Podocyte hypertrophy precedes apoptosis under experimental diabetic conditions

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Directed by Professor Shin-Wook Kang

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I'd like to dedicate this paper to all of you with all of my heart.

Thanks everyone.

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ABSTRACT

Podocyte hypertrophy precedes apoptosis under experimental diabetic conditions

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Background: Diabetic nephropathy, the leading cause of end-stage renal disease worldwide, is characterized pathologically by glomerular hypertrophy and clinically by proteinuria. Apoptosis has also been implicated in the pathogenesis of diabetic nephropathy. To date, however, the sequence of hypertrophy and apoptosis especially of podocytes, two hallmarks in diabetic glomeruli, is still a matter of debate. The aim of this study was to elucidate the consequences of inhibiting hypertrophy on apoptosis and of blocking apoptosis on hypertrophy in podocytes under diabetic conditions.

Methods: *In vitro*, After confirming differentiation of immortalized mouse podocytes and serum restriction for 24 hr, the medium was changed to serum-free RPMI medium containing 5.6 mM glucose (NG), NG+24.4 mM

mannitol (NG+M), or 30 mM glucose (HG) with or without 10 μM PKI 166, an epidermal growth factor receptor (EGFR) inhibitor or 10^{-7} M zAsp-DCB (N-aspartyl-2,6-dichlorobenzoyloxy-methylketone), a pancaspase inhibitor.

In vivo, sixty-four male Sprague-Dawley rats, weighing 250-280 g, were used. Thirty-two rats were injected intraperitoneally with diluent [control (C)] and the other 32 rats with 65 mg/kg streptozotocin (STZ) [diabetes mellitus (DM)]. After confirming DM, 8 rats each from the C and DM groups were treated with 100 mg/kg/day of PKI 166 by gavage for 3 months. Another 8 rats from each group were treated with zAsp-DCB (2 mg/day) via subcutaneously implanted osmotic minipumps for 3 months. 24-hr urinary albumin excretion was checked at the time of sacrifice.

Western blotting for p27, p21, phospho-4EBP1, phospho-p70S6 kinase, Bax, Bcl-2, active fragments of caspase-3, cleaved poly(ADP-ribose)polymerase (PARP), or β-actin were performed with cell lysates and sieved glomeruli. Podocyte hypertrophy was assessed by measurement of cellular protein/cell number and by flow cytometry. Hoechst 33342 staining and TUNEL assay were performed to identify apoptosis.

Results: Compared to NG cells and C glomeruli, there were significant increases in p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression in cultured podocytes exposed to HG medium and DM glomeruli, respectively, and these increases were significantly abrogated by PKI 166, but not by zAsp-DCB treatment. In addition, both cellular protein/cell number and relative cell size and glomerular volume were significantly greater in podocyte cultured under HG conditions and DM glomeruli, respectively, and these changes were significantly ameliorated by PKI 166 treatment, but not by zAsp-DCB. Meanwhile, the increases in the ratio of Bax/Bcl-2 and the protein expression of active fragments of caspase-3 and cleaved PARP in HG-stimulated podocytes and DM glomeruli were significantly attenuated by

not only zAsp-DCB but also PKI 166 treatment. The increase in apoptotic cells assessed by Hoechst 33342 staining and TUNEL assay in podocytes exposed to HG medium and DM glomeruli, respectively, was also significantly abrogated by PKI 166 as well as zAsp-DCB. Moreover, both EGFR inhibitor and pancaspase inhibitor significantly reduced albuminuria in DM rats.

Conclusion: In conclusion, inhibition of podocyte hypertrophy results in less apoptosis, whereas blocking apoptosis has no effect on podocyte hypertrophy under diabetic conditions, suggesting that podocyte hypertrophy precedes apoptosis. Based on these findings, targeting podocyte hypertrophy may be more effective in the treatment of diabetic nephropathy.

Key words: apoptosis, caspase, diabetic nephropathy, epidermal growth factor, hypertrophy, podocyte

Podocyte hypertrophy precedes apoptosis under experimental diabetic conditions

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I. INTRODUCTION

Diabetic nephropathy, the leading cause of end-stage renal disease worldwide, is characterized pathologically by glomerular hypertrophy and clinically by proteinuria.[1] Even though accumulating evidence indicates that the diabetic milieu per se, hemodynamic changes, and local growth factors, such as angiotensin II and transforming growth factor-β (TGF-β), are involved in the pathogenesis of diabetic nephropathy,[2-4] the underlying pathways mediating these processes are not well understood.

Kidney size is typically increased in diabetes, even at the time of diagnosis.[5] This is primarily due to glomerular and tubular hypertrophy, but inflammatory cell infiltration, extracellular matrix (ECM) accumulation, and hemodynamic factors also contribute.[6-9] In addition, some low-grade proliferation of glomerular cells is present in the early stage of diabetic nephropathy.[10,11] Cell

culture experiments using mesangial cells under high glucose conditions and *in vivo* studies using various models of diabetes have suggested a biphasic growth response.[12] Initially, self-limited proliferation occurs, which is then followed by cell cycle arrest and hypertrophy.[13] In contrast to mesangial cells, mature podocytes do not actively synthesize DNA nor proliferate under normal conditions.[14] However, proliferation of podocytes is observed in certain glomerular diseases, such as HIV nephropathy,[15] collapsing glomerulopathy,[16] and the cellular variant of focal segmental glomerulosclerosis.[17] Under diabetic conditions, moreover, podocytes are demonstrated to undergo hypertrophic processes like mesangial cells, resulting in increased cell size.[18,19]

Apoptosis, which is defined as programmed cell death, removes damaged or unwanted cells and has been implicated in the pathogenesis of numerous diseases such as malignancy, lupus erythematosus, and Alzheimer's disease.[20] Furthermore, apoptosis has been documented in the course of various renal diseases including diabetic nephropathy.[21-24] Cell death by apoptosis is believed to be involved in the process of mesangial cell loss in the late stage of diabetic nephropathy, suggesting that apoptosis may be a homeostatic mechanism regulating mesangial cell numbers.[25] Apoptosis is also considered to be one of the underlying causes of podocyte loss, which contributes to the development of albuminuria in diabetic nephropathy.[23,24]

To date, the sequence of hypertrophy and apoptosis especially of podocytes, two hallmarks in diabetic glomeruli, is still a matter of debate. Some have submitted that podocyte hypertrophy in diabetic nephropathy is merely a compensatory phenomenon to cover the denuded glomerular basement membrane (GBM) due to apoptosis-mediated podocyte loss under diabetic conditions.[26,27] In contrast, several previous studies found that the density of the podocytes rather than their absolute number, is reduced in the early stage of diabetic nephropathy in humans as well as in experimental diabetic models.[28] In addition, a recent study by Jung et al[29] showed that the expression of

apoptosis-related molecules and cyclin-dependent kinase inhibitors (CKIs) were differential according to glomerular size in diabetic nephropathy, indicating that the hypertrophic process was ongoing only in less hypertrophied glomeruli and once the glomeruli became sufficiently large, the hypertrophic mechanism was replaced by apoptosis. Moreover, a recent morphometric analysis revealed that podocyte hypertrophy preceded glomerular hypertrophy and glomerulosclerosis in diabetic nephropathy.[30,31] Based on these findings, others have inferred that podocyte hypertrophy is a prominent early feature of diabetic nephropathy prior to other pathologic findings, including podocyte loss.[32]

Therefore, it is imperative to clarify the order of hypertrophy and apoptosis; which comes first in the process of diabetic nephropathy. The aim of this study was to elucidate the consequences of inhibiting hypertrophy on apoptosis and of blocking apoptosis on hypertrophy in podocytes under diabetic conditions. For this purpose, I examined the changes in apoptosis-related molecules and CKIs protein expression, and cell and glomerular size in high glucose-stimulated podocytes and glomeruli from diabetic rats, respectively, treated with a selective epidermal growth factor (EGF) receptor tyrosine kinase inhibitor, which was reported to abrogate glomerular hypertrophy in experimental diabetic rats, or with a pancaspase inhibitor.

II. MATERIALS AND METHODS

1. Podocyte culture

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA) and were cultured as previously described.[33] Briefly, frozen podocytes were first grown under permissive conditions at 33℃ in RPMI-1640 media containing 10% fetal bovine serum (FBS), 50 U/mL interferon-ɣ (INF-ɣ), and 100 U/mL of penicillin/streptomycin in collagen-coated flasks, and the INF-ɣ tapered down to 10 U/mL in successive passages. Cells were then trypsinized and subcultured without INF-ɣ (nonpermissive conditions) and allowed to differentiate at 37℃ with media changed on alternate days. Differentiation of podocytes grown for 14 days at 37℃ was confirmed by the identification of synaptopodin, a podocyte differentiation marker, by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

After confirming differentiation of podocytes and serum restriction for 24 hr, the medium was changed to serum-free RPMI medium containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), or 30 mM glucose (HG) with or without 10 μM PKI 166 (Novartis, Basel, Switzerland), an EGF receptor (EGFR) inhibitor, or 10^{-7} M zAsp-DCB (N-aspartyl-2,6-dichlorobenzoyloxy-methylketone, Alexis Biochemicals, San Diego, CA, USA), a pancaspase inhibitor. At 48 hr after the media change, cells were harvested for protein analyses.

