Heme oxygenase-1 expression is increased to protect podocyte apoptosis under diabetic conditions

Sang Choel Lee

Department of Medicine

The Graduate School, Yonsei University

Heme oxygenase-1 expression is increased to protect podocyte apoptosis under diabetic conditions

Directed by Professor Shin-Wook Kang

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Sang Choel Lee

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This certifies that the Doctoral Dissertation of Sang Choel Lee is approved.

Thesis Supervisor : Shin-Wook Kang

(Hyeon Joo Jeong: Thesis Committee Member#1)

(Soon-Jung Park: Thesis Committee Member#2)

(Koon Ho Rha: Thesis Committee Member#3)

(Soo Young Yoon: Thesis Committee Member#4);

The Graduate School

Yonsei University

June 2008

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ABSTRACT

Heme oxygenase-1 expression is increased to protect podocyte apoptosis under diabetic conditions

Sang Choel Lee

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Shin-Wook Kang)

Background: Diabetic nephropathy, the leading cause of end-stage renal disease in many countries, is clinically characterized by proteinuria. 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 reactive oxygen species-mediated apoptosis is known to be involved in the process of podocyte loss under diabetic conditions. Heme oxygenase-1 (HO-1), a microsomal enzyme, has been regarded as an anti-oxidant and to be

upregulated to alleviate the deleterious consequences of oxidant injury. Previous studies have demonstrated that HO-1 expression is increased in experimental diabetic glomeruli, but the functional significance of this increase has not yet been explored. In this study, to elucidate the role of HO-1 in podocyte apoptosis under diabetic conditions, I investigated not only the expression of HO-1 but also podocyte apoptosis in experimental diabetic glomeruli and in high glucose-stimulated cell before and after inhibition of HO activity.

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 IP Zinc protoporphyrin (ZnPP, 50 μmol/kg/day), an HO-1 inhibitor, for 6 weeks. In vitro, immortalized mouse podocytes were cultured in media containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), or 30 mM glucose (HG) with or without HO-1 siRNA. Real-time PCR for HO-1 mRNA expression and Western blotting for HO-1, Bax, Bcl-2, and active fragments of caspase-3 protein expression were performed with sieved glomeruli and cell lysates. In addition, TUNEL assay and immunofluorescence (IF) staining for active fragments of caspase-3 were conducted with renal tissue and Hoechst 33342 staining with cultured podocytes.

Results: Urinary albumin excretion was significantly higher in DM (1.18±0.11 mg/day) compared to C rats (0.32±0.04 mg/day) ($p<0.05$), and was further increased in DM rats by ZnPP (1.59±0.19 mg/day) ($p<0.05$). HO-1 mRNA and protein expression were increased in diabetic glomeruli and in HG-stimulated podocytes, and these increases were inhibited by ZnPP and HO-1 siRNA, respectively. The ratios of Bax/Bcl-2 protein expression were significantly higher in diabetic glomeruli and in podocytes exposed to HG, which were further increased by ZnPP and HO-1 siRNA, respectively. Western blotting with active fragments of caspase-3 showed a similar pattern to the ratios of Bax/Bcl-2 protein expression. TUNEL positive-stained nuclei were also significantly increased in DM (1.3±0.2) compared to C glomeruli (0.4±0.1) ($p<0.05$), and were further increased in DM glomeruli by ZnPP (2.2±0.3) ($p<0.05$). In addition, double IF staining with active fragments of caspase-3 and synaptopodin revealed that active fragments of caspase-3 expression was most prominent in podocytes of DM+ZnPP rats. Apoptotic cells assessed by Hoechst 33342 staining were also significantly increased in HG-stimulated podocytes compared to NG cells, which were further increased in HG+siRNA group.

Conclusion: This study suggests that HO-1 expression is increased in experimental diabetic glomeruli and in HG-stimulated podocytes, and the

increase in HO-1 expression plays a protective role against podocyte apoptosis under diabetic conditions.

Key words: heme oxygenase-1, diabetic nephropathy, podocyte, high glucose, apoptosis

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Sang Choel Lee

Department of Medicine
The Graduate School, Yonsei University

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I. INTRODUCTION

Diabetic nephropathy, the leading cause of end-stage renal disease in many countries, is clinically characterized by proteinuria[1]. The underlying pathologic change responsible for proteinuria in various glomerular diseases is the loss of size-selective and/or charge-selective properties of the glomerular filtration barrier[2]. The glomerular filtration barrier is comprised of three layers: a fenestrated endothelial layer, a glomerular basement membrane (GBM), and podocyte foot processes connected by a slit diaphragm[3]. Recent studies have shown that podocyte injury plays an important role in the pathogenesis of various proteinuric glomerular diseases, including diabetic nephropathy[4-6].

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 the process of podocyte loss under diabetic conditions[7-9]. Previous studies have demonstrated that high glucose *per se* and transforming growth factor (TGF)-β1, two major mediators in the development and progression of diabetic nephropathy, increase reactive oxygen species (ROS) production and induce apoptosis in cultured podocytes[10-12]. Moreover, podocyte apoptosis under these conditions is prevented by the administration of anti-oxidants, suggesting that oxidative stress with increased ROS is responsible for apoptosis of podocytes under diabetic conditions[12, 13].

Heme oxygenase (HO) is a microsomal rate-limiting enzyme involved in the degradation of heme to biliverdin, which is rapidly converted to bilirubin by biliverdin reductase[14-17]. During this step, iron is released from the heme ring and carbon monoxide (CO) is generated. To date, three distinct isoforms of HO have been identified: HO-1, an inducible form; HO-2, a constitutive form; and HO-3, probably a pseudogene[18]. Among these, HO-1 is the most extensively studied HO isoenzyme and is known to be upregulated in the kidney under various conditions characterized by oxidative stress[19, 20], including toxic nephropathy[21, 22], ischemia-reperfusion injury[23], contrast

nephropathy[24], acute transplant rejection[25], and diabetic nephropathy[26]. Oxidants generating molecules such as angiotensin II (AII)[27], heme[28], proinflammatory cytokine[29], and TGF-β1[30] are also reported to induce HO-1. In addition, pharmacological inhibition of HO-1 activity or deletion of HO-1 gene worsened renal injury induced by toxic substances[31] and ischemia-reperfusion[32], while prior induction of HO-1 protected against renal injury. Based on these findings, HO-1 has been regarded as an anti-oxidant and to be upregulated to alleviate the deleterious consequences of oxidant injury.

Recently, experimental and clinical evidences indicate that excessive oxidative stress may contribute to the initiation and development of diabetic nephropathy. Furthermore, the attenuation of experimental diabetic nephropathy by dietary anti-oxidant supplementation with vitamin E[26], taurine[33], or lipoic acid[13, 34] also supports the role of oxidative stress in the pathogenesis of diabetic nephropathy. Based on these findings, an increase in HO-1 expression has been expected and been demonstrated in experimental diabetic glomeruli, mainly in mesangial cells and podocytes[35], but the functional significance of this increase has not yet been explored. In this study, to elucidate the role of HO-1 in podocyte apoptosis under diabetic conditions, which oxidative stress is known to mediate, I investigated not only the

expression of HO-1 but also podocyte apoptosis in experimental diabetic glomeruli and in high glucose-stimulated cells before and after inhibition of HO activity.

II. MATERIALS AND METHODS

1. Animals

All animal studies were conducted under an approved protocol. Sprague-Dawley rats weighing 250-280 g were injected either with diluent [n=16, Control (C)] or with 65 mg/kg streptozotocin (STZ) intraperitoneally (IP) [n=16, Diabetes (DM)]. Eight rats from each group were treated with IP Zinc protoporphyrin (ZnPP) (50 μmol/kg/day) (Sigma-Aldrich Corp., Saint Louis, MO, USA) (C+ZnPP, DM+ZnPP) 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-weeks study period.

Body weights were checked after 6 weeks, and kidney weights were measured at the time of sacrifice. Serum glucose and 24-hour urinary albumin were measured weekly and at the time of sacrifice, respectively. Blood glucose was measured by glucometer and 24-hour urinary albumin excretion (UAE) was determined by 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. Podocytes culture

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY) 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 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).

