Kallikrein-kinin system is involved in podocyte apoptosis under diabetic conditions

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Directed by Professor Shin-Wook Kang

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ABSTRACT

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Background: Recent studies have shown that podocyte injury plays an important role in the pathogenesis of various proteinuric glomerular diseases, including diabetic nephropathy. The number of podocytes is decreased in diabetic glomeruli and angiotensin II (AII)-mediated apoptosis is known to be involved in the process of podocyte loss under diabetic conditions. The kallikrein-kinin system (KKS) is known to closely interact with the renin-angiotensin system (RAS) and to serve as the physiologic counterbalance to the RAS. Since the RAS is activated in diabetic glomeruli and is considered to play an important role in glomerular injury, the KKS is supposed to have a

protective effect on the pathogenesis of diabetic nephropathy. However, the results of recent studies, which investigated the role of the KKS on diabetic nephropathy, were absolutely contrary. Moreover, the presence of a local KKS in podocytes and the changes of its components under diabetic conditions have never yet been explored. In this study, I examined whether a local KKS existed in podocytes and whether the expression of the components of the KKS and bradykinin (BK) production were changed in diabetic glomeruli and in cultured podocytes exposed to high glucose medium. I also elucidated the functional role of BK in podocyte apoptosis, which is implicated as a potential mechanism of podocyte loss characterized in diabetic nephropathy.

Methods: In vivo, 32 Sprague-Dawley rats were injected either with diluent (n=16, C) or with streptozotocin intraperitoneally (IP) (n=16, DM), and 8 rats from each group were treated with BK (0.5 μg/hour) via subcutaneously implanted osmotic minipumps for 6 weeks. In vitro, immortalized mouse podocytes were cultured in media containing normal glucose (5.6 mM, NG), NG+24.4 mM mannitol (NG+M), NG+10^{-7} M AII (NG+AII), high glucose (30 mM, HG) with or without 6-hour pretreatment of 10^{-8} M BK. BK levels in sieved glomeruli and cell lysates were measured by ELISA. Real-time

PCR and Western blot for kallikrein, kininogen, BK B1-receptor (B1R), and B2-receptor (B2R) mRNA and protein expression, respectively, were performed with sieved glomeruli and cell lysates. For the assessment of apoptosis, Western blot for Bax, Bcl-2, and active fragments of caspase-3 were performed. TUNEL assay and Hoechst 33342 staining were also performed with renal tissue and cultured podocytes.

Results: 24-hour urinary albumin excretion was significantly higher in DM compared to C rats, and this increment was ameliorated by BK treatment in DM rats. Not only kininogen, kallikrein, B1R, and B2R mRNA and protein expression but also BK levels were significantly decreased in DM glomeruli and in cultured podocytes exposed to HG medium. The changes in the expression of apoptosis-related molecules and the increase in the number of apoptotic cells in DM glomeruli and HG- and AII-stimulated podocytes were significantly abrogated by BK treatment. The antiapoptotic effect of BK in experimental diabetic glomeruli and in cultured podocytes under diabetic conditions seemed to be mediated through the proapoptotic p38 mitogen-activated protein kinase pathway.

Conclusion: I demonstrate for the first time that the expression of all

components of the KSS is decreased in diabetic glomeruli and in cultured podocytes exposed to high glucose, and this suppressed KKS is associated with podocyte apoptosis. In addition, BK treatment ameliorated podocyte apoptosis under diabetic conditions. These findings suggest that BK may be beneficial in preventing podocyte loss in diabetic nephropathy.

Key words: diabetic nephropathy, proteinuria, podocyte, apoptosis, kallikrein-kinin system, bradykinin

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I. INTRODUCTION

Podocytes are terminally differentiated and highly specialized cells[1]. They line the urinary side of the glomerular basement membrane and function as a fine filter contributing to ultimate size-selectivity, and permitting permeability to molecules smaller than albumin[2]. Recent studies have shown that podocyte injury plays a role in the pathogenesis of various glomerular

diseases[3], including diabetic nephropathy, the leading cause of end-stage renal disease in many countries[4, 5]. Among the characteristic findings of diabetic nephropathy, podocytes are involved in the development of glomerular hypertrophy, glomerulosclerosis, foot process effacement, and podocytopenia[6]. Even though the molecular and cellular mechanisms responsible for these changes remain incompletely resolved, previous studies have demonstrated that diabetic milieu *per se*, hemodynamic changes, and local growth factors such as angiotensin II (AII) mediate the process of podocyte injury in diabetic nephropathy[6, 7].

AII is the major effector molecule of the renin-angiotensin system (RAS). It is a powerful direct vasoconstrictor, promotes vascular smooth muscle contraction, and induces systemic hypertension[8]. Besides its hemodynamic effects, AII exerts a hypertrophic effect on renal tubular cells[9], mesangial cells[10, 11], and vascular smooth muscle cells[12]. In podocytes, AII is known to be involved in the rearrangement of the actin cytoskeleton[13, 14], depolarization and increase in cytosolic calcium activity[15], and apoptosis[16, 17]. Numerous clinical and experimental studies in diabetic nephropathy have also demonstrated that blockade of the AII generation by angiotensin-converting enzyme inhibitors (ACEi) reduced proteinuria[18-20], and inhibited podocyte

loss[21] and the progression of nephropathy, which cannot be explained merely by their antihypertensive effect. Based on these findings, AII has been considered an important mediator in the pathogenesis of diabetic nephropathy. Recently, however, several investigations have suggested that the aforementioned beneficial effects of ACEi in diabetic nephropathy may be attributed to the less degradation of kinins, resulting in increased bioavailability of kinins, in addition to decreased AII synthesis[22, 23].

Kinins, bradykinin (BK) and kallidin, are mainly synthesized from kininogens by the kininogenase, kallikrein, all of which belongs to the kallikrein-kinin system (KKS)[24, 25]. In the kidney, all components of the KKS are known to exist and to regulate glomerular hemodynamics and tubular function[26, 27]. Accumulating evidence has also shown that there is a close interaction between the KKS and the RAS and that the KKS serves as the physiologic counterbalance to the RAS, which is considered to be largely mediated by nitric oxide and prostaglandins[28-31]. In addition, BK, the major effector molecule of the KKS, has been demonstrated to directly prevent apoptosis in various cells by inhibiting p38 mitogen-activated protein kinase (MAPK) pathway via its two distinct G protein-coupled receptors termed BK B1-receptor (B1R) and/or B2-receptor (B2R)[32-35]. Since the RAS and p38

MAPK pathway are activated in diabetic glomeruli and are considered to play an important role in glomerular injury, the KKS is supposed to have a protective effect on the pathogenesis of diabetic nephropathy. However, the results of recent studies, which investigated the role of the KKS on diabetic nephropathy, were absolutely contrary. The changes of the components of the KKS in diabetic kidney were also not consistent. Moreover, the presence of a local KKS in podocytes and the changes of its components under diabetic conditions have never yet been explored.

This study was undertaken to examine whether a local KKS existed in podocytes and whether the expression of the components of the KKS and BK production were changed in diabetic glomeruli and in cultured podocytes exposed to high glucose medium. I also elucidated the functional role of BK in podocyte apoptosis, which is implicated as a potential mechanism of podocyte loss characterized in diabetic nephropathy.