2. Animals

All animal studies were conducted using a protocol approved by the committee for the care and use of laboratory animals of Yonsei University College of Medicine. Sixty-four male Sprague-Dawley rats, weighing 250-280

g, were used. Thirty-two rats were injected intraperitoneally with diluent [control (C)] and the other 32 rats with 65 mg/kg streptozotocin (STZ) [diabetes mellitus (DM)]. Diabetes was confirmed by tail vein blood glucose levels above 300 mg/dl on the third day post-injection. Sixteen rats from each group were used to evaluate the effect of EGFR inhibitor, PKI 166. After confirming diabetes, 8 rats each from the C and DM groups were treated with 100 mg/kg/day of PKI 166 by gavage (C+PKI 166 or DM+ PKI 166) for 3 months. The dose of PKI 166 was determined based on the previous study by Wassef et al.[34] To study the effect of pancaspase inhibitor, zAsp-DCB, another 16 rats were used. After diabetes confirmation, 8 rats from each group were treated with zAsp-DCB (2 mg/day) via subcutaneously implanted osmotic minipumps (Durect Corp., Cupertino, CA, USA) (C+zAsp-DCB, DM+ zAsp-DCB) for 3 months. A bolus dose of 2 mg zAsp-DCB or vehicle was injected immediately before mini-pump implantation surgery. This dose was obtained from a previous study.[35] The rats were given free access to water and standard laboratory chow during the study period. Body weights were checked monthly, and the kidney weights were measured at the time of sacrifice. Serum glucose concentrations and 24-hr urinary albumin were measured at the time of sacrifice. Blood glucose was measured by a glucometer and 24-hr urinary albumin excretion was determined by enzyme-linked immunosorbent assay (ELISA) (Nephrat II, Exocell, INC., Philadelphia, PA, USA).

3. Glomeruli isolation

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

4. Western blot analysis

Cultured cells harvested from plates and isolated glomeruli were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris-HCl, pH

6.8, 10% (vol/vol) glycerol]. Aliquots of 50 μg protein were treated with Laemmli sample buffer, heated at 100℃ for 5 min, and 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), and the membrane was then incubated in blocking buffer A [1 x phosphate-buffered saline (PBS), 0.1% Tween-20, and 5% nonfat milk] for 1 hr at room temperature, followed by an overnight incubation at 4 ℃ in a 1:1000 dilution of polyclonal antibodies to p27, p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), phospho-eukaryotic elongation factor 4E binding protein 1 (p4EBP1), phospho-p70 S6 ribosomal protein kinase (pp70S6K), cleaved poly(ADP-ribose)polymerase (PARP) (Cell Signaling, Inc., Beverly, MA, USA), Bax, Bcl-2 (Santa Cruz Biotechnology, Inc), active fragments of caspase-3 (Cell Signaling, Inc.), or β-actin (Sigma Chemical Co., St Louis, MO, USA). The membrane was then washed once for 15 min and twice for 5 min 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 donkey anti-goat IgG (Amersham Life Science, Inc., Arlington Heights, IL, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

5. Assessment of podocyte hypertrophy

Hypertrophy of cultured podocytes was assessed by measurement of cellular protein/cell number and by flow cytometry. After seeding podocytes on 100 mm dishes and serum restriction for 24 hr, medium was changed as above, and after 48 hr the cells were harvested with 0.05% trypsin and 0.25 mmol/L ethylenediaminetetraacetic acid, pelleted at 1,500 g for 5 min, and resuspended in PBS. Aliquots of podocytes were used for cell counts using a hemocytometer,

and remaining cells were lysed in 0.5M NaOH and total protein content was measured by using a modified Lowry method. To determine the cell size directly, cells were harvested by trypsinization after 48 hr of treatment as above, fixed with 75% methanol, washed, and incubated with 100 μg/ml RNase and 10 μg/ml propidium iodide in PBS for 1 hr at 37℃. Samples were analyzed by forward light scattering on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and data were processed with Cell Quest Pro software (BD Biosciences).

6. Morphometric measurement of glomerular volume

Glomerular volumes (V_G) of isolated glomeruli were calculated as previously described.[34,36] Briefly, the surface areas of 50 glomeruli cut at the vascular pole on periodic acid-Schiff-stained tissue sections were traced along the outline of the capillary loops using a computer-assisted color image analyzer (Image-Pro Ver. 2.0, Media Cybernetics, Silver Spring, MD, USA). V_G was calculated using the equation: $V_G=\beta/k \times (Area)^{3/2}$, where $\beta=1.38$ is the shape coefficient for spheres, and $k=1.1$ is the size distribution coefficient.

7. Hoechst 33342 staining and TUNEL assay

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

magnification.

8. Statistical analysis

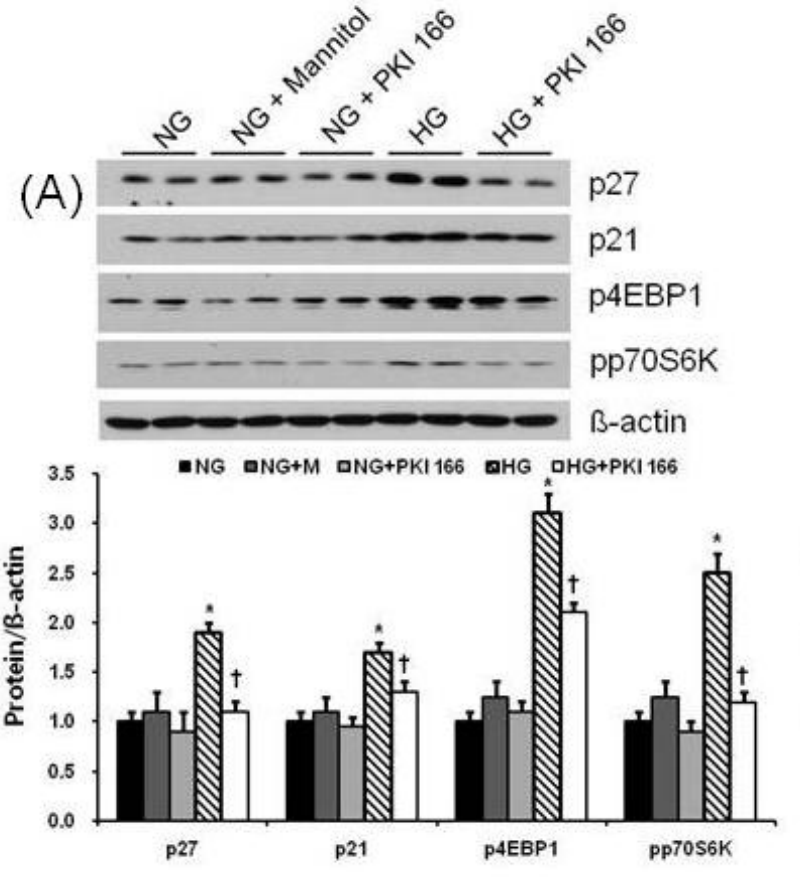
All values are expressed as the mean±SEM. Statistical analysis was performed using the statistical package SPSS for Windows Ver. 15.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were confirmed by the Mann-Whitney U-test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Cultured podocyte studies

A. p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression

To examine the effect of EGFR inhibitor and pancaspase inhibitor on HG-induced podocyte hypertrophy, I first determined the changes in p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression in cultured podocytes. Compared to NG cells, there were 1.9-, 1.7-, 3.1-, and 2.5-fold increases in p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression in cultured podocytes exposed to HG medium ($p<0.05$), and these changes were significantly abrogated by PKI 166 (Figure 1A). In contrast, the increases in the protein expression of p27, p21, phospho-4EBP1, and phospho-p70S6K in HG-stimulated podocytes were not altered by zAsp-DCB treatment (Figure 1B). On the other hand, mannitol had no effect on the expression of these proteins.


(A)
NG
NG + Mannitol
NG + PKI 166
HG
HG + PKI 166
p27
p21
p4EBP1
pp70S6K
ß-actin
NG
NG+M
NG+PKI 166
HG
HG+PKI 166
Protein/ß-actin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p27
p21
p4EBP1
pp70S6K

