorescence score for glomerular type IV collagen was significantly higher in DM relative to C and C+FR rats ($p<0.05$), and this increment was inhibited by the administration of FR167653 ($p<0.05$).

*; $p<0.05$ vs. C, C+FR, and DM+FR groups.

IV. DISCUSSION

Diabetic milieu is known to activate the p38 MAPK pathway in various cells and organs[34-37]; however, the consequences of inhibiting p38 MAPK activation under diabetic conditions have not been well explored. Furthermore, the *in vivo* effect of a p38 MAPK inhibitor on diabetic nephropathy has not yet been investigated. In this study, I demonstrate for the first time that fibronectin and type IV collagen expression and accumulation in diabetic glomeruli were ameliorated with the administration of FR167653, a p38 MAPK inhibitor.

p38 MAPK is a member of the MAPK family and is known as a "stress-activated kinase" along with c-Jun N-amino terminal kinase (JNK)[35]. Recent works have shown that high glucose activated p38 MAPK in cultured vascular smooth muscle cells and mesangial cells[18-20]. Increased p38 MAPK protein as well as activation of p38 MAPK was also observed and characterized in aortas and glomeruli derived from diabetic rats[18]. These findings suggest that activation of the p38 MAPK pathway under diabetic conditions, *in vitro* and *in vivo*, may play an important role in the pathogenesis of diabetic complications. Once p38 MAPK is activated, it phosphorylates several transcription factors at serine and threonine residues, thereby regulating gene expression. Biochemical studies have demonstrated that the p38 MAPK signaling pathway activates various transcription factors, including CREB[38-40]. CREB is a member of a transcription factor family which converts rapid and transient signals into long-term changes in gene expression by binding to the c-AMP response element (CRE). Since the fibronectin promoter contains a CRE located –170 bp of the fibronectin gene, activated CREB can bind the CRE portion of the fibronectin gene, leading to fibronectin mRNA expression[41]. Kreisberg et al[42] have demonstrated that activation of protein kinase C by high glucose plus TGF-β or PMA induced phosphorylation and activation of CREB, resulting in the stimulation of fibronectin transcription in mesangial cells *in vitro*.

Furthermore, Kang et al[43] showed that p38 MAPK was activated in podocytes cultured under high glucose conditions with concomitant increment in collagen α5(IV) mRNA and protein expression. These studies suggest that activation of p38 MAPK in diabetic conditions are involved in ECM synthesis, and treatment with a p38 MAPK inhibitor *in vivo* ameliorated p38 MAPK phosphorylation and the increase in fibronectin and type IV collagen expression were subsequently inhibited.

FR167653 was originally developed as a dual inhibitor of IL-1 and TNF-α production[22]. Since the inflammatory process, which is characterized by induction of numerous cytokines and/or chemokines, and infiltration of macrophages, lymphocytes, and neutrophils, has been implicated in the pathogenesis of various diseases, many investigators have tried to investigate the effect of FR167653 in these conditions. Previous studies[23-28] have demonstrated that FR167653 ameliorated ischemia/reperfusion injury in liver, lung, heart, pancreas, and kidney possibly by inhibition of proinflammatory cytokines production. In an animal model of human lupus erythematosus, renal expression of monocyte chemoattractant protein-1 (MCP-1) and TNF-α were reduced and IgG levels in the diseased kidney were decreased by FR167653, resulting in prolonged survival and attenuated renal pathologic changes[32]. FR167653 also reduced urinary excretion and renal expression of MCP-1 in rat with nephrotoxic serum nephritis, an animal model of human crescentic glomerulonephritis[31]. Six days after the introduction of nephrotoxic serum, the structure of the glomeruli and interstitium appeared almost normal and urinary protein excretion was also normal in rats treated with FR167653 for 6 days. When the experiment was extended to 56 days, glomerulosclerosis and interstitial fibrosis were significantly alleviated, and renal function was well preserved in the FR167653-treated group even though FR167653 was administrated for only 6 days. Since glomerular infiltration of leukocytes has a major role in the pathogenesis of lupus nephritis and nephrotoxic serum

nephritis, the effects of FR167653 seem to be mediated by its anti-inflammatory actions, i.e. inhibition of cytokines production by monocytes and macrophages. In contrast, cellular infiltration within glomeruli is not extensive in diabetic nephropathy, therefore, the effect of FR167653 on the synthesis of proinflammatory cytokines in diabetic glomeruli was not evaluated in this study.