II. MATERIALS AND METHODS

1. Animal study

All animal studies were conducted under an approved protocol. Thirty- two male Sprague-Dawley rats weighing 250-280 g were injected either with diluent [n=16, Control (C)] or with 65 mg/kg streptozotocin intraperitoneally (IP) [n=16, Diabetes (DM)]. Diabetes was confirmed by tail vein blood glucose levels above 300 mg/dl on the third day post-injection. After confirming diabetes, eight rats from each group were treated with BK (0.5 μg/hour) (Sigma-Aldrich Corp., Saint Louis, MO, USA) via subcutaneously implanted osmotic minipumps (Durect Corp., Cupertino, CA, USA) (C+BK, DM+BK) for 6 weeks. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 6-week study period.

Body weights were checked biweekly, and kidney weights were measured at the time of sacrifice. Systolic blood pressure (SBP) was measured by tail-cuff phlethysmography at 6 weeks. Serum glucose and 24-hour urinary albumin were also measured at the time of sacrifice. Blood glucose was measured by a glucometer and 24-hour urinary albumin excretion by enzyme-linked immunosorbent assay (ELISA) (Nephrat II, Exocell, Inc.,

Philadelphia, PA, USA).

2. Glomerular isolation

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

3. Podocyte culture

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY, USA) and were cultured as previously described[36]. Briefly, frozen podocytes were first grown under permissive conditions at 33°C in RPMI 1640 media containing 10% fetal bovine serum, 50 U/ml γ-interferon, and 100 U/ml of penicillin/streptomycin in collagen coated flasks, and the γ-interferon was tapered down to 10 U/ml in successive passages. Cells were then trypsinized and subcultured without γ-interferon (non-permissive conditions) and allowed to differentiate at 37°C with media changed on alternate days. Differentiation of podocytes grown for 10 days at 37°C was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by RT-PCR and Western blotting (data not shown).

After confirming differentiation of podocytes, medium was changed to

serum-free RPMI medium containing normal glucose (5.6 mM, NG), NG+24.4 mM mannitol (NG+M), NG+10^{-7} M AII (NG+AII), high glucose (30 mM, HG), with or without 6-hour pretreatment of 10^{-8} M BK. After 24 hours, cells were harvested for either RNA or protein.

4. Measurement of BK by ELISA

The concentrations of BK in glomerular and podocyte lysates were determined using a commercial ELISA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) according to the manufacturer's protocol. All BK levels were normalized with the total protein content, which had previously been determined by a modified Lowry method. The kit for BK was species-specific and sensitive up to 100ng/ml.

5. Total RNA isolation

Total RNA was extracted as previously described[37]. 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 vortexed and stored for 5 minutes at room temperature, 160 μl of chloroform added, and the mixture shaken vigorously for 30 seconds. After 3 minutes,

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

6. 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. Cellular RNA from each plate was similarly reverse transcribed.

7. Real-time polymerase chain reaction (Real-time PCR)

The primers used for kininogen, kallikrein, B1R, B2R, and GAPDH amplification are summarized in Table 1. Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), the PCR was performed with a total volume of 20 μL in each well, containing 10μL of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μl of cDNA corresponding to 25 ng of RNA, 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 triplicates in separate tubes to permit quantification of the gene normalized to the GAPDH gene. The PCR conditions used were as follows: 35 cycles of denaturation at 94.5°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 minute. Initial heating at 95°C for 9 minutes and final extension at 72°C for 7 minutes were performed for all PCRs. After real time-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 are given as relative expression normalized to the GAPDH gene and expressed in arbitrary units. Signals from C glomeruli and NG cells were assigned a

relative value of 1.0. In pilot experiments, PCR products revealed a single band on agarose gels.

Table 1. Sequences of primers

		Sequence (5'→3')
Kininogen		
Rat	Sense	TCCCGACTGTCAAATGCAAA
	Antisense	CTCTCTTCTTGCACCCTGGC
Mouse	Sense	CTCCAGGCTTTTCTCCTTTCC
	Antisense	ATTCAACCAGCCATTCCCC
Kallikrein		
Rat	Sense	CAGATGGGGTGAAGGTCATC
	Antisense	TCCAACTCTCCTGCACACAG
Mouse	Sense	TCCGCCTCAAAAAGCCTG
	Antisense	ACTGGAGCTCATCTGGGTATTCA
B1R		
Rat	Sense	TCTTCCTGGTGGTGGCTATC
	Antisense	CGTTCAACTCCACCATCCTT
Mouse	Sense	CTGGAAGGAGCTCACAGACC
	Antisense	CTTGGCTCAATGCTGTTTCA
B2R		
Rat	Sense	TTTGTCCTCAGCGTGTTCTG
	Antisense	TCACAAGCATCAGGAAGCAG
Mouse	Sense	CAACGTCACCACACAAGTCC
	Antisense	CGCTGAGGACAAAGAGGTTC
GAPDH		
Rat	Sense	TGCCAAGTATGATGACATCAAGAAG
	Antisense	AGCCCAGGATGCCCTTTAGT
Mouse	Sense	TGTGTCCGTCGTGGATCTGA
	Antisense	CCTGCTTCACCACCTTCTTGA

8. Western blot analysis

Sieved glomeruli and cells harvested from plates 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 min, 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, USA), 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 with a 1:1000 dilution of polyclonal antibodies to kininogen, kallikrein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), B1R, B2R (BD Biosciences, San Jose, CA, USA), Bax, Bcl-2 (Santa Cruz Biotechnology, Inc.), active fragments of caspase-3 (Cell Signaling, Beverly, MA, USA), phospho-specific p38 MAPK, total p38 MAPK (New England Biolabs, Inc., Beverly, MA, USA), or β-actin (Santa Cruz Biotechnology, Inc.). 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.) and the band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

9. TUNEL assay and Hoechst 33342 staining

In addition to the changes in the expression of apoptosis-related molecules, apoptosis was also identified within glomeruli by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) using commercially available kit (Chemicon International, Temecula, CA, USA) and in cultured podocytes seeded on cover slips by Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining. Apoptosis was defined as TUNEL-positive cells within glomeruli and the presence of nuclear condensation on Hoecht staining. TUNEL-positive glomerular cells in formalin-fixed renal tissue and the percentage of podocytes with nuclear condensation were determined by examining at least 30 glomeruli and 300 cells per condition, respectively, at x 400 magnification.

10. Determination of podocyte numbers

Immunohistochemical staining for WT-1 was also performed to determine the number of podocytes as previously described[38]. Briefly, two adjacent 3

μm sections stained with WT-1 were observed in pairs at a magnification of x 400, and the WT-1-positive stained nuclei present in the top but not in the bottom section were counted and summed. Ten glomeruli in five rats from each group and 13 to 15 sections from the midglomerular area were examined.

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 further confirmed by the Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Animal studies

A. Animal data

Body weight and the ratio of kidney weight to body weight were significantly different between DM (295±8 g, 1.18±0.11%) and C rats (411±10, 0.68±0.04%) ($p<0.05$). However, the administration of BK had no effect on either body weight or the ratio of kidney weight to body weight in C and DM rats. The mean blood glucose levels of C, C+BK, DM, and DM+BK were 92.1±5.9 mg/dl, 94.5±4.0 mg/dl, 477.0±13.0 mg/dl, and 488.8±11.2 mg/dl, respectively ($p<0.01$). Compared to the C group (0.32±0.05 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (1.19±0.17 mg/day, $p<0.05$), and the increase in 24-hour urine albumin excretion was significantly abrogated in DM rats by BK treatment (0.63±0.13 mg/day, $p<0.05$) (Table 2).