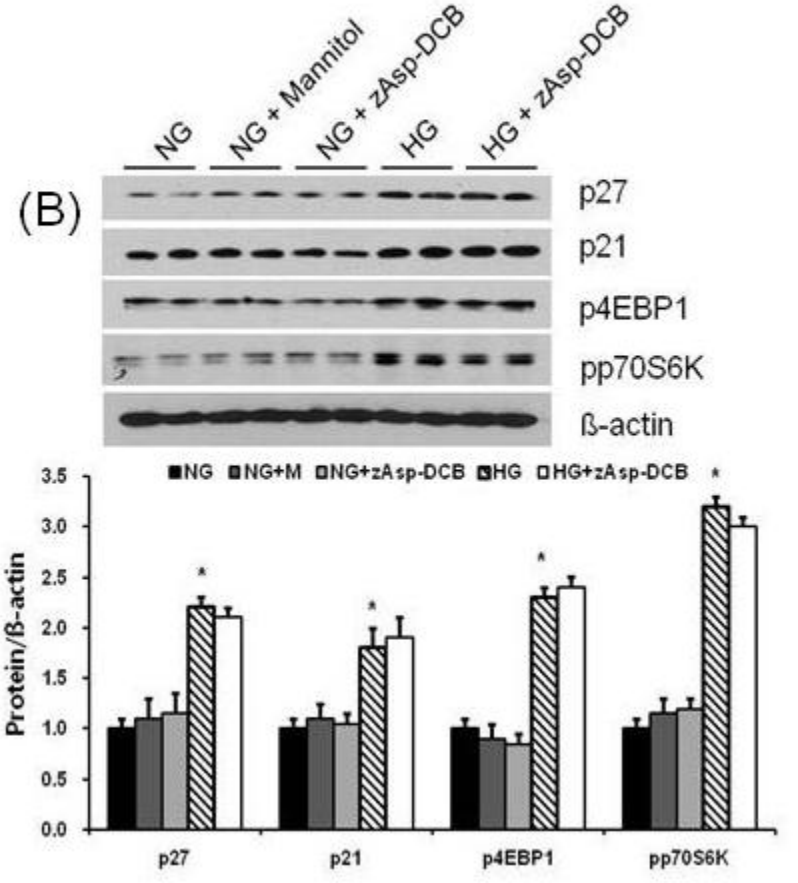
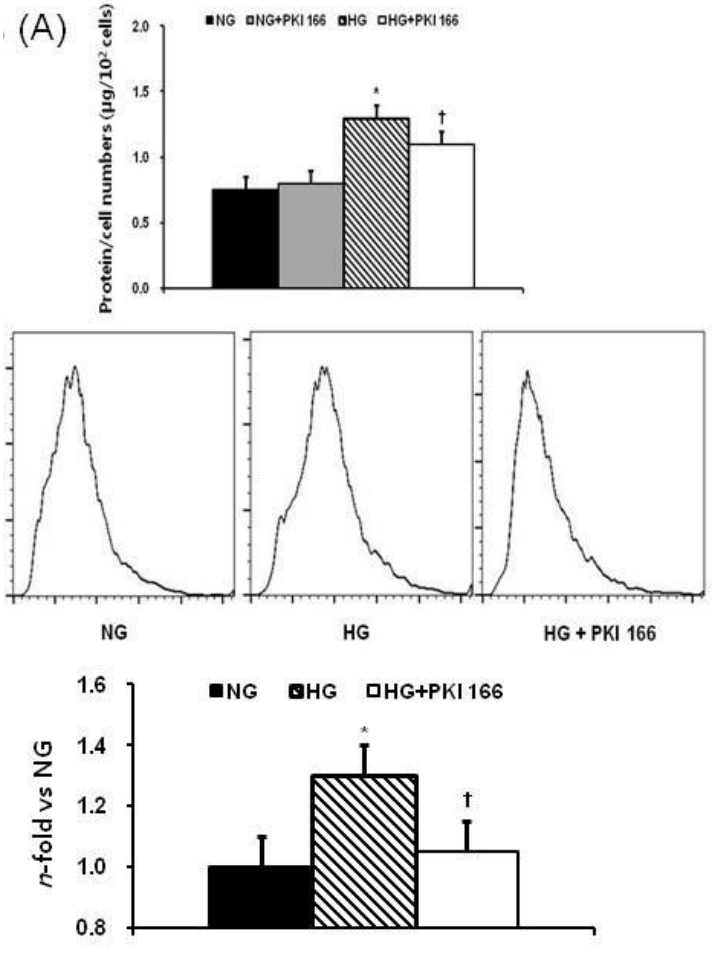


Figure 1. A representative Western blot of p27, p21, phospho-4EBP1, and phospho-p70S6K in cultured podocytes. (A) Compared to NG cells, there were 1.9-, 1.7-, 3.1-, and 2.5-fold increases in p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression in cultured podocytes exposed to HG medium, and these changes were significantly abrogated by PKI 166 treatment. (B) In contrast, the increases in the protein expression of p27, p21, phospho-4EBP1, and phospho-p70S6K in HG-stimulated podocytes were not altered by zAsp-DCB. *; $p<0.05$ vs. NG and NG+M groups, †; $p<0.05$ vs. HG group.

B. Podocyte hypertrophy

To examine the effect of EGFR inhibitor and pancaspase inhibitor on HG-induced podocyte hypertrophy, I next determined cellular hypertrophy by the ratio of total cellular protein content to cell number and relative cell size on FACScan flow cytometer. Both cellular protein/cell number and relative cell size were significantly greater in podocyte cultured under HG conditions compared to NG cells ($p<0.05$), suggesting that HG induced podocyte hypertrophy. These increments were significantly ameliorated by PKI 166 treatment (Figure 2A), while zAsp-DCB had no effect on cellular hypertrophy in HG-stimulated podocytes (Figure 2B).


(A)
NG
NG+PKI 166
HG
HG+PKI 166
Protein/cell numbers (μg/10² cells)
2.0
1.5
1.0
0.5
0.0
*
†
NG
HG
HG + PKI 166
NG
HG
HG+PKI 166
n-fold vs NG
1.6
1.4
1.2
1.0
0.8
*
†

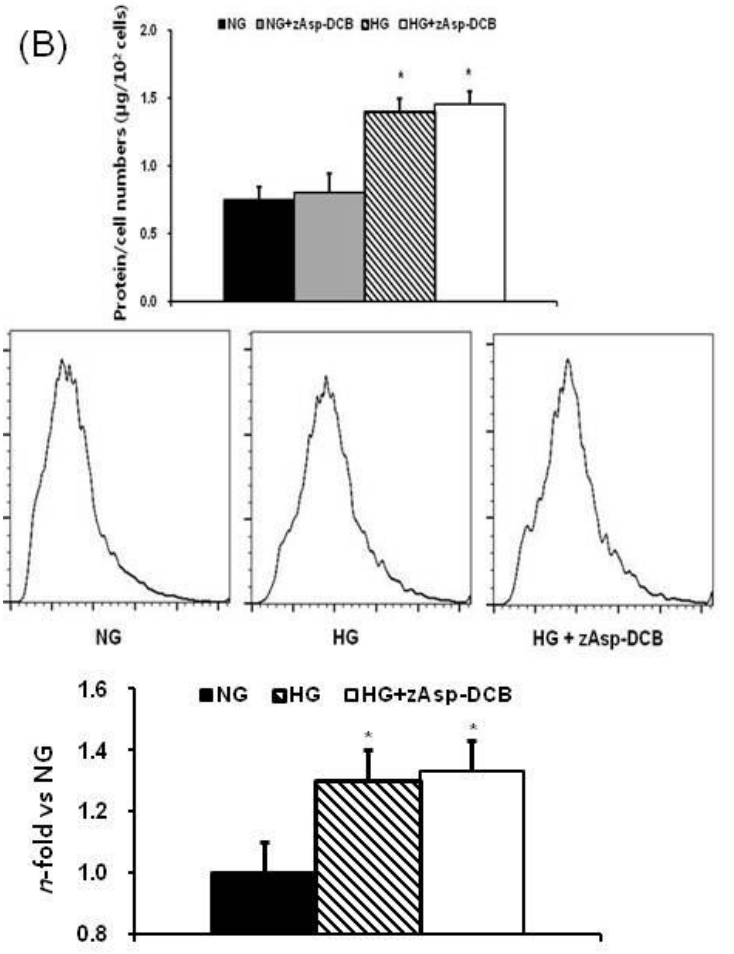
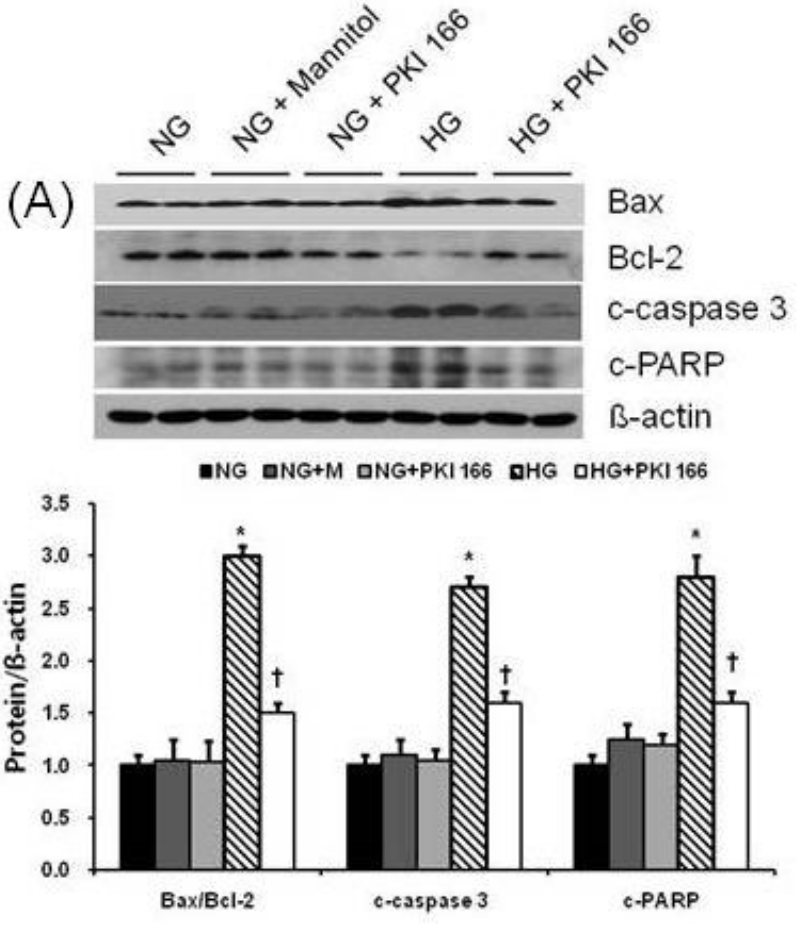


Figure 2. Assessment of cellular hypertrophy in cultured podocytes. (A) Both cellular protein/cell number and relative cell size were significantly greater in podocyte cultured under HG conditions compared to NG cells, and these increments were significantly ameliorated by PKI 166 treatment. (B) In contrast, zAsp-DCB had no effect on cellular hypertrophy in HG-stimulated podocyte. *; $p<0.05$ vs. NG groups, †; $p<0.05$ vs. HG group.