In this study, siRNA for mouse HO-1 was used to inhibit HO-1 expression. The sense and antisense sequences of mouse HO-1 siRNA were as follows: sense, 5'-GGCUUUAAGCUGGUGAUGGTT-3'; and antisense, 5'-CCA UCACCAGCUUAAAGCCTT-3'. The concentrations of siRNA for this study were determined based on the preliminary experiments with three different

concentrations of 10 nM, 20 nM and 50 nM. The HO-1 siRNA were transiently transfected into differentiated podocytes with Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) for 24 hours.

Transfected and nontransfected podocytes were serum-restricted for 24 hours, after which the medium was changed to RPMI medium containing normal glucose (5.6 mM glucose, NG), NG+24.4 mM mannitol (NG+M), NG+20 nM HO-1 siRNA (Ambion, Inc., Austin, Tx, USA) (NG+siRNA), high glucose (30 mM glucose, HG), or HG+20 nM HO-1 siRNA (HG+siRNA). After 24 hours, cells were harvested for either RNA or protein.

4. 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, 400 μl of chloroform added, and the mixture shaken vigorously for 30 seconds. After 3 minutes, the mixture was centrifuged at 12,000 g for 15 minutes at 4°C and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by 400 μl isopropanol and

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

5. Reverse transcription

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

6. Real-time PCR

The primers used for HO-1 and 18s amplifications were as follows: HO-1 sense 5'-GGTGATGCTGACAGAGGAACAC-3', antisense 5'-TAGCAGGC CTCTGACGAAGTG-3'; and 18s sense, 5'-AGTCCCTGCCCTTTGTACA

CA-3', antisense 5'-GATCCGAGGGCCTCACTAAAC-3'. 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 10 μl 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. Each sample was run in triplicate in separate tubes to permit quantification of the HO-1 gene normalized to the 18s gene. The PCR conditions were as follows: for HO-1, 38 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute; and for 18s, 35 cycles of denaturation at 94.5°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. An initial heating at 95°C for 9 minutes and a 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 CT method with $2^{-\Delta\Delta CT}$. The results are given as relative expression normalized to the 18s gene and expressed in arbitrary units. Signals from C glomeruli and NG podocytes were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels revealed a single band.

7. Western blot analysis

Sieved glomeruli and podocytes harvested from plates were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10 mM Tris-HCl, pH6.8, 10% [vol/vol] glycerol). Lysate was centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant was stored at -70°C until all rats were sacrificed. Protein concentrations were determined with a Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Aliquots of 50 μg proteins were treated with Laemmli sample buffer, then heated at 100°C for 5 minutes, and electrophoresed 50 μg/lane in 8% or 12% acrylamide denaturing SDS polyacrylamide gel. Proteins were transferred to Hybond-ECL membrane (Amersham Life Science, Inc., Arlington Heights, IL, USA) 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% non-fat milk) for 1 hour at room temperature, followed by an overnight incubation at 4°C in a 1:1000 dilution of monoclonal antibody to HO-1 (EMD Biosciences, Inc., Darmstadt, Germany), polyclonal antibodies to active fragments of caspase-3 (Cell Signaling, Beverly, MA, USA), Bax, Bcl-2, or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was washed three times and then incubated with a 1:1000 dilution of horseradish peroxidase-linked goat anti-

rabbit IgG (Amersham Life Science, Inc.). The washes were repeated, and the membrane was developed with chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany), and the changes in the optical densities relative to C glomeruli and NG cells were used for analysis.

8. Immunohistochemistry

Slices of kidney for immunohistochemical staining were fixed in 10% neutral buffered formalin, processed in the standard manner, and 4 μm sections of paraffin embedded tissues were utilized. Slides were deparaffinized, hydrated in ethyl alcohol, and washed in tap water. Antigen retrieval was carried out in 10 mM sodium citrate buffer for 20 minutes using a Black and Decker vegetable steamer. For HO-1, the primary monoclonal anti-HO-1 antibody (EMD Biosciences, Inc.) was diluted in 1:200 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added for 20 minutes, and the slides were then washed and incubated with a tertiary rabbit-PAP complex for 20 minutes. DAB was added for 2 minutes and the slides were counterstained with hematolxylin. A semi-quantitative score for measuring the intensity of

HO-1 staining within glomeruli was determined by examining thirty glomeruli in each section and by digital image analysis (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA) as previously described[38]. Briefly, the degree of staining was semi-quantitated on a scale of 0-4+ and a staining score was obtained by multiplying the intensity of staining by the percentage of glomeruli staining for that intensity; these numbers were then added for each experimental animal to give the staining score.

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

Immunohistochemical staining for WT-1 was also performed to determine the number of podocyte as described previously[39]. 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.

9. Immunofluorescence

Slices of kidney for immunofluorescence staining were snap-frozen in optimal cutting temperature (OCT) solution and 4 μm sections of tissues were utilized. Slides were fixed in acetone for 10 minutes at 4°C, air dried for 10

minutes at room temperature, and blocked with 10% donkey serum for 20 minutes at room temperature. For caspase-3 staining, the primary polyclonal antibody to active fragments of caspase-3 (Cell Signaling) was diluted in 1:100 with antibody diluent (DAKO, Glostrup, Denmark) and was applied for 3 hours at room temperature. After washing, Cy3 (red)-conjugated anti-rabbit IgG antibody (Research Diagnostics, Inc., Flanders, NJ, USA) was added for 60 minutes. For synaptopodin double staining, a 1:200 dilution of polyclonal anti-synaptopodin antibody (Santa Cruz Biotechnology, Inc.) was applied, followed by Cy2 (green)-conjugated anti-goat IgG antibody. A semi-quantitative score for measuring intensity of active fragments of caspase-3 was determined as aforementioned.

10. 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 Hoechst 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.

11. Statistical analysis

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

III. RESULTS

1. Animal studies

A. Clinical data

Body weight and the ratio of kidney weight to body weight were significantly different between DM (275±8 g, 1.12±0.11%) and C rats (398±8 g, 0.69±0.04%) ($p<0.05$), but the administration of ZnPP had no effect on body weight and the ratio of kidney weight to body weight in C and DM rats. The mean blood glucose levels of C, C+ZnPP, DM, and DM+ZnPP were 97.2±7.7 mg/dL, 94.1±5.4 mg/dL, 497.2±14.8 mg/dL, and 488.7±17.2 mg/dL, respectively ($p<0.01$). Compared to the C group (0.32±0.04 mg/day), 24-hour urinary albumin excretion was significantly higher in the DM group (1.18±0.11 mg/day, $p<0.05$), and was further increased in DM rats by ZnPP treatment (1.59±0.19 mg/day, $p<0.05$) (Table 1).

Table 1. Animal data

	Control (N=8)	Control+ZnPP (N=8)	DM (N=8)	DM+ZnPP (N=8)
Body Wt (g)	398±8	405±7	275±8*	266±2*
Kidney Wt/Body Wt (%)	0.69±0.04	0.75±0.05	1.12±0.11*	1.20±0.09*
Blood glucose (mg/dl)	97.2±7.7	94.1±5.4	497.2±14.8$^{\#}$	488.7±17.2$^{\#}$
24hr UAE (mg/day)	0.32±0.04	0.29±0.08	1.18±0.11*	1.59±0.19$^{\#,\S}$

* $p<0.05$ vs. Control group; $^{\#}$ $p<0.01$ vs. Control group; § $p<0.05$ vs. DM group. Wt, weight; UAE: urinary albumin excretion

B. Glomerular expression of HO-1 mRNA and protein

Glomerular HO-1 mRNA expression assessed by real-time PCR was significantly higher in DM compared to C rats ($p<0.01$), and ZnPP treatment significantly inhibited the increase in HO-1 mRNA expression in DM rats ($p<0.05$) (Figure 1). Similarly, there was a 2.4 fold increase in HO-1 protein expression in DM compared to C glomeruli ($p<0.05$), and this increase was significantly ameliorated in DM rats by ZnPP treatment ($p<0.05$) (Figure 2). On the other hand, ZnPP had no effect on glomerular HO-1 mRNA and protein expression in C rats. These results suggest that the administration of ZnPP inhibited the increase in HO-1 expression in DM glomeruli.

