High Ambient Glucose Levels Modulates the Production of MMP-9 and $\alpha 5(\text{IV})$ Collagen by Cultured Podocytes

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Key Words

Diabetic nephropathy • Glomerular epithelial cells • Glomerulosclerosis • Extracellular matrix • Glomerular filtration barrier

Abstract

Recent evidences have demonstrated an important role for glomerular visceral epithelial cell (podocyte) in the development and progression of diabetic nephropathy. We investigated the high-glucose (HG)-triggered signaling pathway and its role in matrix metalloproteinase (MMP) production in murine podocytes. The activity of 92-kDa (MMP-9) gelatinase, but not of 72 kDa (MMP-2), in an HG medium significantly increased during incubation of 2 to 3 days and decreased during incubation of more than 5 days revealed by Gelatin zymography. Opposite to the increases in MMP-9 activity, HG medium produced significant decreases in the protein levels of $\alpha 5(\text{IV})$ collagen. Changes in MMP-9 activity were associated with the same pattern as MMP-9 mRNA levels in podocytes exposed to HG media. HG medium rapidly activated ERK1/2 MAPK in podocytes. Moreover, ERK1/2 activation was required for HG-induced enhancement of MMP-9 activity and a decrease in the level of $\alpha 5(\text{IV})$ collagen. HG incubation rapidly induced an increase in the nuclear accumulation of Ets-1 protein. Blocking the ERK pathway suppressed

HG-induced expression and nuclear accumulation of transcriptional factor Ets-1, and MMP-9 mRNA expression. We suggest that short- or long-term exposure to HG concentrations increases or decreases MMP-9 production and $\alpha 5(\text{IV})$ collagen expression in podocytes, this may contribute to the GBM abnormality caused by an imbalance in extracellular matrix (ECM) synthesis and degradation, and may play a critical role in the pathogenesis of proteinuria in diabetic nephropathy.

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Introduction

An early feature of diabetic nephropathy is the alteration of the glomerular basement membrane (GBM), which may result microalbuminuria, subsequent macroproteinuria, and eventual renal failure [1], (the leading cause of morbidity and mortality in patients with diabetes mellitus [DM]). Evidence indicates that the glomerular visceral epithelial cell, also called podocyte is affected by the earliest changes in diabetic glomerulonephrosis, including overproduction of growth factors and cytokines, enhanced excretion of urinary

albumin, and the recruitment of inflammatory cells [2-6], all of which contribute to the initiation and progression of renal disease. Because the onset and progression of diabetic glomerulosclerosis are delayed in patients who achieve an adequate level of glycemic control [7-9], hyperglycemia is thought to be an important regulator of glomerular lesion development. Recent studies have demonstrated that antiproteinuric agents such as angiotensin converting enzyme inhibitor (ACEI) slow the progression of renal disease in patients with DM, presumably by preserving podocyte structure and function [10, 11]. However, the mechanism of action by which hyperglycemia affects podocyte damage remains unclear.

Matrix metalloproteinases (MMPs) are members of a family of Zn^{2+} -dependent and Ca^{2+} -dependent endopeptidases, which are essential for tissue remodeling in both physiologic and pathologic conditions. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are gelatinases that degrade type IV collagen (the major component of the GBM) and are essential in maintaining the integrity of the GBM. In many types of kidney cells, including mesangial cells, gelatinases are synthesized and secreted in a hyperglycemic environment [12], but that phenomenon has not been studied in podocytes. Because the accumulation of matrix protein in the GBM and the mesangium depends on the delicate balance between synthesizing and degradative pathways, the effects of hyperglycemia on the synthetic pathway have been more thoroughly studied in a variety of kidney cells. More recently, attention has focused on the degradative arm of matrix turnover, and increasing evidence suggests that hyperglycemia does affect matrix degradation by the modulation of MMP activity. MMP-2 and MMP-9 gelatinases are reportedly synthesized and secreted by glomerular epithelia cells *in vivo* and *in vitro* [13-16] and could be involved in normal and/or aberrant degradation or remodeling of the glomerular membrane. Thus, the derangement of MMP-2 and MMP-9 regulation is considered the critical factor in the damage of the glomerular filtration barrier that leads to the development of proteinuria and glomerulosclerosis. Elevated levels of circulating glucose *in vivo* may affect MMP activity in several different cell types [17].

We hypothesized that MMP-2 and MMP-9 may be regulated by a hyperglycemic environment in podocytes, thus contributing to the structural alteration of GBM in diabetic nephropathy. We first studied the activity of MMP-2 and MMP-9 in cells cultured in an HG medium for 10 days. In addition, we investigated the possible

pathway for modulating the production of MMP-9 in podocytes by manipulating the HG environment. We provide evidence that the activity of MMP-9, but not MMP-2, is preferentially modulated in podocytes by the HG concentration of the medium, an effect due in part to the regulation of MMP-9 mRNA expression via an ERK-Ets-1 pathway.