Table 2. Animal data

	C (N=8)	C+BK (N=8)	DM (N=8)	DM+BK (N=8)
Body Wt (g)	411±10	398± 12	295±8*	301±10*
Kidney Wt/Body Wt (%)	0.68±0.04	0.70±0.05	1.18±0.11*	1.12±0.08*
Blood glucose (mg/dl)	92.1± 5.9	94.5±4.0	477.0±13.0$	488.8±11.2$
24-hour UAE (mg/day)	0.32±0.05	0.34±0.08	1.19±0.17*	0.63±0.13#
Systolic blood pressure (mm Hg)	110.6±9.1	108.9±7.7	114.6±10.9	111.9±9.7

*, $p<0.05$ vs. C group; #, $p<0.05$ vs. DM group; $, $p<0.01$ vs. C group.

Wt, weight; UAE: urinary albumin excretion

B. Glomerular kininogen, kallikrein, B1R, and B2R mRNA and protein expression

Glomerular kininogen, kallikrein, B1R, and B2R mRNA expression assessed by real-time PCR was significantly decreased in DM compared to C rats ($p<0.05$) (Fig. 1). In addition, the protein expression of these KSS components were also significantly decreased in DM compared to C glomeruli ($p<0.05$) (Fig. 2).

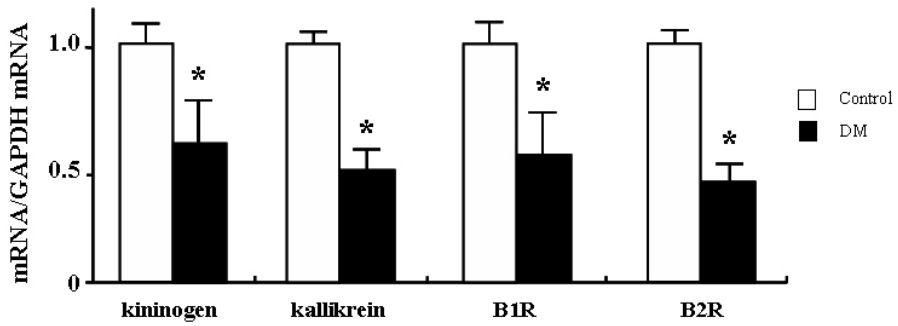

Figure 1. Glomerular kininogen, kallikrein, B1R, and B2R mRNA expression assessed by real-time PCR in C and DM rats. There were significant decreases in the ratios of kininogen, kallikrein, B1R, and B2R mRNA to GAPDH mRNA in DM compared to C glomeruli. *; $p<0.05$ vs. C group.

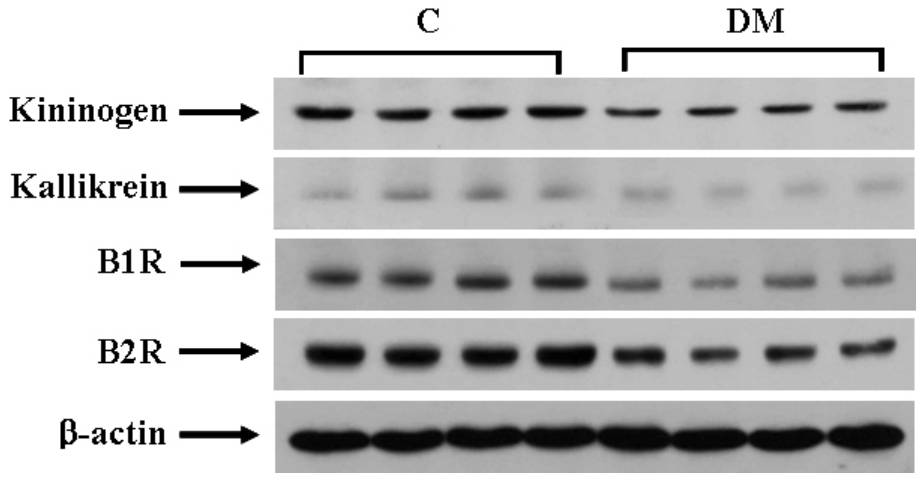

Figure 2. A representative Western blot of glomerular kininogen, kallikrein, B1R, and B2R in C and DM rats (representative of four blots). Kininogen, kallikrein, B1R, and B2R protein expression were significantly decreased in DM relative to C glomeruli. In contrast, there was no difference in β-actin expression between the two groups.

C. BK concentrations in glomerular lysates

Compared to C glomeruli (37.8±5.2 pg/mg protein), there was a significant decrease in the levels of BK in DM glomeruli (15.4±3.8 pg/mg protein) ($p<0.05$).

D. Bax, Bcl-2, and active fragments of caspase-3 protein expression

Bax and active fragments of caspase-3 protein expression were significantly increased, while Bcl-2 protein expression was significantly decreased in DM compared to C glomeruli ($p<0.05$). Administration of BK significantly ameliorated the increases in the ratio of Bax/Bcl-2 ($p<0.05$) and active fragments of caspase-3 protein expression in DM glomeruli ($p<0.05$) (Fig. 3).

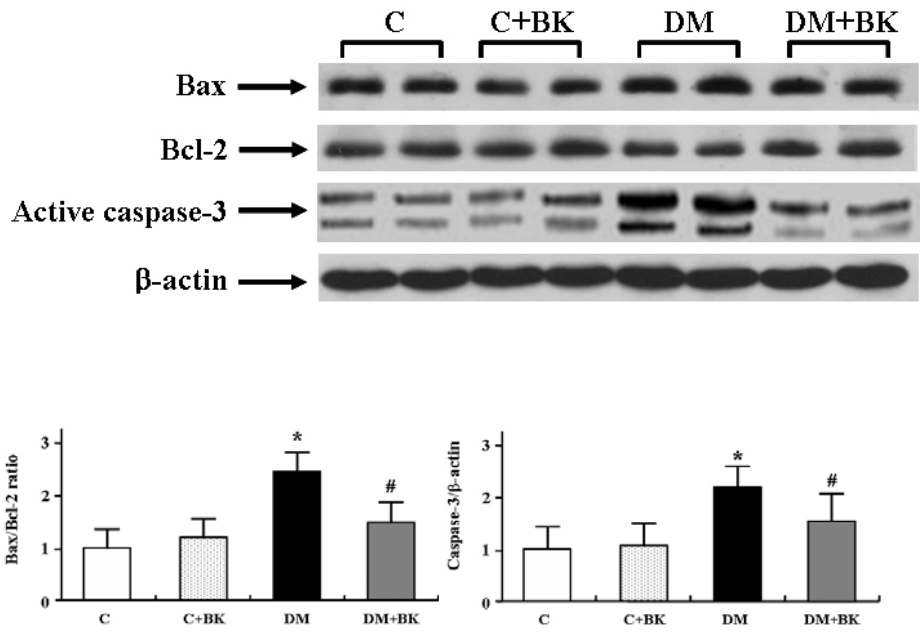

Figure 3. A representative Western blot of glomerular Bax, Bcl-2, and active fragments of caspase-3 in C, C+BK, DM, and DM+BK groups (representative of four blots). Bax and active fragments of caspase-3 protein expression were significantly increased, while Bcl-2 protein expression was significantly decreased in DM compared to C glomeruli. Administration of BK significantly ameliorated the increases in the ratio of Bax/Bcl-2 and active fragments of caspase-3 protein expression in DM glomeruli. *; $p<0.05$ vs. C and C+BK groups, #; $p<0.05$ vs. DM group.