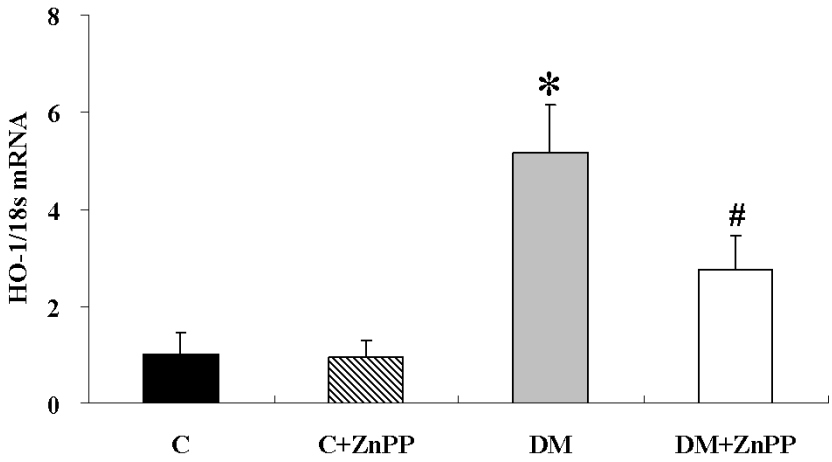

Figure 1. Glomerular HO-1 mRNA expression assessed by real-time PCR in C, C+ZnPP, DM, and DM+ZnPP rats. HO-1/18s mRNA ratio was 5.1-fold higher in DM compared to C glomeruli, and ZnPP treatment significantly inhibited the increase in glomerular HO-1/18s mRNA ratio in DM rats by 46.6%. * $p<0.01$ vs. C and C+ZnPP groups; $^{\#}$ $p<0.05$ vs. Other groups.

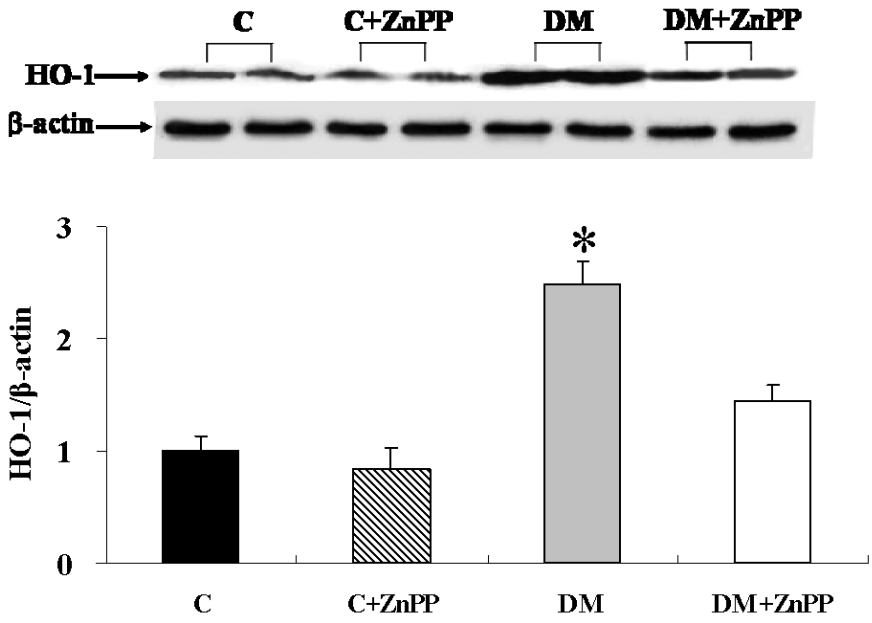

Figure 2. A representative Western blot of glomerular HO-1 in C, C+ZnPP, DM, and DM+ZnPP rats (representative of four blots). HO-1 protein expression was 2.4-fold higher in DM compared to C glomeruli, and this increase was significantly ameliorated in DM rats by ZnPP treatment. * $p<0.05$ vs. Other groups.

C. Immunohistochemical staining for glomerular HO-1

Immunohistochemical staining for glomerular HO-1 confirmed the Western blot findings. Glomerular HO-1 staining was significantly stronger in DM than in C rats, and ZnPP treatment abrogated the increase in HO-1 staining in DM glomeruli (Figure 3). The mean semi-quantitative staining score for glomerular HO-1 was significantly higher in DM (75.7±9.0) compared to C rats (22.4±2.4) ($p<0.05$), and this increase was attenuated in DM glomeruli by the administration of ZnPP (37.8±5.4) ($p<0.05$).

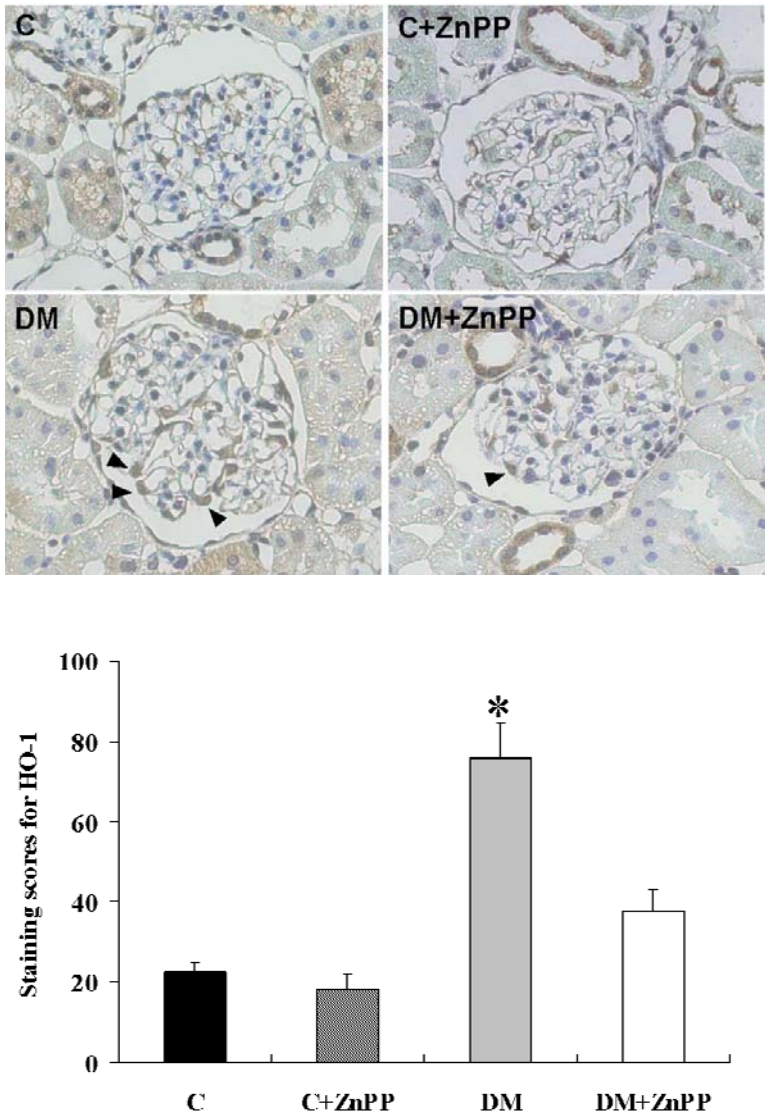

Figure 3. Immunohistochemical staining for HO-1 in C, C+ZnPP, DM, and DM+ZnPP rats. Glomerular HO-1 staining (arrow heads) was significantly stronger in DM compared to C and C+ZnPP rats, and this increase was attenuated in DM glomeruli by the administration of ZnPP. (x 400) * $p<0.05$ vs. Other groups.

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 ($p<0.05$). The administration of ZnPP significantly aggravated the increases in the ratios of Bax/Bcl-2 and active fragments of caspase-3 protein expression in DM glomeruli ($p<0.05$) (Figure 4).