Besides the anti-inflammatory effects, recent studies[26-28] have revealed that FR167653 is a selective p38 MAPK inhibitor, and the pyridine and fluorophenyl rings within FR167653 are essential for binding to p38 MAPK. In addition, previous study[44] demonstrated that FR167653 did not affect the activities of other protein kinases, such as extracellular signal-regulated kinase-1/2 (ERK1/2), JNK, protein kinase C, or protein kinase A. In contrast to SB203580, another p38 MAPK inhibitor, it is known that FR167653 has no effect on cyclooxygenase (COX)-1 or COX-2 activity[44]. By inhibiting the p38 MAPK pathway, renal NAD(P)H oxidase expression and superoxide formation were suppressed and renal damage was ameliorated by FR167653 in Dahl salt-sensitive rats with heart failure[45], suggesting that the renoprotective effect of FR167653 was associated with the inhibition of oxidative stress. In addition, the study by Koshikawa et al[33] demonstrated that p38 MAPK activation played an important role in podocyte injury in proteinuric glomerulopathies, including rat puromycin aminonucleoside (PAN) nephropathy and mouse ADR nephropathy. They showed that inhibition of the p38 MAPK pathway by FR167653, which was introduced 2 days before the induction of nephropathy and for 14 days thereafter, completely suppressed proteinuria and reversed the changes in nephrin and connexin43 expression. Early treatment with FR167653 also effectively prevented glomerulosclerosis and renal dysfunction even in the chronic phase of ADR nephropathy. Furthermore, pretreatment with FR167653 prevented actin reorganization induced by PAN or H_2O_2 in immortalized mouse podocytes. Taken together, it seems that FR167653 can exert renoprotective effects by inhibiting the p38 MAPK pathway per se in various kidney diseases,

even in which inflammatory cell infiltration is minimal or insignificant.

In contrast to the study by Koshikawa et al[33], there have been some studies suggesting that p38 MAPK activation is rather an adaptive process to maintain cytoskeleton in podocytes. Aoudjit et al[46] demonstrated that treatment with a p38 MAPK inhibitor significantly augmented complement-mediated cytotoxicity *in vitro* and observed an increment in proteinuria in a rat model of passive Heymann nephritis (PHN) *in vivo*, when FR167653 was administrated from 7 to 14 days after the induction of nephropathy. In another study by Dai et al[47], inhibition of p38 MAPK decreased fibrillar actin (FA)/G-actin (GA) ratio, disrupted and clumped FA filaments, and increased cytoplasmic GA monomers in high glucose-stimulated podocytes, suggesting that p38 MAPK activation was necessary in maintenance and preservation of the actin cytoskeleton in podocytes cultured under diabetic condition. I observed a significant decrease in 24-hour urinary albumin excretion in FR167653-treated DM rats compared to DM rats. The impact of p38 MAPK inhibition on proteinuria has been inconsistent. Although the reasons for the divergent effects are unclear, species differences and differences in cells and nephropathies, p38 MAPK inhibitors used in the experiments, or treatment duration *in vivo* may contribute.

Since ECM synthesis was focused on in this study, I did not evaluate the changes in slit diaphragm-associated molecules or structural changes of podocytes. Additional studies are needed to verify the *in vivo* effect of a p38 MAPK inhibitor on podocytes.

In summary, FR167653 inhibited p38 MAPK activation and ameliorated fibronectin and type IV collagen expression in diabetic glomeruli. These findings suggest that p38 MAPK could be a potential target for preventing nephropathy in diabetes.

V. CONCLUSION

I investigated the effects of FR167653, a p38 MAPK inhibitor, on p38 MAPK and CREB activities, and fibronectin and type IV collagen expression in isolated glomeruli from diabetic rats.

Thirty-two Sprague-Dawley rats were divided into four groups; control (C), C+FR, DM, and DM+FR. The ratio of kidney weight to body weight and 24-hour urinary albumin excretion were determined after 3 months. Glomerular p38 MAPK and CREB activities were determined by Western blot with phospho-specific antibodies, and glomerular fibronectin and type IV collagen mRNA and protein expression were determined by real-time PCR and Western blot, respectively, with sieved glomeruli.

1. The ratio of kidney weight to body weight (KW/BW) in DM (1.54±0.13%) was significantly higher than that in C rats (0.53±0.04%; $p<0.01$), and the increase in KW/BW was ameliorated by FR167653 administration (0.84±0.07%; $p<0.01$).

2. 24-hour urinary albumin excretion was significantly higher in the DM group (1.99±0.22 mg/day) compared to the C group (0.40±0.06 mg/day; $p<0.05$), and FR167653 treatment significantly reduced albuminuria in DM rats (1.04±0.19 mg/day; $p<0.05$).

3. Glomerular phospho-specific p38 MAPK protein expression in DM rats was 1.8-fold higher than that in C rats ($p<0.05$), and FR167653 treatment nearly normalized the increase in glomerular p38 MAPK activity in DM rats ($p<0.05$).

4. Glomerular phospho-specific CREB protein expression in DM rats was 2.3-fold higher than that in C rats ($p<0.01$) and FR167653 treatment ameliorated the increase in glomerular CREB activity in DM rats ($p<0.01$).

5. Glomerular fibronectin mRNA and protein expression were significantly increased in DM compared to C rats ($p<0.05$), and these increments in DM glomeruli were inhibited by 86.8% and 82.7%, respectively, with FR167653 treatment ($p<0.05$).

6. Glomerular type IV collagen mRNA and protein expression were significantly increased in DM compared to C rats ($p<0.05$), and these increments in DM glomeruli were inhibited by 79.9% and 64.9%, respectively, with FR167653 treatment ($p<0.05$).