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

To examine the effect of EGFR inhibitor and pancaspase inhibitor on HG-induced podocyte apoptosis, Bax, Bcl-2, active fragments of caspase-3, and cleaved PARP protein expression were determined in cultured podocytes. Compared to NG-stimulated podocytes, the protein expression of Bax, active fragments of caspase-3, and cleaved PARP were significantly increased, while Bcl-2 protein expression was significantly decreased in cultured podocytes exposed to HG medium ($p<0.05$). The increases in the ratio of Bax/Bcl-2, and the expression of active fragments of caspase-3 and cleaved PARP protein in HG-stimulated podocytes were significantly attenuated by not only PKI 166 but also zAsp-DCB treatment ($p<0.05$), indicating that both drugs inhibited podocyte apoptosis under HG conditions (Figure 3).


NG
NG + Mannitol
NG + PKI 166
HG
HG + PKI 166
(A)
Bax
Bcl-2
c-caspase 3
c-PARP
ß-actin
NG NG+M NG+PKI 166 HG HG+PKI 166
Protein/ß-actin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
†
Bax/Bcl-2
c-caspase 3
c-PARP

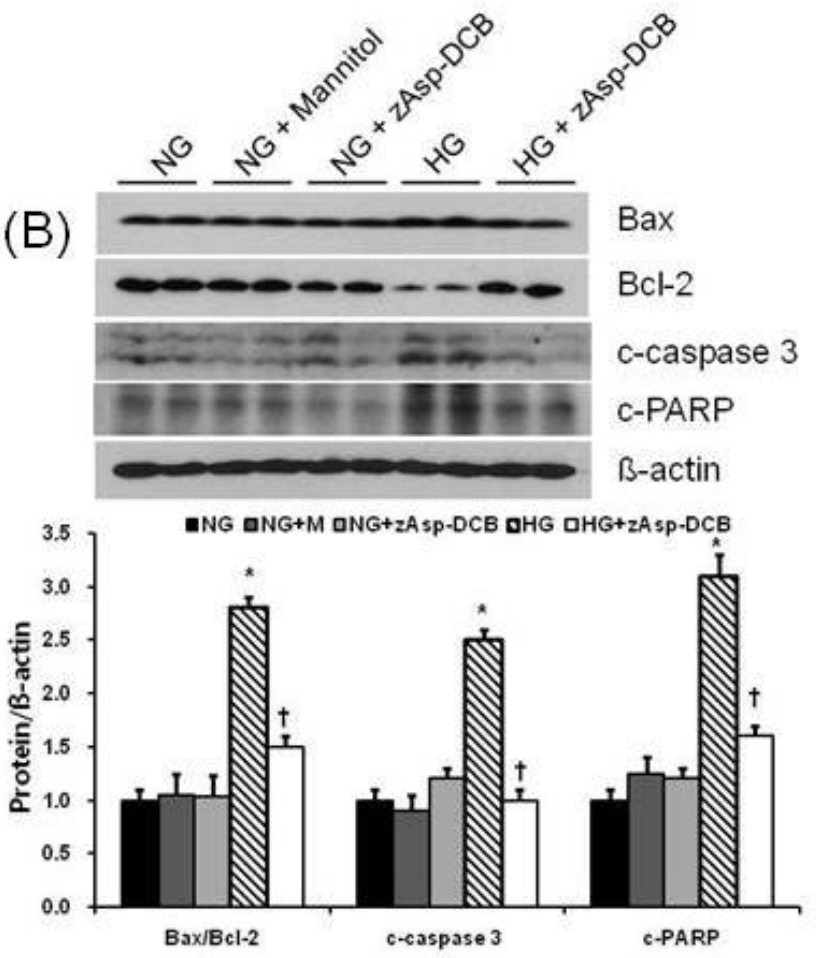
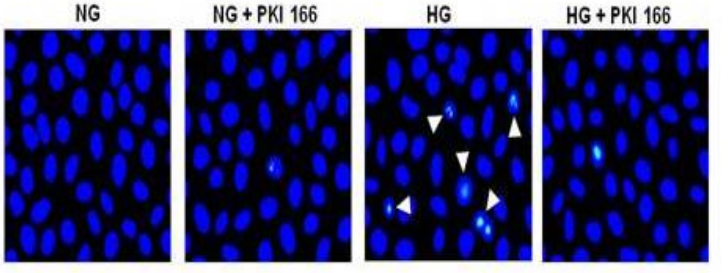


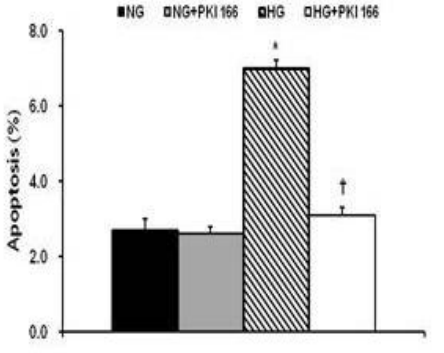
Figure 3. A representative Western blot of Bax, Bcl-2, active fragments of caspase 3 (c-caspase 3), and cleaved poly(ADP-ribose)polymerase (c-PARP) in cultured podocytes. (A) HG significantly increased the ratio of Bax/Bcl-2, and the protein expression of c-caspase 3 and c-PARP, and these increases were significantly attenuated by PKI 166 treatment. (B) zAsp-DCB also inhibited the increases in the ratio of Bax/Bcl-2, and the protein expression of c-caspase 3 and c-PARP in HG-stimulated podocytes. *; $p<0.05$ vs. NG and NG+M groups, †; $p<0.05$ vs. HG group.

D. Hoechst 33342 staining

To examine the effect of EGFR inhibitor and pancaspase inhibitor on HG-induced podocyte apoptosis, apoptosis was also determined by Hoechst 33342 staining in cultured podocytes. The number of apoptotic cells was significantly increased in podocytes cultured under HG conditions ($p<0.01$), and this increase was significantly abrogated by PKI 166 as well as zAsp-DCB treatment ($p<0.05$) (Figure 4).


NG
NG + PKI 166
HG
HG + PKI 166

(A)


NG
NG+PKI 166
HG
HG+PKI 166
Apoptosis (%)
8.0
6.0
4.0
2.0
0.0
*
†

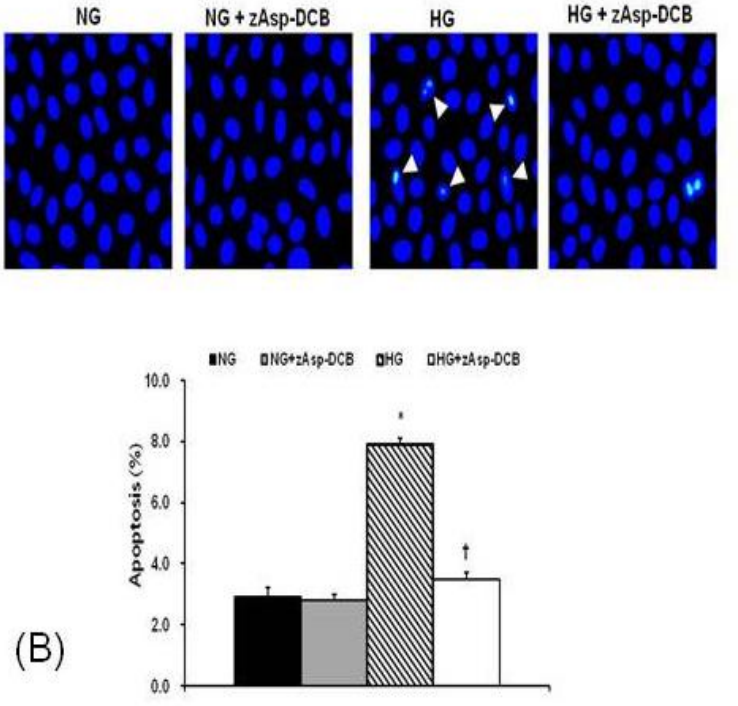


Figure 4. Apoptosis assessed by Hoechst 33342 staining in cultured podocytes. (A) The number of apoptotic cells was significantly increased in HG-stimulated podocytes compared to NG cells, and this increase was significantly abrogated by PKI 166 treatment. (B) zAsp-DCB also inhibited the increase in apoptotic podocytes induced by HG. *; $p<0.01$ vs. NG groups, †; $p<0.05$ vs. HG group.

2. Animal studies

A. Animal data

After the 3-month experimental period, body weight was significantly greater in C rats compared to the DM group (620±9 vs. 324±9 g, $p<0.001$). In contrast, the ratios of kidney weight to body weight in the DM group were significantly higher than those in C rats (1.19±0.12 vs. 0.52±0.06%, $p<0.01$), and administration of PKI 166 but not zAsp-DCB significantly ameliorated this increase in kidney weight to body weight ratios in DM rats (0.98±0.09%, $p<0.05$; 1.13±0.08%, $p=0.78$). Moreover, an increase in albuminuria in the DM group (2.51±0.27 mg/day) was significantly reduced by not only PKI 166 but also zAsp-DCB treatment (0.89±0.12 and 1.07±0.11 mg/day, respectively, $p<0.05$). On the other hand, neither PKI 166 nor zAsp-DCB had any effect on body weight and blood glucose levels in C and DM rats (Table 1).