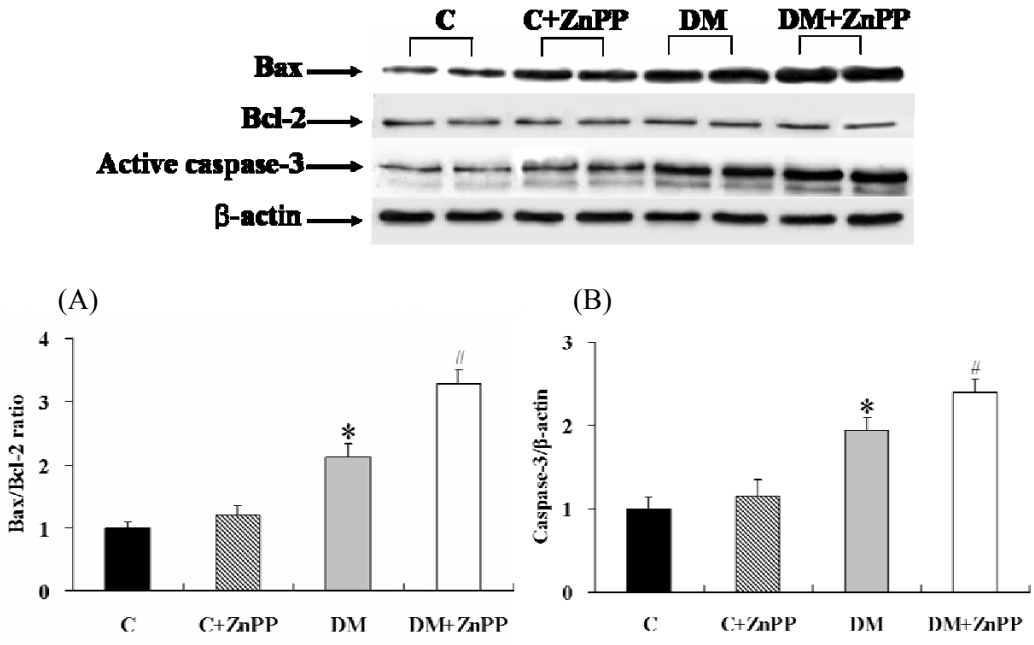

Figure 4. A representative Western blot of Bax, Bcl-2, and active fragments of caspase-3 protein expression in C, C+ZnPP, DM, and DM+ZnPP glomeruli (representative of four blots). The ratios of Bax/Bcl-2 (A) and active fragments of caspase-3 protein expression (B) were significantly increased in DM compared to C glomeruli, and the administration of ZnPP significantly aggravated these increases in DM glomeruli. * $p<0.05$ vs. Other groups; # $p<0.01$ vs. C and C+ZnPP groups.

E. TUNEL assay and double immunofluorescence staining

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+ZnPP rats ($p<0.05$), and was further increased in DM rats by ZnPP treatment ($p<0.05$) (Figure 5). To identify podocyte apoptosis in glomeruli, double immunofluorescence staining with antibodies to synaptopodin and active fragments of caspase-3 was performed. As seen in Figure 6, C and C+ZnPP rats showed minimal staining of active fragments of caspase-3 with no colocalization with synaptopodin, while the colocalization signal was increased in DM glomeruli and was highest in the glomeruli of DM+ZnPP rats.

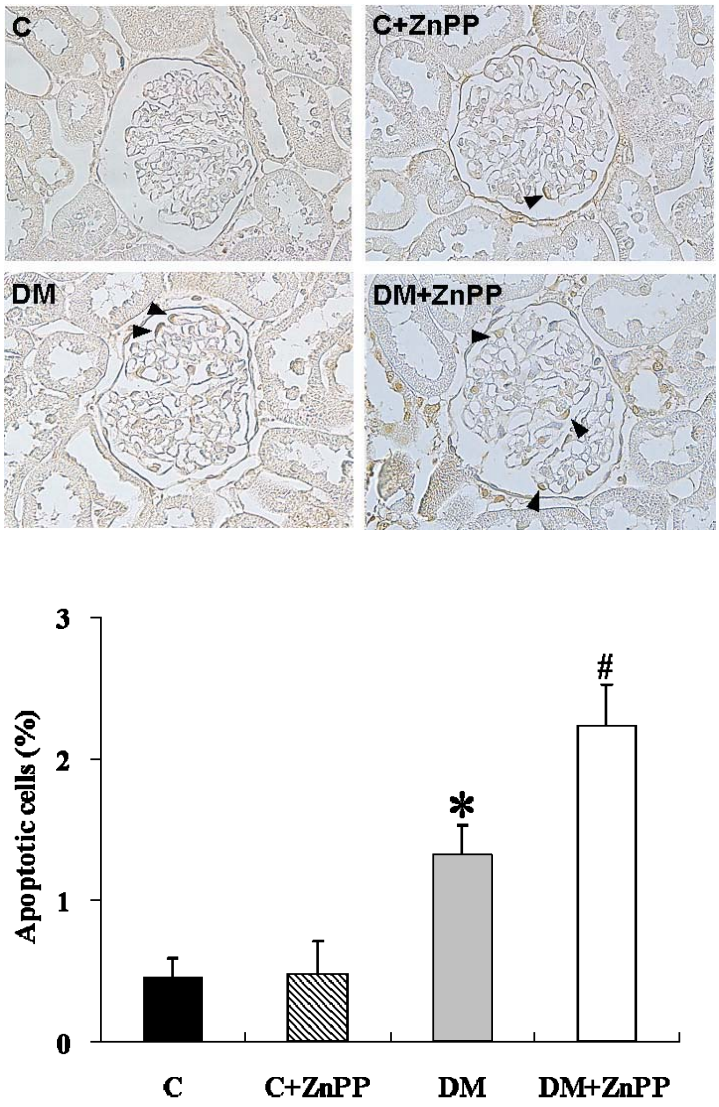

Figure 5. Apoptosis assessed by TUNEL assay in C, C+ZnPP, DM, and DM+ZnPP rats. There was a significant increase in apoptotic cells (arrow heads) in DM compared with C and C+ZnPP glomeruli, and this increase in apoptotic cells was accentuated with ZnPP treatment. (x 400) * $p<0.05$ vs. Other groups; # $p<0.01$ vs. C and C+ZnPP groups.

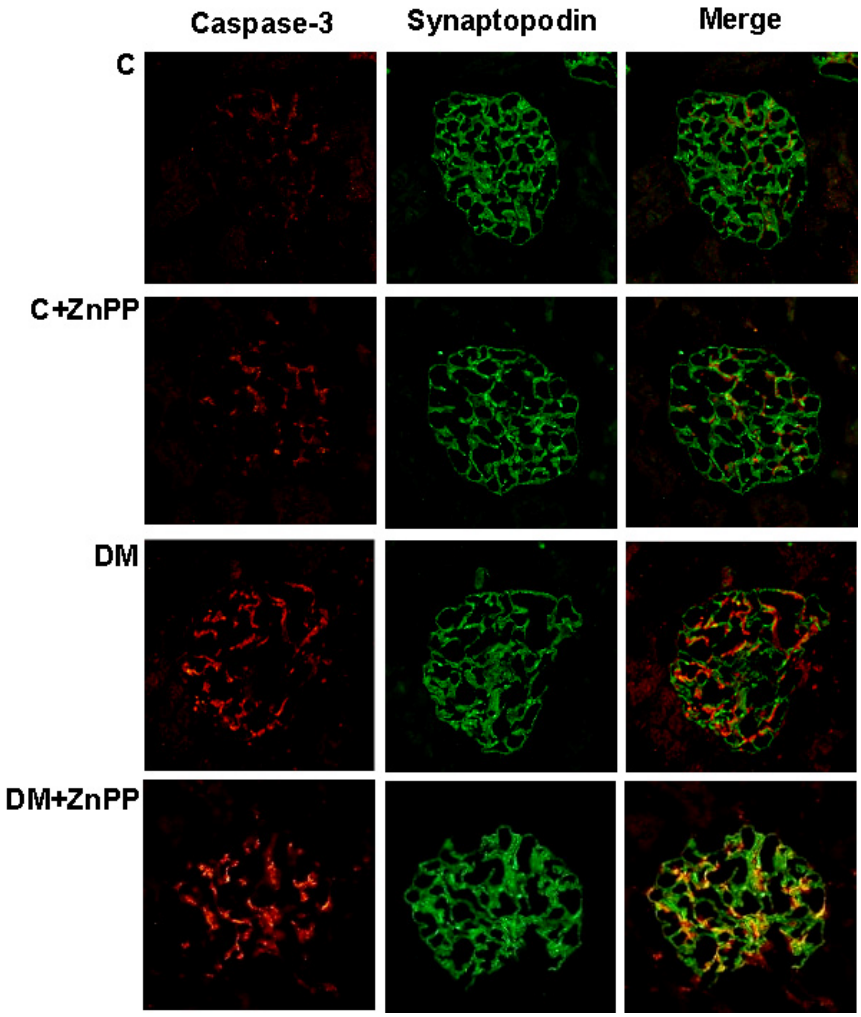

Figure 6. Double immunofluorescence staining for active fragments of caspase-3 (red) and synaptopodin (green). Compared to C and C+ZnPP rats, the colocalization signal (yellow) was increased in DM glomeruli, and was highest in the glomeruli of DM+ZnPP rats. (x 400)

F. Podocyte numbers

Compared to C (170.0±4.2) and C+ZnPP rats (168.3±5.9), the number of podocyte tended to be decreased in DM rats (159.1±3.7), and the reduction in podocyte numbers was aggravated in DM rats treated with ZnPP (142.9±3.2) ($p<0.05$) (Figure 7).