Materials and Methods

Cell culture and cell treatment

An immortalized mouse podocyte clone kindly provided by Professor Peter Mundel (Albert Einstein College of Medicine, New York) was cultivated according to the methods described by Mundel *et al* [18]. The murine podocytes were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA). The cells were propagated at 33°C under permissive conditions and were treated with 10 U/ml of mouse recombinant γ -interferon (Gibco BRL, Gaithersburg, MD, USA). To induce the differentiation of podocytes, the cells were maintained at 37°C in the same medium without γ -interferon under nonpermissive conditions for 14 days before the experiments were conducted.

Podocytes were grown for 10 days in either normal medium (the NG group, glucose 100 mg/dl), mannitol-added medium (the MN group, glucose 100 mg/dl and D-mannose 350 mg/dl), or high-glucose medium (the HG group, glucose 450 mg/dl) and were cultured for a 24 hours. Each medium was collected daily and was assessed for gelatinase activity and α 5(IV) collagen production. Inhibition studies were performed with podocytes cultured in media to which inhibitors had been added 30 minutes before high-glucose medium.

Gelatin zymography

Gelatinolytic activities of conditioned media of cultured cells were analyzed by electrophoresis in SDS-polyacrylamide gels containing gelatin 1 mg/ml [19]. Samples were applied to the gel in a sample buffer containing SDS 2.0%, Tris-HCl 50 mM (pH, 6.8), and glycerol 10%. After electrophoresis, the gels were washed twice with Triton-X 100 2.5% for 15 minutes and were then incubated overnight at 37°C in Tris-HCl 50 mmol/l (pH, 7.4), $CaCl_2$ 10 mmol/l, and Brij solution 0.05%. The gels were then stained with 0.25% Coomassie brilliant blue R-250, after which destaining was performed by means of methanol: acetic acid:water (30:10:60, v/v/v) until clear bands were seen against the blue background of stained gel. Band densities representing enzyme activity were quantified by means of the KODAK Gel Logic 100 Imaging System and 1D Image Analysis Software (New Haven, CT, USA).

Precipitation of protein in cultured medium

Precipitation of protein in cultured medium was modified from the procedure as reported by Artishevsky and colleagues [20]. Briefly, the collected medium was clarified and subjected

Fig. 1. Gelatinic activity of differentiated mouse podocytes incubated in various media for 10 days. A: The normal-glucose medium (NG group) did not affect MMP-9 or MMP-2 activity. B: and the mannitol-added medium (MN group) did not affect MMP-9 or MMP-2 activity. C: A high-glucose medium (HG group) induced dynamic changes in MMP-9 activity in podocytes, and the MMP-2 activity was constant during the 10-day incubation (upper panel). This is a representative of three independent experiments. Quantifications of the activities of MMP-9 were shown with the mean $\pm$ SEM.

to precipitation by the addition of trichloroacetic acid 10% (w/v) for overnight incubation at 4°C and then to centrifugation at 10,000g for 30 minutes. The precipitate was resuspended in 50 μ l of buffer (Tris-HCl 100 mM [pH, 8.0] and NaCl 150 mM) containing protease inhibitors, and the Bradford assay was used to determine the protein concentration.

Isolation of cytoplasmic and nuclear fraction protein

The separated cytoplasmic and nuclear extracts were obtained by means of the previously described procedures [21] with minor modifications. Cultured podocytes were washed 3 times with ice-cold PBS (pH, 7.4) and were then scraped with small pliceman into 1 milliliter of cold PBS contained in a 1.5-ml Eppendorf tube. Cell pellets were then collected by centrifugation at 4°C for 5 minutes at 2500 rpm. Supernatants

were aspirated, and the pellets were saved for resuspension in 200-400 μ l of buffer A (HEPES 10 mM [pH, 7.9], MgCl₂ 1.5 mM, KCl 10 mM, DDT 1 mM, Na₃VO₄ 1 mM, and PMSF 1 μ g/ml) and were incubated on ice for 20 minutes. After the addition of detergent NP-40 0.1% to each tube and subsequent vortexing, the nuclei were recovered by centrifugation at 4°C for 5 minutes, and the supernatant containing the cytoplasmic protein fraction was stored on ice and saved. Pellets of nuclei were resuspended in buffer B (HEPES 20 mM [pH, 7.9], NaCl 420 mM, MgCl₂ 1.5 mM, glycerol 25%, and DTT 1 mM) and were incubated on ice for 10 minutes. The suspension was then centrifuged for 45 minutes at 4°C and 13,000 rpm. The supernatants saved were crude nuclear extracts. The protein concentration of isolated cytoplasmic and nuclear fraction proteins was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA).