Table 1. Physiological and renal parameters of control (C) and diabetic rats (DM) treated with PKI 166 and zAsp-DCB

	C (n=16)	C+PKI 166 (n=8)	C+zAsp-DCB (n=8)	DM (n=16)	DM+PKI 166 (n= 8)	DM+zAsp-DCB (n= 8)
Body weight (Bwt, g)	620±9	612±10	622±10	324±9*	335± 7*	329± 9*
Kidney Wt (g)	3.22±0.10	3.06±0.11	3.11±0.14	3.86±0.11#	3.28±0.14†	3.72±0.13
Kidney Wt/Bwt (%)	0.52±0.06	0.50±0.08	0.50±0.08	1.19±0.12#	0.98±0.09†	1.13±0.08
Glucose (mg/dL)	108±4	113±6	115±6	486±13*	495±11*	478±11*
24-hr UAE (mg/day)	0.40±0.03	0.36±0.05	0.45±0.04	2.51±0.27*	0.89±0.12†	1.07±0.11†

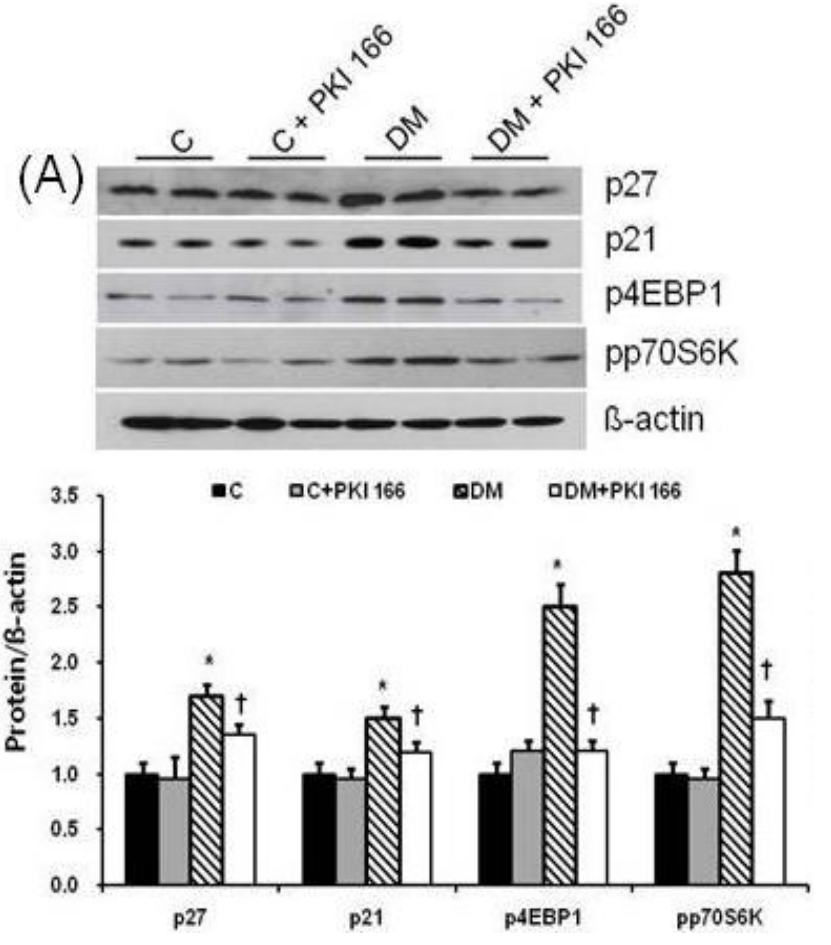
*; $p<0.001$ vs. C, C+PKI 166, and C+zAsp-DCB groups.

#; $p<0.01$ vs. C, C+PKI 166, and C+zAsp-DCB groups.

†; $p<0.05$ vs. DM group.

B. Glomerular p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression

To examine the effect of EGFR inhibitor and pancaspase inhibitor on glomerular hypertrophy in DM rats, I determined the changes in p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression in isolated glomeruli. Figure 5 shows a representative Western blot analysis with the lysates of sieved glomeruli from each group. Compared to C rats, there were 1.6-, 1.4-, 2.5-, and 2.8-fold increases in glomerular p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression, respectively, in the DM group ($p<0.05$), and these increases were significantly attenuated in DM rats by PKI 166 treatment ($p<0.05$). In contrast, the increases in the protein expression of p27, p21, phospho-4EBP1, and phospho-p70S6K in DM glomeruli were not altered by the administration of zAsp-DCB .


(A)
C
C + PKI 166
DM
DM + PKI 166
p27
p21
p4EBP1
pp70S6K
ß-actin
C
C+PKI 166
DM
DM+PKI 166
Protein/ß-actin
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p27
p21
p4EBP1
pp70S6K

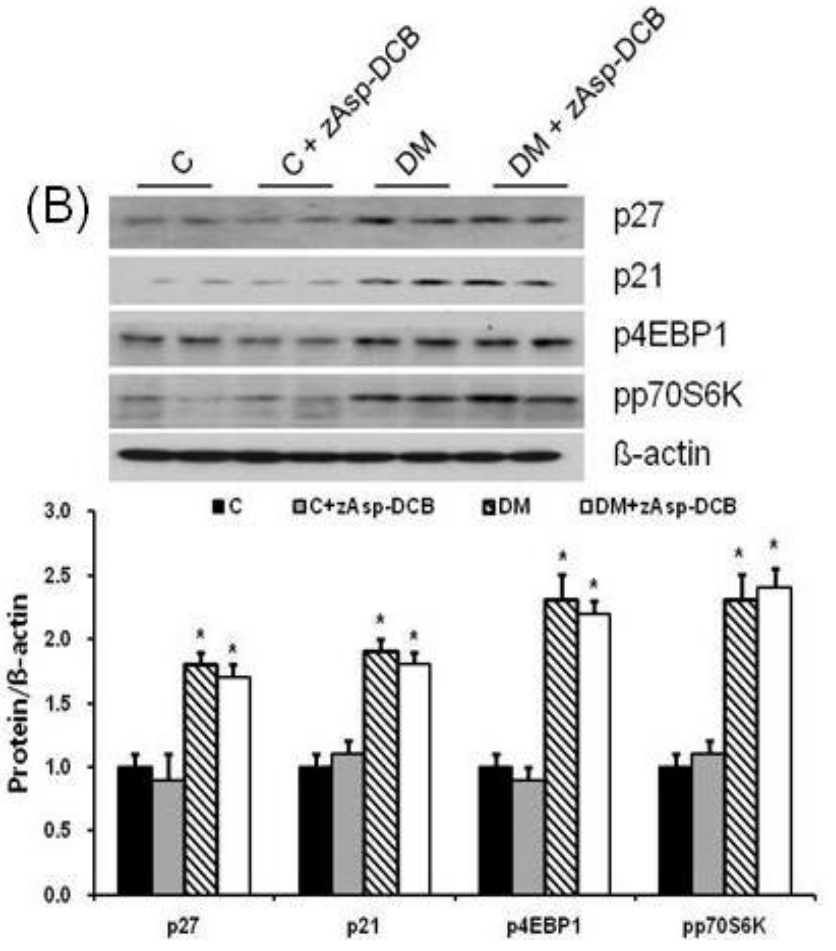


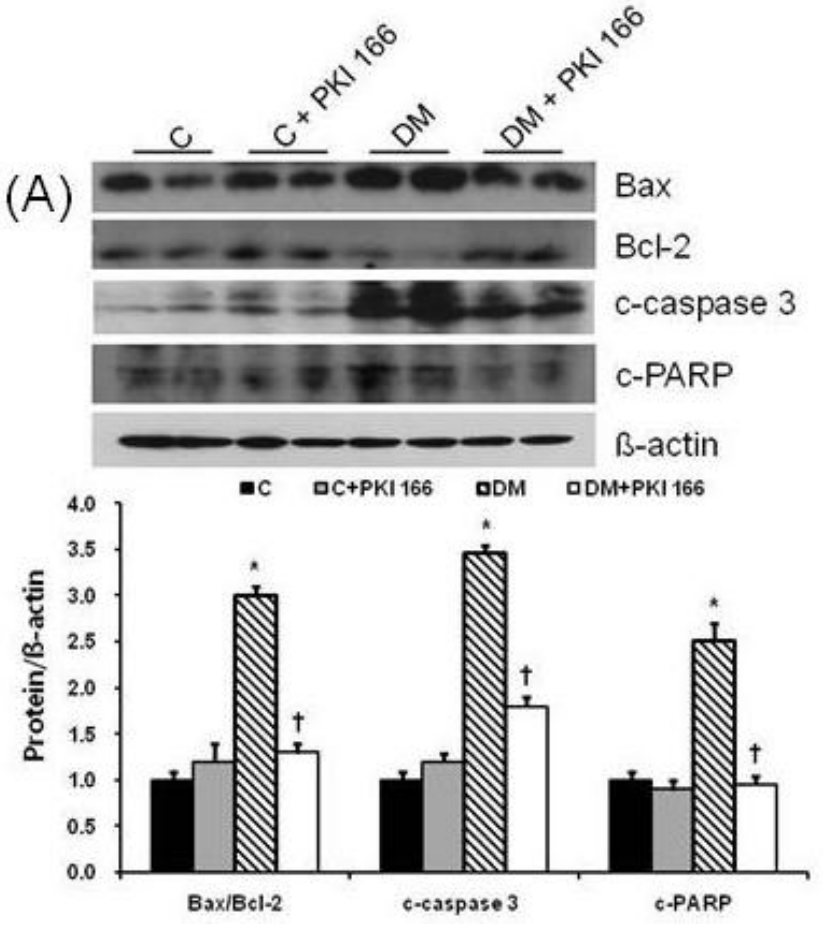
Figure 5. A representative Western blot of glomerular p27, p21, phospho-4EBP1, and phospho-p70S6K. (A) Compared C rats, there were 1.6-, 1.4-, 2.5-, and 2.8-fold increases in glomerular p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression, respectively, in the DM group, and these changes were significantly attenuated by PKI 166 treatment. (B) In contrast, the increases in the protein expression of p27, p21, phospho-4EBP1, and phospho-p70S6K in DM glomeruli were not altered by zAsp-DCB . *; $p<0.05$ vs. C group, †; $p<0.05$ vs. DM group.