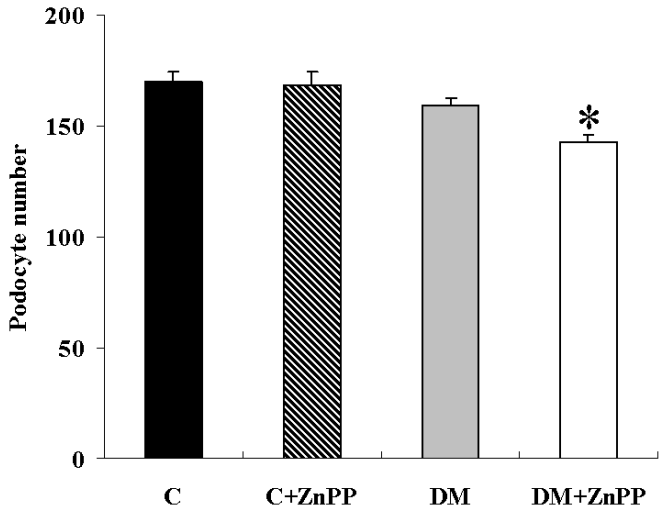

Figure 7. Podocyte numbers in C, C+ZnPP, DM, and DM+ZnPP rats. The number of podocyte tended to be decreased in DM rats, and the reduction in podocyte numbers was aggravated in DM rats treated with ZnPP. * $p<0.05$ vs. Other groups.

2. Cultured podocytes studies

A. HO-1 protein expression

Western blot analysis revealed that HO-1 siRNA inhibited HO-1 protein expression in a dose-dependent manner (Figure 8). On the other hand, high glucose significantly increased HO-1 protein expression in cultured podocytes by 131% compared to NG and NG+M cells ($p<0.05$) (Figure 9).

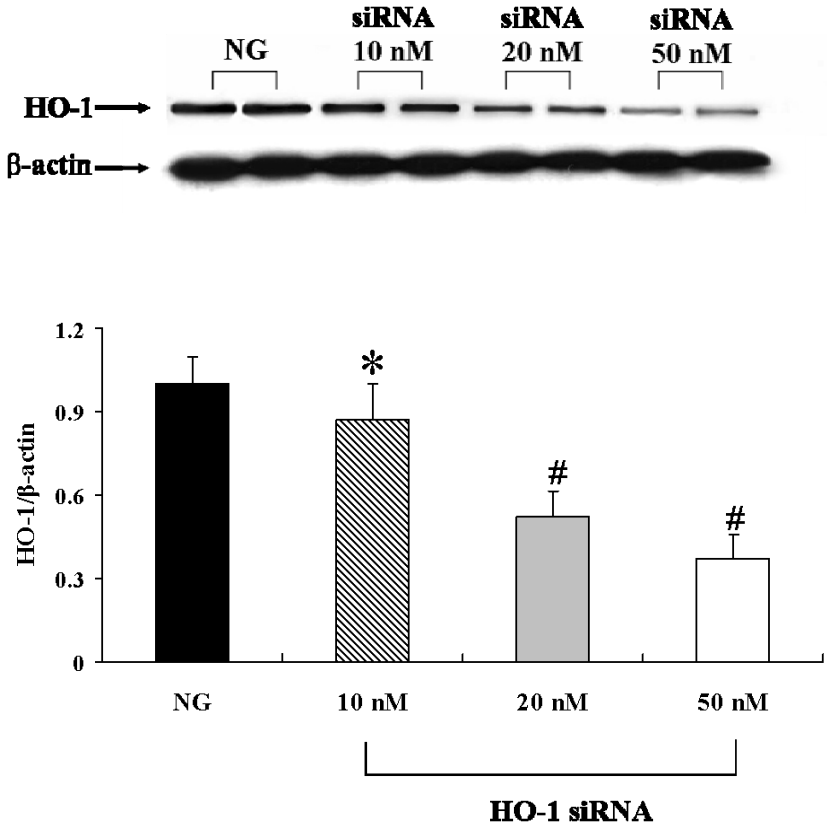

Figure 8. A representative Western blot of HO-1 in cultured podocytes treated with three different concentrations of HO-1 siRNA (representative of four blots). HO-1 siRNA inhibited HO-1 protein expression in a dose-dependent manner. * $p<0.05$ vs. NG group; # $p<0.01$ vs. NG group.

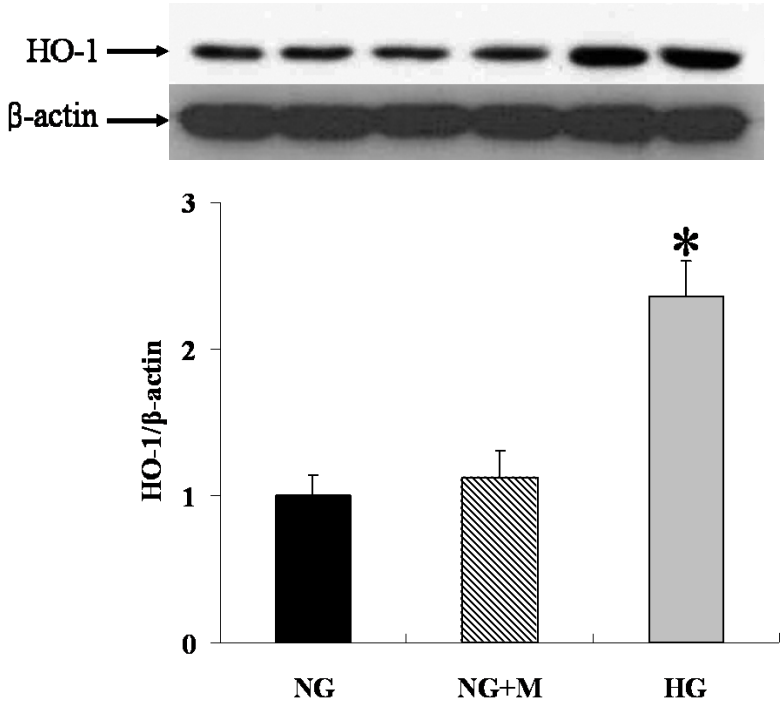

Figure 9. A representative Western blot of HO-1 in cultured podocytes exposed to NG, NG+M, and HG medium (representative of four blots). There was a significant increase in HO-1 expression in HG-stimulated podocytes compared to NG and NG+M cells. * $p<0.05$ vs. NG and NG+M groups.

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

The ratios of Bax/Bcl-2 and active fragments of caspase-3 protein expression were significantly increased in HG-stimulated podocyte compared to podocytes exposed to NG and NG+M medium ($p<0.05$), and these increases were accentuated in HG cells by HO-1 siRNA ($p<0.05$). Bax/Bcl-2 protein ratios and active fragments of caspase-3 protein expression were also increased in HO-1 siRNA-treated NG cells, but did not reach statistical significance (Figure 10).