E. TUNEL assay

In addition to Bax, Bcl-2, and active fragments of caspase-3 protein expression, apoptosis in glomeruli was assessed by TUNEL assay. The number of glomerular apoptotic cells was significantly increased in DM compared to C and C+BK rats ($p<0.01$), and the increase in apoptotic cells in DM glomeruli was significantly attenuated by BK treatment ($p<0.05$) (Fig. 4).

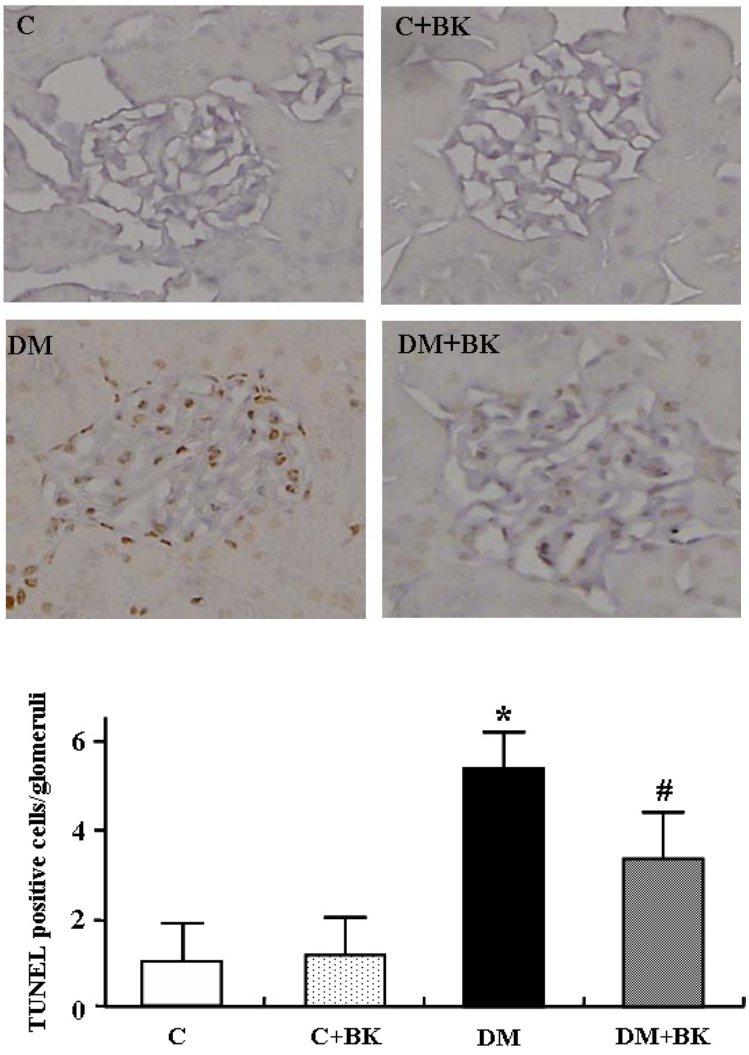

Figure 4. Apoptosis assessed by TUNEL assay in C, C+BK, DM, and DM+BK rats. The number of glomerular apoptotic cells was significantly increased in DM compared to C and C+BK rats, and the increase in apoptotic cells in DM glomeruli was significantly attenuated by BK treatment. *; $p<0.01$ vs. C and C+BK groups, #; $p<0.05$ vs. DM group.

F. Podocyte numbers

Compared to C (170.0±3.8) and C+BK rats (173.8±4.9), the number of podocytes tended to be decreased in DM rats (152.7±4.2), and the reduction in podocyte numbers was inhibited in DM rats treated with BK (166.0±6.1).

G. p38 MAPK activity and protein expression

To elucidate the underlying mechanism how BK treatment inhibited apoptosis in DM glomeruli, we examined the changes in p38 MAPK activation in the four groups. The expression of phospho-p38 MAPK protein (activity) was significantly increased in DM compared to C glomeruli ($p<0.05$), and the increase in glomerular p38 MAPK activity was significantly abrogated in DM rats by the administration of BK ($p<0.05$). In contrast, there was no difference in total p38 MAPK protein expression among the four groups (Fig. 5).

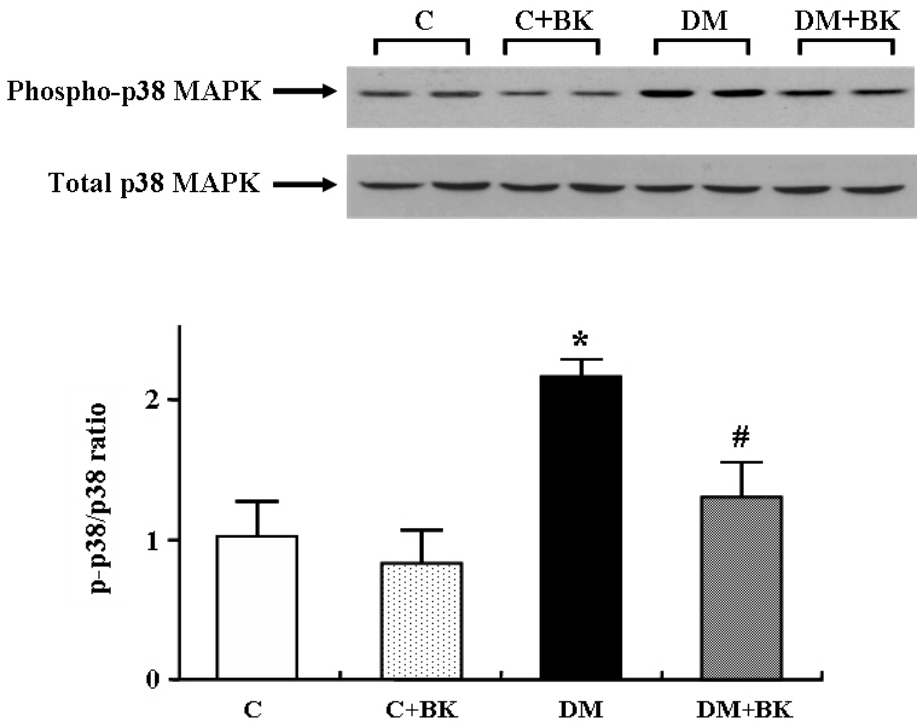

Figure 5. A representative Western blot of glomerular phospho-p38 MAPK and total p38 MAPK in C, C+BK, DM, and DM+BK groups (representative of four blots). The expression of phospho-p38 MAPK (activity) was significantly increased in DM compared to C glomeruli ($p<0.05$), and the increase in glomerular p38 MAPK activity was significantly inhibited in DM rats by the administration of BK ($p<0.05$). In contrast, there was no difference in total p38 MAPK protein expression among the four groups. *; $p<0.05$ vs. C and C+BK groups, #; $p<0.05$ vs. DM group.