7. Immunohistochemical staining for fibronectin revealed a significant increase in glomerular fibronectin protein expression in DM compared to C, and this increase was ameliorated by FR167653 treatment ($p<0.05$).

8. Immunofluorescence staining for type IV collagen revealed a significant increase in glomerular type IV collagen protein expression in DM relative to C rats, and this increase was ameliorated by FR167653 treatment ($p<0.05$).

In conclusion, these findings suggest that p38 MAPK could be a potential target for preventing nephropathy in diabetes.

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< ABSTRACT (IN KOREAN)>

p38 mitogen-activated protein kinase 억제제인 FR167653이 당뇨 백서 사구체 내 fibronectin 및 제 4형 collagen의 발현에 미치는 영향

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배 경: 당뇨병성 신병증은 병리학적으로 사구체 비후와 세포외 기질의 축적이 특징적인 소견으로, 당뇨 조건 하에서 p38 mitogen-activated protein kinase (MAPK) 경로의 활성화를 통하여 fibronectin과 제 4형 collagen의 발현이 증가하는 것으로 보고되고 있다. FR167653 {1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8(4-pyridylpyrazolo(5,1-c)(1,2,4)triazin-2-yl]-2-phenylethanedion sulfate monohydrate}은 interleukin-1과 tumor necrosis factor-α 생성을 억제시키는 효과를 가진 약제로, p38 MAPK 경로의 억제를 통한 항염증 작용으로 인하여 만성 이식 신병증, 반월상 사구체신염, 그리고 자가면역 신질환 등에서 신손상을 감소시켰다는 보고는 있으나, 당뇨병성 신병증에서의 효과에 대한 연구는 현재까지 전무한

실정이다. 이에 본 연구에서는 당뇨 백서를 대상으로 p38 MAPK 억제제인 FR167653가 24시간 뇨알부민 배설량과 사구체 내 fibronectin과 제 4형 collagen의 발현에 미치는 영향을 알아보고자 하였다.

방 법: 32 마리의 Sprague-Dawley 백서를 대조군 (16 마리)과 streptozotocin 을 복강내로 투여하여 당뇨를 유발시킨 당뇨군 (16 마리)으로 나누어, 각 군에서 8 마리씩은 p38 MAPK 억제제인 FR167653 을 1 일 10 mg/kg 용량으로 3 개월간 매일 경구 투여하였으며 (C+FR, DM+FR), 나머지 8 마리씩은 위약을 투여하였다 (C, DM). 당뇨 유발 3 개월 후 24 시간 소변을 채취하여 알부민 배설량을 ELISA 로 측정하였으며, 희생시킨 후 sieve 를 이용하여 분리한 사구체를 실험에 사용하였다. 사구체 내 p38 MAPK와 p38 MAPK에 의하여 조절되는 전사인자인 c-AMP-responsive element binding protein (CREB)의 단백 발현 및 활성도는 Western blot 으로, fibronectin 과 제 4 형 collagen mRNA 와 단백 발현은 각각 real time-PCR 과 Western blot 으로 분석하였다. 또한 신장 조직을 이용하여 fibronectin에 대한 면역조직화학 염색과 제 4형 collagen에 대한 면역형광 염색도 시행하였다.

결 과: 당뇨 유발 3 개월 후 체중에 대한 신장 무게비는 DM 군에서 1.54±0.13%로, C 군의 0.53±0.04%와 C+FR 군의 0.59±0.06%에 비하여 유의하게 증가되었으며, 이러한 증가는 FR167653 투여로 의미있게 억제되었다 ($p<0.01$). 또한, FR167653 은 DM 군에서 의의있게 증가된 24 시간 뇨알부민 배설량을 유의하게 감소시켰다 (C, 0.40±0.06 mg/day; DM, 1.99±0.22 mg/day; DM+FR, 1.04±0.19

mg/day; p<0.05). Phospho-specific p38 MAPK 와 phospho-specific CREB 을 이용한 Western blot 분석상 p38 MAPK 와 CREB 의 활성도는 C 군에 비하여 DM 군에서 각각 1.8 배 (p<0.05), 2.3 배 증가되었으며 (p<0.01), 이러한 증가는 FR167653 투여로 각각 77.8% (P<0.05), 63.4% (p<0.01) 억제되었다. 반면에 총 p38 MAPK 와 총 CREB 의 단백 발현은 네 군 사이에 의미있는 차이가 없었다. Fibronectin 과 제 4 형 collagen 의 mRNA 발현은 C 군에 비하여 DM 군에서 각각 2.1 배, 1.9 배 증가되었으며, FR167653 은 DM 군에서 증가된 fibronectin 과 제 4 형 collagen 의 mRNA 발현을 의의있게 억제시켰다. Western blot 와 신조직 염색으로 분석한 fibronectin 과 제 4 형 collagen 의 단백 발현도 mRNA 발현 양상과 유사하였다.

결 론: 이상의 결과로, p38 MAPK 억제제가 당뇨병성 신병증의 발생 예방에 도움이 될 수 있을 것으로 생각된다.

핵심되는 말: 당뇨병성 신병증, 단백뇨, p38 MAPK, fibronectin, 제 4 형 collagen