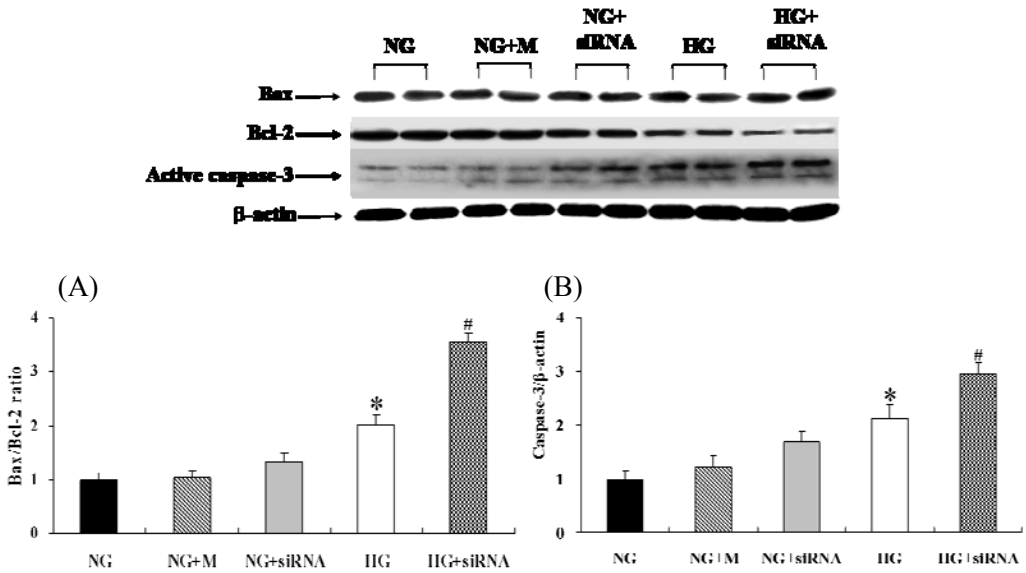

Figure 10. A representative Western blot of Bax, Bcl-2, and active fragments of caspase-3 in cultured podocytes (representative of four blots). The ratios of Bax/Bcl-2 (A) and active fragments of caspase-3 protein expression (B) were significantly increased in HG-stimulated podocyte compared to NG and NG+M groups, and these increases were accentuated in HG cells by HO-1 siRNA (20 nM). * $p<0.05$ vs. Other groups; $^{\#}$ $p<0.01$ vs. NG, NG+M, and NG+siRNA groups.

C. Hoechst 33342 staining

Apoptotic cells assessed by Hoechst 33342 staining were significantly increased in HG-stimulated podocytes compared to NG cells ($p<0.05$), and apoptosis in HG podocytes was significantly augmented by HO-1 siRNA ($p<0.05$) (Figure 11).

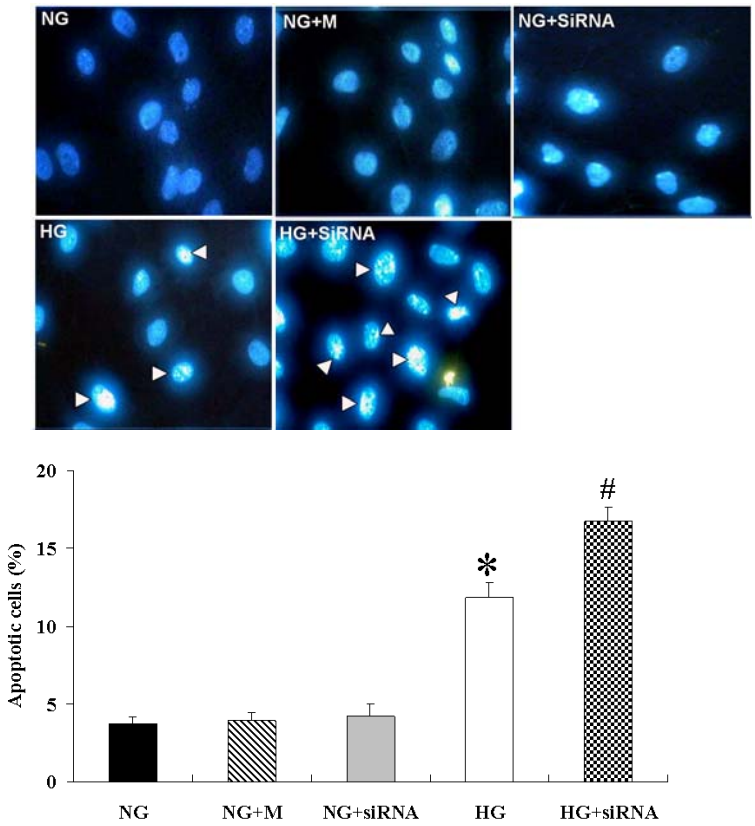

Figure 11. Apoptosis assessed by Hoechst 33342 staining. There was a significant increase in apoptotic cells (arrow heads) in HG-stimulated podocytes compared to NG cells, and apoptosis in HG podocytes was significantly augmented by HO-1 siRNA. * $p<0.05$ vs. Other groups; $^{\#}$ $p<0.01$ vs. NG, NG+M, and NG+siRNA groups.

IV. DISCUSSION

Previous studies have demonstrated that HO-1 plays a protective role in various kidney diseases, of which oxidative stress is implicated in the pathogenesis. However, its functional significance in terms of podocyte apoptosis in diabetic nephropathy has never been elucidated. In this study, I demonstrate for the first time that HO-1 expression is increased in experimental diabetic glomeruli and in high glucose-stimulated podocytes and that podocyte apoptosis under diabetic conditions is partly protected by this increase in HO-1 expression.

Although the term 'oxidative stress' has been of wide use, no clear definition exists yet. A number of researchers, however, see oxidative stress as a status where production of the short-lived, highly-reactive molecules such as superoxide anion, hydrogen peroxide, and peroxynitrite is increased[40]. Recently, accumulating clinical and experimental evidences indicate that excessive oxidative stress contributes to the initiation and development of diabetic nephropathy. High glucose *per se*[41], TGF-β1[42, 43], and AII[44], major mediators involved in the development of diabetic nephropathy, are all reported to increase ROS in cultured mesangial cells. ROS is known to mediate both in vivo and in vitro activation of protein kinase C, nuclear factor

(NF)-κB, TGF-β1, fibronectin, and plasminogen activator inhibitor (PAI)-1 under diabetic conditions[11, 45]. In high glucose-stimulated mesangial cells, anti-oxidant treatment normalized PKC and NF-κB activation as well as PAI-1 expression, suggesting that ROS might mediate the high glucose-induced changes in cultured mesangial cells[10, 42, 46]. Activation of glomerular PKC-δ and PKC-ε and increased glomerular TGF-β1 and fibronectin mRNA expression in diabetic animals were also inhibited by the administration of anti-oxidant, resulting in amelioration of glomerular basement membrane thickening, mesangial expansion, and proteinuria[47].

On the other hand, the number of podocytes is decreased in the glomeruli of patients with type 1 diabetes of all ages, even in diabetes of short duration[48]. Analysis of kidney biopsies from Pima Indians with type 2 diabetes demonstrated that subjects with clinical nephropathy exhibited broadening of foot processes associated with a reduced number of podocytes per glomerulus[49]. Podocyte number usually reflects the balance between podocyte loss and proliferation. Therefore, a decrease in podocyte number can occur by increased loss and/or lack of proliferation. Previous studies have demonstrated that podocyte apoptosis is involved in the process of podocyte loss under diabetic conditions, and that high glucose *per se*[12], TGF-β1[50], and high

concentrations of AII[51] induce apoptosis in cultured podocytes. While AII-induced podocyte apoptosis was partly TGF-β1-dependent, high glucose and TGF-β1 increased ROS production and apoptosis in cultured podocytes via proapoptotic p38 mitogen-activated protein kinase (MAPK) pathway[12]. Systemic inhibition of NADPH oxidase also prevented podocyte apoptosis, reduced loss of podocytes, and ameliorated urinary albumin excretion in *db/db* mice[12]. Furthermore, the decrease in podocyte density in diabetic rat with early diabetic nephropathy was prevented by anti-oxidant therapy with lipoic acid[13]. Taken together, increased ROS generation may constitute a major mediator of podocyte apoptosis under diabetic conditions. In this study, I found that the expression of apoptosis-related molecules, Bax and active fragments of caspase-3, were increased in experimental diabetic glomeruli and in high glucose-stimulated podocytes, leading to a decrease in podocyte number, which was consistent with previous studies[12].