C. Glomerular hypertrophy

To examine the effect of EGFR inhibitor and pancaspase inhibitor on glomerular hypertrophy in DM rats, glomerular volume was determined by morphometric measurement of glomeruli in the rats. The mean glomerular volume at 3-month was significantly larger in the DM group compared to the C group ($1.24\pm0.07x10^6$ vs. $0.92\pm0.04x10^6$ μm^3, $p<0.05$), and PKI 166 treatment significantly abrogated the increase in glomerular volume in DM rats ($0.98\pm0.05x10^6$ μm^3, $p<0.05$). On the contrary, glomerular hypertrophy in the DM group was not ameliorated by the administration of zAsp-DCB ($1.25\pm0.05x10^6$ μm^3).

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

To examine the effect of EGFR inhibitor and pancaspase inhibitor on podocyte apoptosis under diabetic conditions, Bax, Bcl-2, active fragments of caspase-3, and cleaved PARP protein expression were determined in isolated glomeruli. Compared to the C group, the protein expression of glomerular Bax, active fragments of caspase-3, and cleaved PARP were significantly increased, while Bcl-2 protein expression was significantly decreased in DM rats ($p<0.05$). The increases in the ratio of Bax/Bcl-2, and the expression of glomerular active fragments of caspase-3 and cleaved PARP protein in the DM group were significantly attenuated by not only PKI 166 but also zAsp-DCB treatment ($p<0.05$), indicating that both drugs inhibited apoptosis in DM glomeruli (Figure 6).


C
C + PKI 166
DM
DM + PKI 166
(A)
Bax
Bcl-2
c-caspase 3
c-PARP
ß-actin
C
C+PKI 166
DM
DM+PKI 166
Protein/ß-actin
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Bax/Bcl-2
c-caspase 3
c-PARP

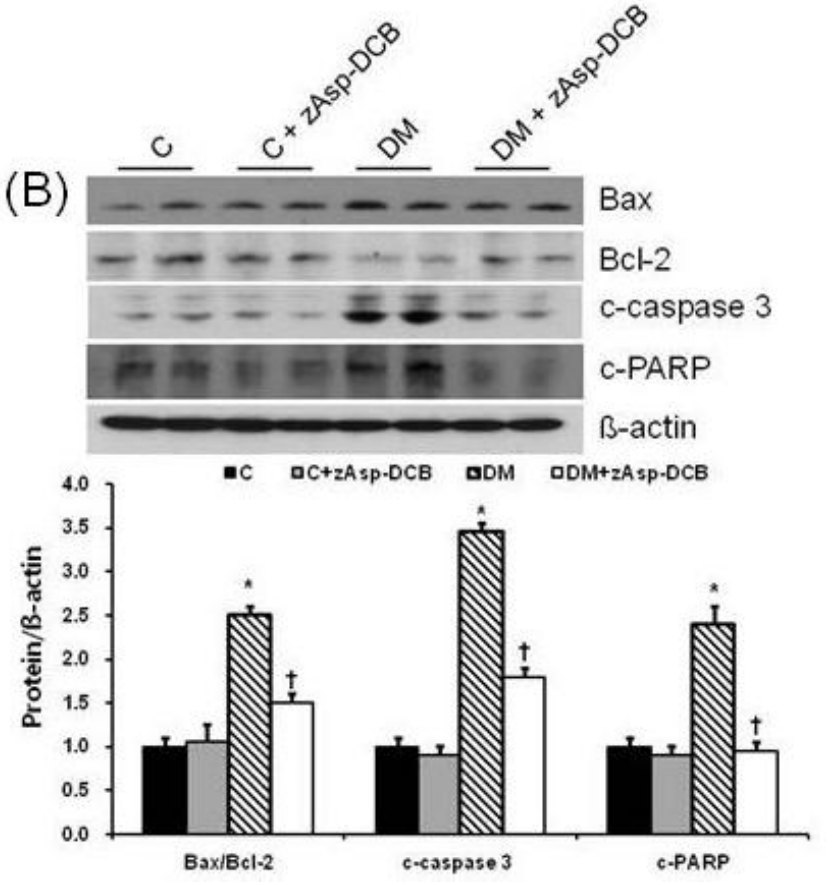
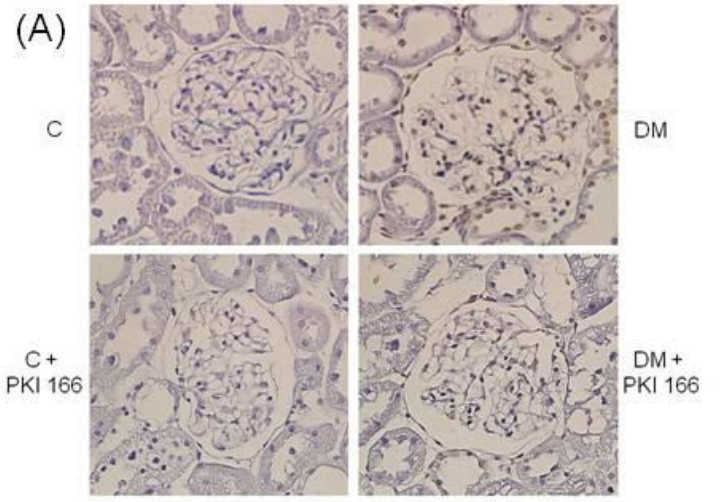
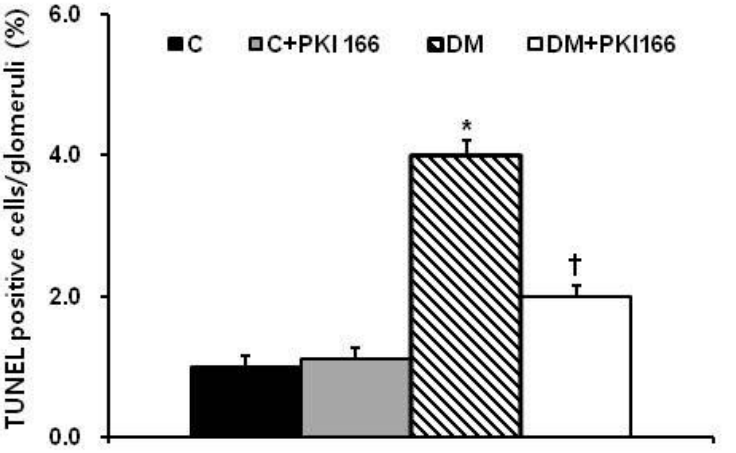


Figure 6. A representative Western blot of glomerular Bax, Bcl-2, active fragments of caspase 3 (c-caspase 3), and cleaved poly(ADP-ribose)polymerase (c-PARP). (A) There were significant increases in the ratio of Bax/Bcl-2, and the protein expression of c-caspase 3 and c-PARP in DM glomeruli, and these increases were significantly attenuated by PKI 166 treatment. (B) zAsp-DCB also significantly inhibited the increases in the ratio of Bax/Bcl-2, and the protein expression of c-caspase 3 and c-PARP in DM glomeruli. *; $p<0.05$ vs. C group, †; $p<0.05$ vs. DM group.

E. TUNEL assay

To examine the effect of EGFR inhibitor and pancaspase inhibitor on podocyte apoptosis under diabetic conditions, apoptosis in glomeruli was also assessed by TUNEL assay. The number of apoptotic cells in glomeruli was significantly increased in the DM group compared to the C group ($p<0.01$), and this increase in apoptotic cells in DM glomeruli was significantly abrogated by both PKI 166 and zAsp-DCB treatment ($p<0.05$) (Figure 7).