2. Cultured podocytes studies

A. Kininogen, kallikrein, B1R, and B2R mRNA and protein expression in cultured podocytes

Kininogen, kallikrein, B1R, and B2R mRNA expression assessed by real-time PCR was significantly decreased in podocytes exposed to HG compared to NG cells ($p<0.05$) (Fig. 6). In addition, the protein expression of these KSS components showed similar patterns to their mRNA expression ($p<0.05$) (Fig. 7). On the other hand, there were no differences in the expression of these molecules between NG and NG+M groups.

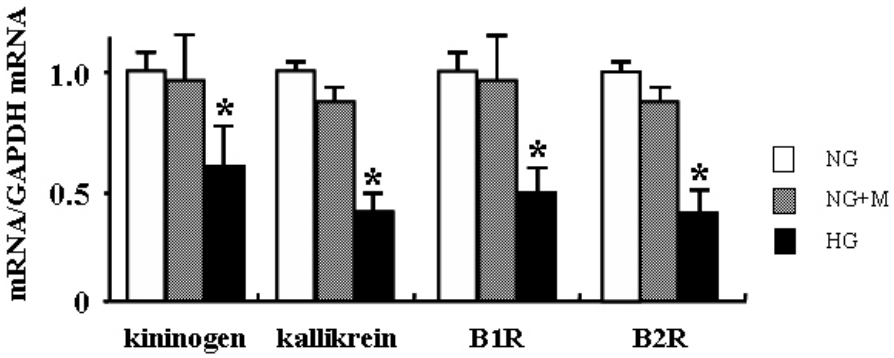

Figure 6. Kininogen, kallikrein, B1R, and B2R mRNA expression assessed by real-time PCR in cultured podocytes exposed to NG, NG+M, and HG. There were significant decreases in the ratios of kininogen, kallikrein, B1R, and B2R mRNA to GAPDH mRNA in cultured podocytes exposed to HG compared to NG and NG+M cells. *; $p<0.05$ vs. NG and NG+M groups.

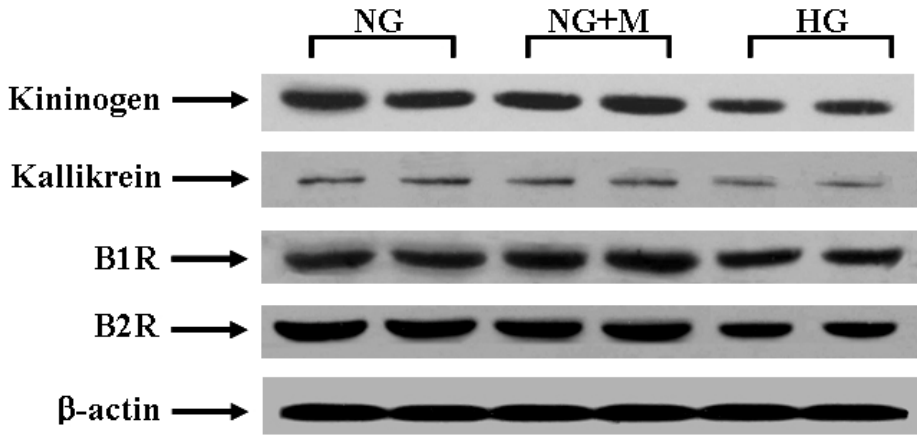

Figure 7. A representative Western blot of kininogen, kallikrein, B1R, and B2R in cultured podocytes exposed to NG, NG+M, and HG (representative of four blots). Kininogen, kallikrein, B1R, and B2R protein expression were significantly decreased in podocytes cultured under high glucose medium relative to NG and NG+M cells. In contrast, there was no difference in β-actin expression between the two groups. *; $p<0.05$ vs. NG and NG+M groups.

B. BK concentrations in cultured podocytes

Compared to NG (56.3±5.9 pg/mg protein) and NG+M podocytes (53.6±4.7 pg/mg protein), there was a significant decrease in the levels of BK in podocytes cultured under high glucose medium (25.2±3.6 pg/mg protein) ($p<0.05$).

C. Bax, Bcl-2, and active fragments of caspase-3 protein expression

The effects of high glucose and AII on apoptosis-related molecules and BK on high glucose- and AII-induced changes in these molecules in cultured podocytes are shown in Figure 8. High glucose significantly increased Bax and active fragments of caspase-3 protein expression and significantly reduced Bcl-2 protein expression in cultured podocytes ($p<0.05$). Bax and active fragments of caspase-3 protein expression were also significantly increased, while Bcl-2 protein expression was significantly decreased in podocytes exposed to AII compared to NG cells ($p<0.05$). These changes induced by high glucose and AII were significantly abrogated by BK pretreatment ($p<0.05$).

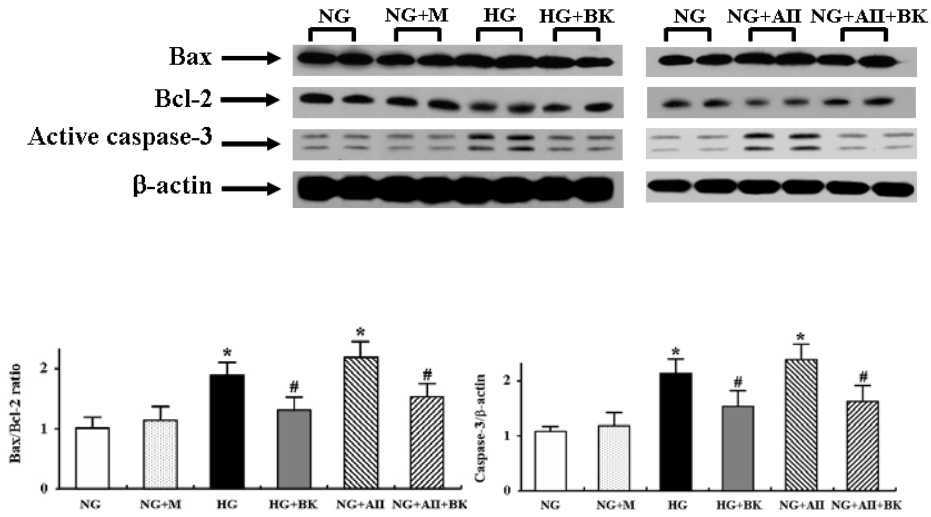

Figure 8. A representative Western blot of glomerular Bax, Bcl-2, and active fragments of caspase-3 in cultured podocytes exposed to NG, NG+M, NG+AII, and HG with or without 6-hour pretreatment of 10^{-8} M BK (representative of four blots). Bax and active fragments of caspase-3 protein expression were significantly increased, while Bcl-2 protein expression was significantly decreased in cultured podocytes exposed to HG and AII compared to NG cells. Pretreatment of BK significantly abrogated the increases in the ratio of Bax/Bcl-2 and active fragments of caspase-3 protein expression in HG- and AII-stimulated podocytes. *; $p<0.05$ vs. NG and NG+M groups, #; $p<0.05$ vs. HG and NG+AII groups.

D. Hoechst 33342 staining

Apoptotic cells assessed by Hoechst 33342 staining were significantly increased in HG- and AII-stimulated cells compared to NG cells ($p<0.01$), and these increments in apoptotic cells by HG and AII were significantly ameliorated by the administration of BK ($p<0.05$) (Fig. 9).