HO is a catalyzing enzyme that cleaves the heme ring, converts heme to biliverdin, and releases iron and CO[14-16]. By this process, HO regulates the cellular levels of heme, which is known to act as a pro-oxidant and to induce cell dysfunction. Therefore, when cellular heme levels are increased by denaturation or destabilization of heme protein during cell injury, HO is

supposed to be increased to remove heme and to replace it by bilirubin, a potent anti-oxidant. The cytoprotective role of HO was first suggested by Keyse et al[52]. They identified sodium arsenite-induced 32-kDa protein in cultured human skin fibroblasts as HO and showed that HO gene is also induced by UVA radiation, hydrogen peroxide, and heavy metal salts. Based on these findings, they suggested that the induction of HO may be a general response to oxidant stress and constitutes an important cellular defense mechanism against oxidative damage. After this report, numerous studies have investigated the expression and the functional role of HO, especially of HO-1, which is the largely induced isoform of HO under stressed conditions. In the kidney, HO-1 is weakly expressed in proximal and distal tubules, in Henle's loop, and in medullary collecting ducts under normal state, but its expression is increased under various conditions[14]. The functional role of increased HO-1 expression has been elucidated in toxic nephropathy[22] and in renal ischemia-reperfusion injury[32, 53-55], of which oxidative stress plays a role in the pathogenesis, by inducing or inhibiting HO-1 expression. Upregulation of HO-1 reduced renal injury induced by cyclosporine[22] and potassium dichromate[56], and protected tubular cell injury induced by cisplatin[21], whereas inhibition of HO-1 expression exacerbated renal injury in maleate nephropathy[31] and worsened cisplatin-induced cell injury[57]. In addition,

induction of HO-1 by hemolysate[53], tin chloride[54], or cobalt[55] ameliorated ischemic acute renal injury in rats, while mild renal ischemia exerted severe renal dysfunction and increased mortality in HO-1 knockout mice compared to control mice[53-55]. Taken together, HO-1 seems to be induced to play an important cytoprotective role in oxidative stress-induced renal injury.

Based on these previous reports, HO-1 expression was surmised to be increased in diabetic nephropathy in response to oxidative stress. Recently, Hayashi et al.[35] and Koya et al.[26] demonstrated that HO-1 mRNA and protein expression were induced in diabetic glomeruli and anti-oxidant treatment nearly normalized the increase in HO-1 expression, suggesting that glomerular HO-1 expression was increased secondary to diabetes-induced oxidative stress. The former investigators also showed that the glomerular cells with increased HO-1 protein expression were mesangial cells and podocytes. However, the two aforementioned studies did not examine the biologic significance of increased glomerular HO-1 expression under diabetic conditions. The present study also demonstrated that HO-1 expression was increased not only in experimental diabetic glomeruli but also in high glucose-stimulated podocytes, which was in accordance with previous studies[26, 35].

The cytoprotective effect of HO-1 seems to be attributed to the anti-apoptotic and anti-inflammatory effects of the HO system. Shiraishi et al.[21] showed that renal failure and renal injury characterized by tubular apoptosis were more severe in transgenic mice deficient in HO-1 compared to wild type mice treated with cisplatin. Cisplatin-induced apoptosis in cultured proximal tubular epithelial cells was significantly attenuated by inducing HO-1 expression, whereas inhibition of HO-1 enzyme activity reversed the anti-apoptotic effect. Based on these findings, they postulated that HO-1 had a cytoprotective effect by preventing apoptosis induced by oxidative stimuli. The anti-apoptotic effect of HO-1 seems to be mediated by upregulating the expression of p21, an anti-apoptotic cell cycle protein, in renal tubular epithelial cells[58]. In some cells, CO, a metabolite of the HO system, is responsible for the anti-apoptotic effect of HO-1, while augmentation of iron efflux by HO-1 contributes to its anti-apoptotic property in cultured fibroblasts[59]. The present study demonstrated for the first time that apoptosis in experimental diabetic glomeruli and in high glucose-stimulated podocytes was further accentuated in HO-1-suppressed state both in vivo and in vitro, suggesting that podocyte HO-1 expression seemed to be induced to protect podocyte apoptosis under diabetic conditions. Moreover, double immunofluorescence staining with active fragments of caspase-3 and

synaptopodin in ZnPP-treated diabetic rats revealed that glomerular cells with augmented apoptosis were podocytes. Based on these findings, the further increase in albuminuria in diabetic rats treated with ZnPP may also be attributed to exacerbated apoptosis of podocytes under HO-1-inhibited state.

On the other hand, HO-1 is also known to suppress inflammation in models of tubulointerstitial disease by inhibiting the expression of monocyte chemoattractant protein-1 (MCP-1), the most important mediator of inflammatory cells infiltration[60]. Ischemic insult, which exerted little effect in wild type mice, induced MCP-1 mRNA and caspase-3 expression, activated NF-κB, and increased mortality in HO-1 knockout mice. Diabetic nephropathy is generally considered a nonimmune renal disorder, however, recent studies have suggested that an inflammatory mechanisms also contribute to the pathogenesis of diabetic nephropathy and that MCP-1 plays a critical role in the pathogenesis of diabetic nephropathy via inducing inflammatory cells infiltration[61, 62]. In this study, I did not examine the expression of MCP-1 in podocytes under diabetic conditions and the changes in MCP-1 expression after suppressing HO-1 expression, because MCP-1 expression was not detected in podocytes and inflammatory cells infiltration was mainly demonstrated in diabetic tubulointerstitium.

To clarify the functional role of HO-1 in diabetic nephropathy, ZnPP and HO-1 siRNA were used to inhibit HO-1 in vivo and in vitro, respectively, in this study. The majority of previous in vivo studies[27, 63, 64] used ZnPP to suppress HO-1 activity, but ZnPP were known to inhibit not only HO-1 but also HO-2 at transcription levels[63, 65]. For this reason, the results of the in vivo study could not definitely determine whether the increase in podocyte apoptosis was due to the blockade of HO-1 and/or HO-2 activity. For the in vitro experiments, therefore, HO-1 siRNA was introduced to selectively inhibit HO-1, which led to an increase in apoptosis of podocytes under diabetic conditions, which was consistent with the animal study. On the other hand, since the consequences of HO-1 induction on podocyte apoptosis under diabetic conditions were not examined in this study, further study will be needed to elucidate whether upregulation of HO-1 by inducers such as hemin and cobalt protoporphyrin confers cytoprotection in diabetic nephropathy. However, it should be noted that it is difficult to determine the degree of HO-1 induction because overexpression of HO-1 can be deleterious in certain conditions[66].

In conclusion, HO-1 expression was increased in both experimental diabetic glomeruli and high glucose-stimulated podocytes. Moreover,

inhibition of HO-1 expression further increased albuminuria, reduced podocyte numbers in diabetic rats, and accentuated podocyte apoptosis in diabetic glomeruli and in high glucose-stimulated podocytes. Taken together, these results suggest that podocyte HO-1 expression is induced to protect podocyte against apoptosis under diabetic conditions.

V. CONCLUSION

In this study, I investigated whether HO-1 expression was increased in experimental diabetic glomeruli and in high glucose-stimulated podocytes. In addition, to elucidate the functional role of HO-1, I examined podocyte apoptosis under diabetic conditions before and after inhibition of HO-1 activity.

1. 24-hour urinary albumin excretion was significantly higher in DM (1.18±0.11 mg/day, $p<0.05$) compared to C rats (0.32±0.04 mg/day), and was further increased in DM rats by ZnPP treatment (1.59±0.19 mg/day, $p<0.05$).
2. Glomerular HO-1 mRNA and protein expression were significantly higher in DM compared to C rats ($p<0.01$), and ZnPP treatment significantly inhibited the increase in HO-1 expression in DM rats ($p<0.05$).
3. Glomerular HO-1 staining was significantly stronger in DM relative to C and C+ZnPP rats ($p<0.05$), and this increase was attenuated in DM glomeruli by the administration of ZnPP.
4. The ratios of Bax/Bcl-2 and active fragments of caspase-3 protein

expression were significantly increased in DM compared to C glomeruli ($p<0.05$), and the administration of ZnPP significantly accentuated these increases in DM glomeruli ($p<0.05$).