(A)
C
DM
C +
PKI 166
DM +
PKI 166


TUNEL positive cells/glomeruli (%)
6.0
4.0
2.0
0.0
C
C+PKI 166
DM
DM+PKI166
*
†

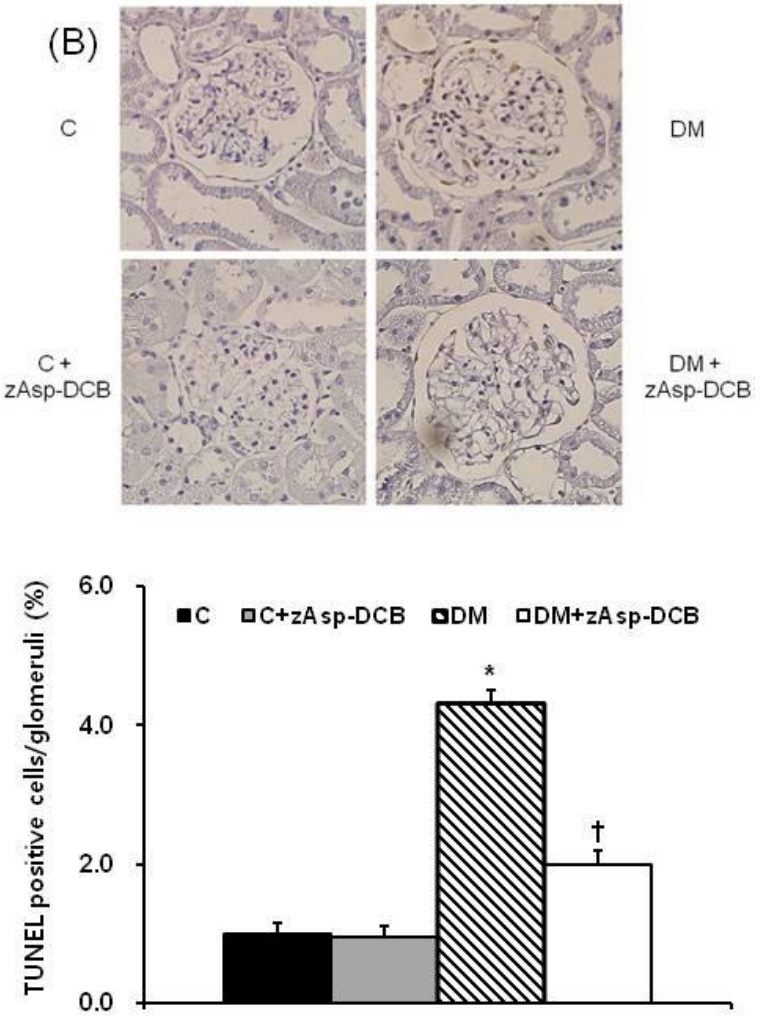


Figure 7. Apoptosis assessed by TUNEL assay. (A) The number of apoptotic cells in glomeruli was significantly increased in DM rats compared to C rats, and this increase in apoptotic cells in DM glomeruli was significantly abrogated by PKI 166 treatment. (B) zAsp-DCB also significantly inhibited the increase in apoptotic cells in glomeruli of DM rats. *; $p<0.01$ vs. NG groups, †; $p<0.05$ vs. HG group.

IV. DISCUSSION

Podocyte hypertrophy and apoptosis play an important role in the pathogenesis of diabetic nephropathy. However, little is known which of them comes first in the course of diabetic nephropathy. In this study, I demonstrate that inhibition of hypertrophy results in less podocyte apoptosis, whereas blocking apoptosis has no effect on podocyte hypertrophy both *in vitro* and *in vivo*, suggesting that podocyte hypertrophy precedes apoptosis under diabetic conditions.

Glomerular hypertrophy is a characteristic feature of diabetic nephropathy, and is mainly attributed to glomerular cell hypertrophy and ECM accumulation.[37] Unlike glomerular mesangial cells, which are known to proliferate in the early stage of diabetic nephropathy,[38] mature podocytes do not actively undergo proliferation under diabetic conditions.[39,40] Instead, their individual size is increased in diabetic nephropathy. Some investigators inferred that podocyte hypertrophy was merely a compensatory phenomenon to cover the denuded GBM after diabetes-induced podocyte injury.[26] However, accumulating evidence indicated that renal enlargement is a very early finding in experimental diabetic nephropathy. Seyer-Hansen[41] found that the kidney weight was increased by 15% at 3-day after diabetes induction and by 90% after 42 days of diabetes. In addition, another study revealed that the increase in kidney weight was 62% at 6-days after STZ-induced diabetes.[42] They also showed that hyperplasia and hypertrophy contributed equally to renal enlargement. Recently, Jung et al[29] found that not only the protein expression of Bax and cleaved caspase-3 were significantly increased but also the number of podocytes was significantly decreased in more hypertrophied diabetic glomeruli but not in less hypertrophied glomeruli, suggesting that podocyte loss followed glomerular hypertrophy. The results of the current study demonstrate inhibition

of hypertrophy results in less podocyte apoptosis, whereas blocking apoptosis has no effect on podocyte hypertrophy, confirming that podocyte hypertrophy is an earlier event than podocyte apoptosis.

In the present study, PKI 166, an EGFR blocker, was used to examine the consequence of inhibiting podocyte hypertrophy on apoptosis. Rather than the EGF-EGFR axis, TGF-β and angiotensin II have been considered key molecules implicated in the pathogenesis of diabetic nephropathy.[3,4,43] However, these two molecules are involved in both podocyte hypertrophy and apoptosis under diabetic conditions, making it impossible to investigate the impact of blocking one event on the other. Therefore, I tried to seek a molecule, which had a hypertrophic but not an apoptotic effect on podocyte, and determined to use PKI 166. EGFR was found to be present in podocytes and to be activated by angiotensin II, an end product of the renin-angiotensin system which was activated within podocytes under diabetic conditions.[44] Moreover, previous studies suggested that the EGF-EGFR system played a role in diabetes-related renal and glomerular hypertrophy in experimental rats.[45] There was a 3-fold increase in urinary EGF excretion and 3 to 6-fold increase in renal EGF mRNA expression in STZ-induced diabetic rats compared to control rats. In contrast, serum EGF levels were comparable between the two groups. Furthermore, administration of PKI 166 significantly abrogated the increase in kidney weight in experimental diabetic rats by 30% at 3-week of diabetes along with reduced albuminuria.[34] Glomerular hypertrophy was also significantly ameliorated in STZ-induced diabetic TGR(mRen-2)27 rats treated with PKI 166 for 16 weeks, which was associated with podocyte preservation and reduction of albuminuria.[46] The results of this study showed that PKI 166 treatment significantly inhibited the increase in albuminuria and glomerular volume and the decrease in podocyte number, which was consistent with most of the previous studies. In addition, considering my findings that podocyte apoptosis

was significantly attenuated in diabetic rats treated with PKI 166, it was surmised that preservation of podocyte number was attributed to reduced apoptosis-mediated podocyte loss under diabetic conditions.

Meanwhile, Wassef et al[34] demonstrated that apoptosis in the kidney, assessed by TUNEL staining and active caspase-3 immunohistochemical staining, was significantly increased in diabetic rats treated with PKI 166 compared to vehicle-treated diabetic rats, which was contrary to the results of the current study. In that study, however, apoptosis was almost restricted in the tubulointerstitial area. Moreover, the expression of proliferating cell nuclear antigen and 5-bromo-2'-deoxyuridine were significantly increased in renal tubules in diabetic rats. Based on these findings and the results of a number of previous studies showing an apoptotic effect of PKI 166 on various cancer cell lines, including epidermoid carcinoma,[47] renal cell cancer,[48] and pancreatic cancer,[49] it was submitted that PKI 166 had an anti-proliferative effect on actively proliferating cells by inducing apoptosis. In contrast, in the presence of TGF-β, EGF is known to exert hypertrophic rather than hyperplastic effect.[50] Since TGF-β expression is significantly increased in diabetic glomeruli and TGF-β is regarded as a principal mediator implicated in the pathogenesis of diabetic glomerulopathy, I postulated that the EGF-EGFR axis might be involved in podocyte hypertrophy under diabetic conditions, and it was verified by the results of the present study demonstrating that diabetes-induced podocyte hypertrophy both *in vitro* and *in vivo* was significantly abrogated by PKI 166. Furthermore, similar to the results of the study by Wassef et al, an increase in apoptosis in glomeruli was not found in my diabetic rats treated with PKI 166 may be due to minimal or no actively proliferating cells in diabetic glomeruli.

The number of podocytes is decreased in the glomeruli of human diabetic patients and experimental diabetic animals.[51,52] Podocyte number usually

reflects the balance between podocyte loss and proliferation, and apoptosis has been considered an important cause underlying podocyte loss in diabetic nephropathy. In addition, administration of angiotensin II receptor blocker, aldosterone antagonist, or anti-oxidants reduced albuminuria along with less podocyte apoptosis in experimental diabetic animals,[53-55] suggesting that podocyte apoptosis is closely associated with the development of albuminuria in diabetes. In this study, the increase in albuminuria in diabetic rats was significantly ameliorated by not only PKI 166 but also a pancaspase inhibitor. However, pancaspase inhibitor significantly attenuated podocyte apoptosis but not podocyte hypertrophy. Nevertheless, albuminuria was significantly reduced in diabetic rats treated by a pancaspase inhibitor. Taken together, it is suggested that albuminuria is more closely associated with podocyte apoptosis than hypertrophy in diabetic nephropathy.