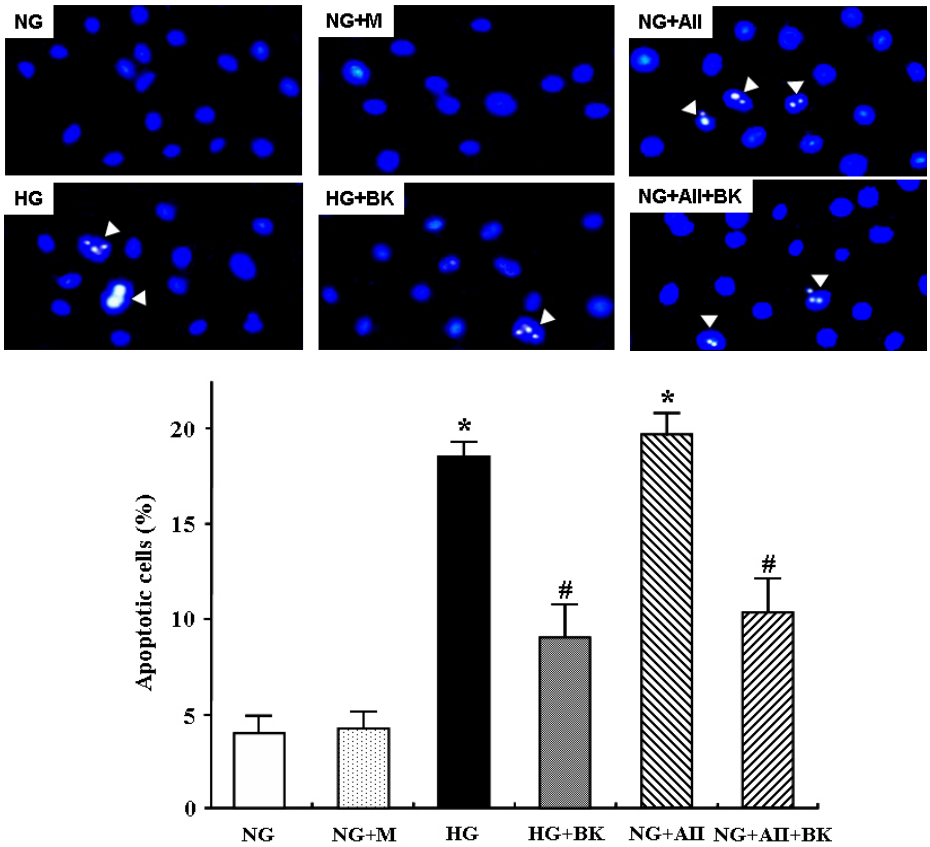

Figure 9. Apoptosis assessed by Hoechst 33342 staining. There were significant increases in apoptotic cells (arrow heads) in HG- and AII-stimulated podocytes compared to NG cells, and these increments in apoptotic cells by HG and AII were significantly ameliorated by the administration of BK. *; $p<0.01$ vs. NG and NG+M groups, #; $p<0.05$ vs. HG and NG+AII groups.

E. p38 MAPK activity and protein expression

Similar to the results of in vivo experiments, p38 MAPK was activated in cultured podocytes under diabetic conditions ($p<0.05$), and this increase in the activity of p38 MAPK in HG-stimulated podocytes was attenuated by BK pretreatment ($p<0.05$). In contrast, there was no difference in total p38 MAPK protein expression among the groups (Fig. 10).

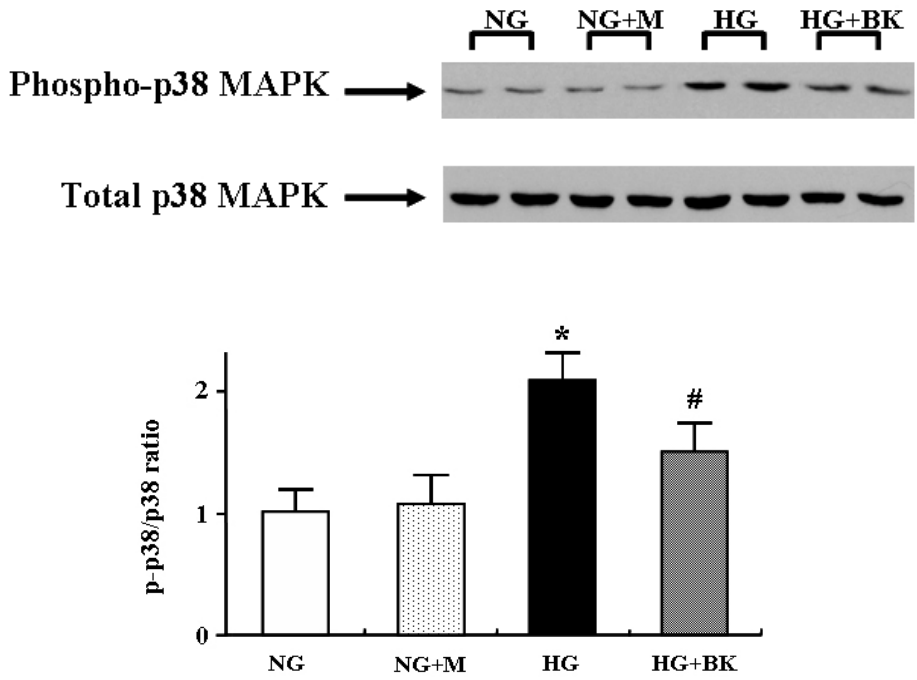

Figure 10. A representative Western blot of glomerular phospho-p38 MAPK and total p38 MAPK in cultured podocytes exposed to NG, NG+M, and HG with or without 6-hour pretreatment of 10^{-8} M BK (representative of four blots). The expression of phospho-p38 MAPK (activity) was significantly increased in cultured podocytes under diabetic conditions, and this increase in the activity of p38 MAPK in HG-stimulated podocytes was attenuated by BK pretreatment. In contrast, there was no difference in total p38 MAPK protein expression among the groups. *; $p<0.05$ vs. NG and NG+M groups, #; $p<0.05$ vs. HG group.

IV. DISCUSSION

Previous studies have demonstrated that the KKS plays a protective role in various kidney diseases including diabetic nephropathy[33, 34, 39-40]. However, its functional significance in terms of podocyte apoptosis under diabetic conditions has never been explored. This study demonstrates for the first time that all components of the KKS exist in podocytes and their expression are decreased in experimental diabetic glomeruli and in high glucose-stimulated podocytes. In addition, the results of the present study showing that administration of BK inhibits podocyte apoptosis both in vivo and in vitro suggest that this suppressed KKS within podocytes under diabetic conditions may contribute to podocyte apoptosis.

The KKS is considered the physiologic counterbalance to the RAS and accumulating evidence has suggested that renal KKS also plays an important role in glomerular hemodynamic regulation[28-31, 41-42]. In addition, local RAS in glomeruli has recently been demonstrated[43]. Moreover, specific binding sites for kinins have been found in intact glomeruli[44-46]. These findings suggest that local KKS may exist within glomeruli, but most previous studies on renal KKS have been performed with whole kidney or renal cortical tissue rather than isolated glomeruli[39, 47, 48]. In this study, I show for the first time

that all components of the KKS are present in not only glomeruli but also podocytes.