5. TUNEL positive-stained nuclei were also significantly increased in DM relative to C glomeruli ($p<0.05$), and this increase was significantly augmented in DM rats with ZnPP treatment ($p<0.05$).
6. Double IF staining with active fragments of caspase-3 and synaptopodin revealed that the colocalization signal was increased in DM glomeruli and was highest in the glomeruli of DM+ZnPP rats.
7. The number of podocyte was decreased in DM (159.1±3.7) compared to C (170.0±4.2) and C+ZnPP rats (168.3±5.9), and the reduction in podocyte numbers was aggravated in DM rats treated with ZnPP (142.9±3.2) ($p<0.05$).
8. HO-1 siRNA inhibited HO-1 protein expression in cultured podocytes in a dose-dependent manner.
9. High glucose significantly increased HO-1 protein expression in cultured podocytes by 131% compared to NG and NG+M cells ($p<0.05$).
10. The ratios of Bax/Bcl-2 and active fragments of caspase-3 protein expression were significantly increased in HG-stimulated podocytes

relative to NG and NG+M groups ($p<0.05$), and these increases were significantly accentuated in HG cells by HO-1 siRNA ($p<0.05$).

11. Apoptotic cells assessed by Hoechst 33342 staining were also significantly increased in HG-stimulated podocytes compared to NG cells, and apoptosis in HG podocytes was significantly augmented by HO-1 siRNA ($p<0.05$).

In summary, HO-1 expression was increased in both experimental diabetic glomeruli and high glucose-stimulated podocytes. Moreover, inhibition of HO-1 expression further increased albuminuria, reduced podocyte numbers in diabetic rats, and accentuated podocyte apoptosis in diabetic glomeruli and in high glucose-stimulated podocytes. Taken together, these results suggest that podocyte HO-1 expression is induced to protect podocyte against apoptosis under diabetic conditions.

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ABSTRACT (in Korean)

당뇨병성 신병증에서 heme oxygenase-1이 족세포 사멸에 미치는 영향

<지도교수 강 신 욱>

연세대학교 대학원 의학과

이 상 철

서론: 당뇨병성 신병증은 국내외적으로 말기 신부전증의 가장 흔한 원인 질환으로, 초기에는 단백뇨가 대표적인 임상적 특징이다. 최근의 보고들에 의하면 단백뇨의 발생에 사구체 여과 장벽을 구성하는 세포의 하나인 족세포가 중요한 역할을 하는 것으로 알려져 있다. 당뇨병성 신병증에서 족세포의 수 및 밀도가 감소되어 있으며 이는 사구체 여과장벽의 손상을 일으켜 결국 사구체 경화증을 초래하는 요인이 되는데, 이 과정에 당뇨 조건 하에서 생성이 증가된 ROS가 관여하는 것으로 알려져 있다. Heme oxygenase-1 (HO-1)은 미소체 효소로서 산화성 스트레스에 의한 각종 조직 손상에서 발현이 증가되며, 항산화 효과, 항염증 작용, 그리고 항세포사멸 효과가 있는 것으로 보고되고 있다. 당뇨병성

신병증에서도 HO-1의 발현 증가가 보고된 바는 있으나, 그 기능적 역할에 대한 연구는 전무한 실정이다. 이에 본 연구자는 당뇨병성 신병증에서 HO-1의 발현 증가 여부를 확인하고, 당뇨병성 신병증에서 발현이 증가되는 HO-1의 기능적 의미를 알아보기 위하여 생체 내외의 실험을 통하여 HO-1 발현 억제 전후에 족세포 사멸의 변화를 알아보고자 하였다.

방법: 32마리의 Sprague-Dawley 백서를 대상으로 대조군 (16마리)과 복강 내로 streptozotocin을 투여한 당뇨군 (16마리)으로 나누었으며 각 군에서 8마리에는 HO-1의 억제제인 Zinc protoporphyrin (ZnPP, 50 μmol/kg/day)를 6주간 복강 내로 투여하였다. 또한, 불멸 생쥐 족세포 (immortalized mouse podocytes)를 정상 포도당군 (5.6 mM), 정상 포도당+만니톨군 (24.4 mM 만니톨), 정상 포도당+HO-1 siRNA 처리군, 고포도당군 (30 mM), 그리고 고포도당+HO-1 siRNA 처리군으로 나누어 배양하였다. 실험 동물로부터 분리한 사구체와 배양 족세포에서 HO-1 mRNA와 단백 발현은 각각 real-time PCR과 Western blot을 이용하여 분석하였으며, 신장 조직 내 HO-1의 단백 발현은 면역조직화학 염색을 시행하여 확인하였다. 세포사멸은 Bax, Bcl-2, 그리고 active fragments of caspase-3에 대한 Western blot을 이용하여 분석하였으며, 신장 조직을 이용한 TUNEL 염색과 배양 세포를 이용한 Hoechst 33342 염색으로도 확인하였다.

결과: 24시간 뇨알부민 배설량은 대조군 (0.32±0.04 mg/day)에 비하여 당뇨군 (1.18±0.11 mg/day)에서 의미있게 많았으며 ($p<0.05$), ZnPP 투여 당뇨군에서의 뇨알부민 배설량이 유의하게 더 증가하였다 (1.59±0.19 mg/day) ($p<0.05$). 사구체 내 HO-1의 mRNA 발현은 대조군에 비하여 당뇨군에서 5.1배 증가되었으며 ($p<0.01$), ZnPP 투여로 당뇨군에서 발현 증가가 46.6% 억제되었다 ($p<0.05$). 사구체 내 HO-1의 단백 발현도 mRNA 발현 양상과 유사하였다. 세포 실험상 고포도당으로 자극한 족세포에서 HO-1의 단백 발현은 정상 포도당군에 비하여 2.3배 증가되었으며 ($p<0.05$), HO-1 siRNA는 HO-1의 단백 발현을 농도 의존적으로 억제시켰다. 세포사멸의 지표인 Bax/Bcl-2 단백 발현의 비와 active fragments of caspase-3의 단백 발현은 당뇨 사구체와 고포도당으로 자극한 족세포에서 의미있게 높았으며 ($p<0.05$), ZnPP 투여 및 HO-1 siRNA 처치로 의의있게 더 증가되었다 ($p<0.01$). 사구체 내 TUNEL-양성 세포 수는 대조군 (0.4±0.1)에 비하여 당뇨군 (1.3±0.2)에서 유의하게 많았으며 ($p<0.05$), ZnPP 투여 당뇨군 (2.2±0.3)에서 의미있게 더 증가되었다 ($p<0.05$). WT-1에 대한 면역조직화학 염색을 이용하여 측정한 족세포 수는 대조군 (170.0±4.2)에 비하여 당뇨군 (159.1±3.7)에서 적었으며, ZnPP를 투여한 당뇨군 (142.9±3.2)에서 의의있게 더 감소되었다 ($p<0.05$). Active fragments of caspase-3와 synaptopodin에 대한 이중

면역형광염색을 시행한 결과, 세포사멸이 동반된 세포가 주로 족세포임을 알 수 있었다. 배양 족세포에서 Hoechst 33342 염색을 이용하여 측정한 세포사멸이 동반된 족세포 수도 정상 포도당군 (3.7±0.1%)에 비하여 고포도당군 (11.1±0.2%)에서 유의하게 많았으며 ($p<0.05$), HO-1 siRNA 처리군 (17.2±0.2%)에서 의미있게 더 증가되었다 ($p<0.05$).

결론: 당뇨병성 신병증의 동물 모델과 고포도당으로 자극한 족세포에서 HO-1의 발현은 의의있게 증가되었으며, HO-1의 발현 증가를 억제시킬 경우 족세포의 세포사멸이 유의하게 더 증가되었다. 이상의 결과들을 종합해 볼 때, 당뇨 조건 하에서 족세포 내 HO-1의 발현 증가는 세포사멸에 대한 보호 역할을 할 것으로 생각된다.

핵심 되는 말: heme oxygenase-1, 당뇨병성 신병증, 족세포, 고포도당, 세포사멸