There are two different mechanisms underlying apoptosis; caspase-dependent and -independent.[56,57] Caspase-independent apoptosis is associated with the translocation of apoptogenic factors, such as apoptosis inducing factor, Omi/Htra2, or endonuclease G, from the cytoplasm to the nucleus and has been found in numerous cells under various conditions.[58,59] Previous studies have revealed that hippocampal neuronal and retinal cell apoptosis under diabetic conditions are induced by a caspase-independent pathway.[60,61] In podocytes, puromycin aminonucleoside was also shown to induce apoptosis through a caspase-independent mechanism.[62] To date, however, caspase-independent apoptosis has never been demonstrated in podocyte under diabetic conditions. Moreover, since TGF-β-mediated apoptosis was mainly caspase-dependent,[63] I inferred that podocyte apoptosis under diabetic conditions might occur mainly via a caspase-dependent mechanism, and thus used pancaspase inhibitor to suppress podocyte apoptosis in the current study. In the near future, it will be necessary to clarify the involvement of a caspase-independent pathway in

diabetes-induced podocyte apoptosis.

In the present study, I found that inhibiting podocyte hypertrophy by PKI 166 resulted in less podocyte apoptosis. As aforementioned, PKI 166 is known to induce rather apoptosis in actively proliferating cells. In mature podocytes, even though there has been no evidence that PKI 166 has a direct proapoptotic or antiapoptotic effect, the possibility cannot be completely excluded that PKI 166 directly inhibits podocyte apoptosis under diabetic conditions. However, considering the results of previous studies by Jung et al,[29] it is surmised that reduced podocyte apoptosis might be a consequence of abrogating podocyte hypertrophy under diabetic conditions. On the other hand, recently, accumulating evidence indicates an involvement of endoplasmic reticulum (ER) stress in cardiomyocyte apoptosis in an animal model of cardiac hypertrophy.[64,65] Therefore, further studies are needed to elucidate the underlying conceivable mechanisms of a transition from podocyte hypertrophy to apoptosis under diabetic conditions, including ER stress.

In conclusion, inhibition of podocyte hypertrophy results in less apoptosis, whereas blocking apoptosis has no effect on podocyte hypertrophy under diabetic conditions, suggesting that podocyte hypertrophy precedes apoptosis. Based on these findings, targeting podocyte hypertrophy may be more effective in the treatment of diabetic nephropathy.

V. CONCLUSION

To clarify the order of podocyte hypertrophy and apoptosis in the process of diabetic nephropathy, I elucidated the consequences of inhibiting hypertrophy on apoptosis and of blocking apoptosis on hypertrophy in podocytes under diabetic conditions by using EGFR inhibitor and pancaspase inhibitor, respectively.

1. Compared to NG cells, there were significant increases in p27, p21, phospho-4EBP1, and phospho-p70S6K protein expression in cultured podocytes exposed to HG medium, and these changes were significantly abrogated by PKI 166, but not by zAsp-DCB treatment.
2. Both cellular protein/cell number and relative cell size were significantly greater in podocyte cultured under HG conditions compared to NG cells. These increments were significantly ameliorated by PKI 166 treatment, while zAsp-DCB had no effect on cellular hypertrophy in HG-stimulated podocytes.
3. The increases in the ratio of Bax/Bcl-2, and the protein expression of active fragments of caspase-3 and cleaved PARP in HG-stimulated podocytes were significantly attenuated by not only PKI 166 but also zAsp-DCB treatment.
4. The increase in apoptotic cells, assessed by Hoechst 33342 staining, in podocytes cultured under HG conditions was significantly abrogated by PKI 166 as well as zAsp-DCB treatment.
5. The ratios of kidney weight to body weight in the DM group were significantly higher than those in C rats, and administration of PKI 166 but not zAsp-DCB significantly ameliorated this increase in kidney weight to body weight ratios in DM rats.
6. The increase in albuminuria in the DM group was significantly reduced by not only PKI 166 but also zAsp-DCB treatment.
7. Compared to C rats, there were significant increases in glomerular p27, p21,

phospho-4EBP1, and phospho-p70S6K protein expression in the DM group, and these increases were significantly attenuated in DM rats by PKI 166, but not by zAsp-DCB treatment.

8. The mean glomerular volume was significantly larger in the DM group compared to the C group. Glomerular hypertrophy in DM rats was significantly abrogated by PKI 166 treatment, whiles zAsp-DCB had no effect on glomerular hypertrophy in the DM group.

9. The increases in the ratio of Bax/Bcl-2, and the protein expression of active fragments of caspase-3 and cleaved PARP in DM glomeruli were significantly ameliorated by not only PKI 166 but also zAsp-DCB treatment.

10. The increase in apoptotic cells, assessed by TUNEL assay, in DM glomeruli was significantly attenuated by PKI 166 as well as zAsp-DCB treatment.

In conclusion, inhibition of podocyte hypertrophy results in less apoptosis, whereas blocking apoptosis has no effect on podocyte hypertrophy under diabetic conditions, suggesting that podocyte hypertrophy precedes apoptosis. Based on these findings, targeting podocyte hypertrophy may be more effective in the treatment of diabetic nephropathy.

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ABSTRACT (IN KOREAN)

당뇨 조건 하에서 족세포의 비후가 세포사멸에 선행한다

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배경: 당뇨병성 신병증은 전세계적으로 말기 신부전증의 가장 중요한 원인 질환으로, 사구체의 비후와 단백뇨를 특징으로 한다. 또한, 최근의 많은 연구를 통하여 족세포의 세포사멸이 당뇨병성 신병증의 병인과 밀접한 관련이 있음이 밝혀졌다. 그러나, 족세포의 비후와 세포사멸 중에서 어떤 변화가 선행하는 지에 대해서는 아직까지 논란이 많은 실정이다. 이에 본 연구자는 당뇨 조건 하에서 족세포의 비후를 억제하였을 때 세포사멸의 변화와 세포사멸 억제가 비후에 미치는 영향을 관찰함으로써, 당뇨병성 신병증에서 족세포의 비후와 세포사멸의 선행 관계를 밝히고자 하였다.

방법: 불멸 생쥐 족세포를 정상 포도당군 (5.6 mM), 정상 포도당+만니톨군 (24.4 mM 만니톨), 그리고 고포도당군 (30 mM)으로 나누어 배양하였으며, 이와 동시에 표피 성장 인자 (epidermal growth factor) 수용체 억제제인 PKI 166 (10 μM)과 pancaspase 억제제인 zAsp-DCB (N-aspartyl-2,6-dichlorobenzoyloxy-methylketone) (10^{-7} M)로 전처리한 실험도 시행하였다. 생체 내 실험으로는 64마리의 Spague-Dawley 백서를 대상으로 대조군 (32마리)과 steptozotocin으로 당뇨를 유발시킨 당뇨군 (32마리)으로 나눈 후, 각 군에서 8마리씩은 PKI 166을 100 mg/kg/day 용량으로 경구로, 그리고 8마리씩은 zAsp-DCB를 2 mg/day 용량으로 소형펌프를 이용하여 피하주사로 3개월간 투여하였다. 배양 족세포와 실험 동물로부터 분리한 사구체 내 p27, p21, phospho-4EBP1, phospho-p70S6 kinase, Bax, Bcl-2, active fragments of caspase-3,

cleaved poly(ADP-ribose)polymerase, 그리고 β-actin 단백의 발현은 Western blot을 이용하여 분석하였다. 족세포의 비후는 단백량/세포 수 및 유세포 분석으로, 그리고 사구체의 비후는 morphometry를 이용하여 평가하였다. 또한, 세포사멸은 배양 족세포를 이용한 Hoechst 33342 염색과 신장 조직을 이용한 TUNEL 분석을 이용하여 추가적으로 확인하였다.

결과: 정상 포도당으로 자극한 배양 족세포와 대조군 사구체에 비하여, 고포도당으로 자극한 족세포와 당뇨 사구체에서 p27, p21, phospho-4EBP1, 그리고 phospho-p70S6K의 단백 발현이 의미있게 증가되었으며, 이러한 변화는 PKI 166 처치에 의하여 의의있게 억제되었다. 그러나, zAsp-DCB 치료는 이러한 변화에 영향을 미치지 않았다. 단백량/세포 수 및 유세포 분석으로 평가한 족세포와 사구체 크기도 당뇨 조건 하 배양 족세포와 당뇨 사구체에서 증가되었으며, 이러한 증가는 PKI 166에 의하여 유의하게 억제되었던 반면, zAsp-DCB 에 의해서는 변화가 없었다. 또한, 고포도당으로 자극한 배양 족세포와 당뇨 사구체 내 Bax/Bcl-2 비 및 active fragments of caspase-3와 cleaved PARP의 단백 발현이 증가되었으며, 이러한 증가는 PKI 166뿐만 아니라 zAsp-DCB 처치에 의하여 의미있게 억제되었다. 당뇨 조건 하에서 Hoechst 33342 염색와 TUNEL 분석을 이용하여 확인한 세포사멸의 증가도 PKI 166와 zAsp-DCB 모두에 의하여 의의있게 억제되었다. PKI 166와 zAsp-DCB는 당뇨 백서에서 증가된 뇨알부민 배설량도 유의하게 감소시켰다.

결론: 당뇨 조건 하에서 족세포의 비후를 억제하였을 때 세포사멸은 억제되었던 반면, 족세포의 세포사멸을 억제하였을 때 비후에는 전혀 영향이 없었다. 이러한 결과는 족세포의 비후가 세포사멸에 선행함을 시사하며, 당뇨병성 신병증의 치료에 있어서 족세포의 비후 억제를 목표로 하는 치료가 좀 더 효과적일 것으로 생각된다.

핵심되는 말: 당뇨병성 신병증, 족세포, 세포 비후, 세포 사멸