The changes in the components of renal KKS under diabetic conditions have been examined by a number of investigators[22, 23, 40, 49-51]. It has been demonstrated that renal kallikrein production is reduced in patients with severe renal failure[52, 53] and in streptozotocin-induced diabetic rats with decreased glomerular filtration rates, while its activity is increased in insulin-treated streptozotocin-induced diabetic rats[54], suggesting that insulin may modulate the expression of renal kallikrein activity. The results of the present study reveal that kallikrein expression was decreased in glomeruli from diabetic rats without decreased renal function and in high glucose-stimulated cultured podocytes, which is not clear whether these changes are attributed to lack of insulin treatment or not. On the other hand, renal kininogen, B1R, and B2R expression are known to be increased in diabetic kidney. These are completely contrary to my results. Since a recent study showed that there was a differential expression pattern of ACE2 in early diabetic rats[55]; decreased expression in diabetic glomeruli, but increased expression in the tubules of diabetic kidney, and I used isolated glomeruli in contrast to whole kidney or renal cortical tissue in most previous studies, it is supposed that the

expression of kininogen, B1R, and B2R may also be different between glomeruli and tubulointerstitium in diabetic kidney. Therefore, further experiments were performed with the tubulointerstitial tissue to determine whether the expressions of these genes in the tubulointerstitium were really different from those of glomeruli, and revealed that the expression of kininogen, B1R, and B2R were increased in diabetic tubulointerstitium, which was totally opposite to the changes of these genes' expression in diabetic glomeruli (data not shown). Further studies will be needed to clarify the functional role of activated KKS within the tubulointerstitium of diabetic kidney. In addition, since ACE expression is known be to unchanged in diabetic glomeruli and in high glucose-stimulated podocytes[43], the decrease in BK levels under diabetic conditions seems to be mainly due to the decreases in the substrate, kininogen, and the enzyme regulating its synthesis, kallikrein.

In human and animal studies, administration of ACEi inhibits the development and progression of various kidney diseases, such as diabetic nephropathy[56-58], ischemic-reperfusion renal injury[59], and hypertensive glomerulosclerosis[60]. It is well known that ACEi exert its numerous actions by reducing both AII synthesis and BK degradation. Based on previous

studies showing that the beneficial effects of ACEi were abrogated by a B2R antagonist in streptozotocin-induced diabetic rats[22, 23] and in db/db mice[61], it has been suggested that the foregoing beneficial effects of ACEi on the kidney is at least partly mediated by accumulated BK and consequent activation of B2R. The direct protective role of the KKS *per se* has also been demonstrated in animal models of type 1 and type 2 diabetes. Kakoki et al[39] showed that the amount of proteinuria and an increase in glomerular mesangial sclerosis were significantly greater in Akita diabetic mice lacking the B2R compared to wild-type diabetic littermates. In addition, a recent study revealed that a two-fold significant increase in albuminuria was observed in streptozotocin-induced diabetic tissue kallikrein-knockout mice compared to wild-type mice[40]. In contrast, Tan et al[62] used streptozotocin-induced diabetic mice with targeted deletion of B2R to elucidate the functional role of the KKS in the development of diabetic nephropathy and found that diabetic B2R knockout mice displayed reduced albuminuria as well as reduced glomerular and tubular injury compared to wild-type diabetic mice. In the study by Tan et al, however, urinary albumin excretion was increased in B2R-/- diabetic mice at the later stage, which became comparable with B2R+/+ diabetic mice. These conflicting results on the effect of the KKS on renal injury may be partly due to the differences in

genetic background, extent of B1R activation, severity of hyperglycemia, and the sample used for experiments (whole kidney vs. renal cortex). Even though previous studies have shown that renal KKS plays a critical role in the pathogenesis of diabetic nephropathy in terms of proteinuria, the effect of the KKS on podocyte, the most important cell responsible for proteinuria, has not been fully explored.

The number of podocytes is decreased in the glomeruli of diabetic patients and animal models of diabetes, and apoptosis is known to be involved in podocyte loss under diabetic conditions[3, 63, 64]. Previous studies have demonstrated that AII, an important mediator in the development and progression of diabetic nephropathy, increase reactive oxygen species production and induce apoptosis in cultured podocytes[3, 6, 17, 65]. Moreover, recent studies have demonstrated that local RAS within podocytes is activated in diabetes both in vivo and in vitro[43]. Since there is a close interaction between the RAS and the KKS, and kinin is known to increase nitric oxide levels, consequently resulting in a decrease in oxidative stress[31], I supposed that local KKS may also present within podocytes and this local KKS may protect against podocyte apoptosis under diabetic conditions. The results of the present study show for the first time that administration of BK

reduces urinary albumin excretion in diabetic rats and that podocyte apoptosis is ameliorated by BK both in vivo and in vitro, suggesting that local KKS within podocytes may be protective against podocyte injury under diabetic conditions.

Numerous studies reveal protein kinase C (PKC) activation in diabetic glomeruli[66] and in podocytes cultured under high-glucose conditions[67-70]. PKC propagates the physiologic responses of receptor-ligand interactions via an array of downstream signals, such as MAPK. Ultimately, these transmitted signals regulate the transcription of genes responsible for key cellular responses such as proliferation, differentiation, and apoptosis[71-73]. The MAPK family includes the extracellular signal-regulated kinase-1/2 (ERK1/2) or p42/44 MAPK[73, 74], stress-activated c-Jun N-amino terminal kinase (JNK/stress-activated protein kinase or SAPK)[72, 73, 75], p38 MAPK[76-78], and big MAPK (ERK 5)[79]. The classic MAPK ERK1/2 plays a pivotal role in growth factor-induced mitogenesis and differentiation, whereas p38 MAPK is known as a "stress-activated kinase," along with JNK. p38 MAPK is strongly activated by environmental stressors, including osmolality changes[80, 81], oxidants[80, 82], and proinflammatory cytokines[80, 81], leading to cellular differentiation and apoptosis[83]. Previous works have shown that p38 MAPK

activity is increased in diabetic glomeruli[84] in vivo and in high glucose-stimulated mesangial cells[85] and podocytes[86] in vitro and that administration of p38 MAPK inhibitor ameliorates apoptosis in diabetic glomeruli and in cultured mesangial cells exposed to high glucose medium[87]. In addition, p38 MAPK has been demonstrated to be required for TGF-β1-induced podocyte apoptosis[88]. These findings suggest that p38 MAPK activation is closely linked to apoptosis of mesangial cells and podocytes under diabetic conditions.

Prior studies have shown that the KKS directly protect against apoptosis in various cells under diverse conditions[89-92]. Pretreatment with BK partly prevented AII-induced apoptosis in cultured vascular smooth muscle cells[93]. In addition, stretch-induced apoptosis in cultured alveolar type II cells was inhibited by BK[94]. The antiapoptotic effect of the KKS was also demonstrated in vivo[33, 95]. Immediate tissue kallikrein infusion in rats after myocardial infarction reduced myocardial infarct size and inhibited cardiomyocyte apoptosis along with decreased p38 MAPK phosphorylation[96]. Moreover, BK infusion in Dahl salt-sensitive rats fed with high-salt diet[33] and tissue kallikrein infusion in rats with gentamicin-induced nephrotoxicity ameliorated tubular cell apoptosis[97], which was associated with the

attenuation of increased phosphorylation of p38 MAPK and of decreased phosphorylation of Akt, respectively. The functional role of the KKS on the inhibition of apoptosis was further documented in animals with targeted deletion of B2R with or without B1R. Mice lacking both the B1R and B2R or mice lacking B2R only had more severe tubular injury and more TUNEL-positive cells in the renal cortex after ischemia-reperfusion renal injury compared with wild-type mice[34]. Based on these findings, the KKS seems to directly exert its antiapoptotic effect through inhibiting the proapoptotic p38 MAPK pathway. The results of the present study also shows that the activation of p38 MAPK in podocytes under diabetic conditions is abrogated by BK treatment both in vivo and in vitro, resulting in less apoptosis, which are in agreement with most of the previous studies.

In conclusion, I demonstrate that the expression of all components of the KKS are decreased in diabetic glomeruli and in podocytes cultured under high glucose medium, and this suppressed KKS under diabetic conditions is associated with podocyte apoptosis. In addition, substitution of BK abrogated podocyte apoptosis under diabetic conditions via inhibiting proapoptotic p38 MAPK pathway. These findings suggest that local KKS within podocytes may play an important protective role against podocyte injury under diabetic

conditions.

V. CONCLUSION

In this study, I investigated whether a local KKS existed in podocytes and whether the expression of the components of the KKS and BK production were changed in cultured podocytes exposed to high glucose medium and in diabetic glomeruli. In addition, I elucidated the functional role of BK in podocyte apoptosis, which is implicated as a potential mechanism of podocyte loss characterized in diabetic nephropathy.

1. Twenty-four hour urinary albumin excretion at 6 weeks was significantly higher in DM compared to C rats, and this increment was ameliorated by BK treatment in DM rats.
2. Glomerular kininogen, kallikrein, B1R, and B2R mRNA and protein expression were significantly decreased in DM compared to C rats.
3. BK levels were significantly lower in DM compared to C glomeruli.
4. BK significantly attenuated the changes in the expression of apoptosis-related molecules and reduced the number of apoptotic cells in DM glomeruli.
5. The expression of all components of the KKS were significantly decreased and BK concentrations were significantly lower in cultured podocytes exposed to HG compared to NG cells.

6. BK significantly abrogated the changes in the expression of apoptosis-related molecules and inhibited the increase in apoptotic cells in HG- and AII-stimulated podocytes.
7. The antiapoptotic effect of BK in experimental DM glomeruli and in cultured podocytes under HG medium seemed to be mediated through the proapoptotic p38 MAPK pathway.

In conclusion, the expression of all components of the KSS is decreased in diabetic glomeruli and in podocytes cultured under high glucose medium, and this suppressed KKS is associated with podocyte apoptosis. In addition, BK treatment abrogated podocyte apoptosis under diabetic conditions. These findings suggest that BK may be beneficial in preventing podocyte loss in diabetic nephropathy.

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<Abstract (in Korean)>

당뇨 조건 하에서 kallikrein-kinin계가 족세포의 세포사멸에 미치는 영향

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곽 승 재

서론: 당뇨병성 신병증은 국내외적으로 말기 신부전증의 가장 흔한 원인 질환으로, 단백뇨가 대표적인 임상적 특징이다. 최근의 보고들에 의하면 당뇨병성 신병증을 포함하여 다양한 사구체 질환에서 단백뇨의 발생에 사구체 여과 장벽을 구성하는 세포의 하나인 족세포가 중요한 역할을 하는 것으로 알려져 있다. 당뇨병성 신병증에서 족세포의 수는 감소되어 있는데, 이러한 수적 감소는 고혈당이나 안지오텐신 II에 의한 세포사멸에 기인하는 것으로 보고되고 있다. Kallikrein-kinin 계는 레닌-안지오텐신 계와 밀접한 연관이 있으면서 레닌-안지오텐신 계와는 생리학적으로 길항 작용을 하는 것으로 알려져 있다. 사구체 내 국소 레닌-안지오텐신 계의 활성화가 당뇨병성 신병증의 병태생리와 밀접한

연관이 있기 때문에 당뇨병성 신병증에서 사구체 내 국소적 kallikrein-kinin 계의 보호적 역할이 기대되었으나, 기존의 연구 결과는 서로 상반되어 있다. 또한, 족세포 내 kallikrein-kinin 계의 존재 및 당뇨 조건 하에서의 변화에 대한 연구는 전무한 실정이다. 이에, 본 연구자는 실험적 당뇨 사구체와 고포도당으로 자극한 족세포에서 kallikrein-kinin 계의 변화를 관찰하였으며, 당뇨 조건 하에서 bradykinin 투여가 족세포의 세포사멸에 미치는 영향을 규명하고자 하였다.

방법: 생체 외 실험으로는 32마리의 Sprague-Dawley 백서를 대상으로 대조군 (16마리)과 streptozotocin으로 당뇨를 유발시킨 당뇨군 (16마리)으로 나누었으며, 각 군에서 8마리씩은 bradykinin을 infusion pump를 이용하여 6주간 투여하였다. 생체 내 실험으로는 불멸 생쥐 족세포를 정상 포도당군 (5.6 mM), 정상 포도당+만니톨군 (24.4 mM 만니톨), 정상 포도당+안지오텐신 II군 (10^{-7} M), 그리고 고포도당군 (30 mM)으로 나누어 배양하였으며, 이와 동시에 bradykinin (10^{-8} M)으로 전처리한 실험도 시행하였다. 실험 동물로부터 분리한 사구체와 배양 족세포에서 kallikrein-kinin 계의 mRNA와 단백 발현은 각각 real-time PCR과 Western blot을 이용하여 분석하였으며, bradykinin 농도는 ELISA로 측정하였다. 세포사멸은 Bax, Bcl-2, 그리고 active fragments of caspase-3에 대한

Western blot을 이용하여 확인하였으며, 신장 조직을 이용한 TUNEL 염색과 배양 족세포를 이용한 Hoechst 33342 염색으로도 관찰하였다.

결과: 24시간 뇨알부민 배설량은 대조군에 비하여 당뇨군에서 의미있게 증가되었으며, bradykinin 투여로 당뇨군에서의 뇨알부민 배설량이 의의있게 감소되었다. Kallikrein-kinin 계의 구성 요소인 kininogen, kallikrein, 그리고 제 1형 및 제 2형 bradykinin 수용체의 mRNA와 단백 발현뿐만 아니라 bradykinin 농도도 당뇨 사구체 및 고포도당으로 자극한 배양 족세포에서 유의하게 감소되어 있었다. 당뇨 사구체와 고포도당 또는 안지오텐신으로 자극한 배양 족세포에서 세포사멸의 지표인 Bax/Bcl-2 단백 발현의 비와 active fragments of caspase-3의 단백 발현이 의미있게 증가되었으며, 이러한 증가는 bradykinin 투여로 의의있게 억제되었다. TUNEL 염색과 Hoechst 33342 염색으로 확인한 사구체 내 및 배양 족세포에서 세포사멸이 동반된 세포 수 역시 당뇨군과 고포도당 또는 안지오텐신으로 자극한 군에서 유의하게 많았으며, bradykinin 투여로 세포사멸이 동반된 세포 수는 의미있게 감소되었다.

결론: 당뇨 사구체와 고포도당으로 자극한 족세포에서 kallikrein-kinin 계 구성 요소의 발현이 감소되어 있었으며, 이러한 발현 감소와 당뇨 조건 하 족세포의 세포사멸 사이에는 밀접한 관련이 있었다. 이상의 결과를 종합해 볼 때, bradykinin은 당뇨 조건 하에서

족세포의 소실 예방에 유용할 것으로 생각된다.

핵심되는 말: 당뇨병성 신병증, 단백뇨, 족세포, 세포사멸, kallikrein-kinin계, bradykinin

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