Fig. 2. The protein levels of secreted $\alpha 5(\text{IV})$ collagen in the cultured medium in podocytes were assessed by Western blot analysis. A: The levels of secreted $\alpha 5(\text{IV})$ collagen in the normal-glucose medium (NG group) did not change significantly. B: The levels of secreted $\alpha 5(\text{IV})$ collagen in the mannitol-added medium (MN group) did not change significantly. C: A high-glucose medium (HG group) induced a decrease in the level of secreted $\alpha 5(\text{IV})$ collagen at 2, 3, and 4 days of incubation, after which (on day 5) that value returned to its basal level. This is a representative of three independent experiments. Data are shown with the mean $\pm$ SEM.

Western blot analysis

After the addition of Laemmli reducing sample buffer, the precipitated medium proteins were heated at 95°C for 5 minutes and were then separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) 10% gel. Western blot analysis with anti- $\alpha 5(\text{IV})$ collagen antibody (Wieslab AB, Lund, Sweden) enabled the visualization of $\alpha 5(\text{IV})$ collagen.

Ten micrograms of cytoplasmic or nuclear protein extracts containing Laemmli reducing sample buffer per lane was subjected to electrophoresis in 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel. Ets-1, GAPDH, and histone H4 were visualized by Western blot analysis with monoclonal anti- $\alpha 5(\text{IV})$ collagen, monoclonal anti-GAPDH antibody (Bioscience, Saco, ME, USA), and antihistone H4 antibody

(Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively.

Whole-cells lysates were prepared in lysis buffer (Tris-Cl 50 mM [pH, 8.0], NaCl 150 mM, Nonidet P-40 1%, SDS 0.1%, and Triton-X 100 1%) containing protease inhibitors. The total protein concentration was determined by the Bradford assay. Equal amounts of protein in Laemmli reducing sample buffer were separated by SDS-PAGE 12%. Western blot analysis enabled the visualization of phospho-ERK1/2, ERK1/2, Ets-1 with anti-phospho-ERK1/2, anti-ERK1/2, and anti-Ets-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively.

The separated protein was electrophoretically transferred to Hybond-ECL nitrocellulose membranes at a constant 350 milliamperes for 2 hours. The membranes, which were blocked

Fig. 3. The levels of MMP-9 mRNA in podocytes cultured for 2 days or 5 days were analyzed by reverse transcription-PCR. The high-glucose (HG) medium incubated for 2 days stimulated MMP-9 mRNA expression, but in the HG medium incubated for 5 days, the level of MMP-9 mRNA returned to the basal level. Data are expressed as the mean $\pm$ SEM of three independent experiments.

with blocking buffer (5% nonfat dry milk) for 1 hour at room temperature, were then incubated overnight at 4°C with various primary antibodies. After extensive washing in PBS containing Tween-20 0.1%, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS at 1:6000 for 1 hour at room temperature. The membranes were then washed with PBS containing Tween-20 0.1%, and the signals were visualized by means of an enhanced chemiluminescence (ECL) system (PerkinElmer Life Sci, Boston, MA, USA).

Immunocytochemistry

Podocytes were planted and cultured on glass cover slides, and were harvested and washed three times with phosphate-buffered saline (PBS) and fixed with 100% methanol at -20°C for 10 min after different treatment, and then the cells were permeabilized with 0.1% Triton-X 100 in PBS at room temperature for 5 min, and 3% H₂O₂ was used to quench endogenous peroxidase within the cells. After blocking with 1% bovine serum albumin (BSA) for 30 min, the cells were incubated with the rabbit anti-Ets-1 antibody (1:100) overnight at 4°C. For staining the Histostain-Plus streptavidin-horseradish peroxidase (HRP) kit (Zymed Lab, Inc. South San Francisco, CA) was used with 3, 3'-diaminobenzidine (DAB) chromogen as substrate. After finishing up the staining procedures, the cells were sequentially dehydrated and mounted. As negative controls, primary antibody was omitted. The positive staining

signals were visualized using a Olympus BX51 microscope with high-power fields.

Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed with total RNA isolated from podocytes grown in the conditions described above with the Promega RNeasy kit (Promega, Madison, WI, USA). cDNA synthesis was performed as follows: Two micrograms of total RNA primed with random hexamers, reverse transcriptase buffer (Tris-HCl 50 mM [pH, 8.3], KCl 50 mM, MgCl₂ 10 mM, spermidine 0.5 mM, and DTT 10 mM) was denatured at 70°C for 5 minutes, and the reaction mixture was chilled on ice. AMV reverse transcriptase (Promega, Madison, WI, USA) 20 units, each deoxyribonucleoside triphosphate (dNTP) 2.5 mM, and RNasin 40 units were added to a total volume of 25 μ l and the mixture was incubated at 42°C for 1 hour. Five microliters of the cDNA was used in buffer system (MgCl₂ 1.5 mM, each dNTP 2 mM, Taq polymerase 1 unit, Tris-HCl 10 mM, KCl 50 mM, and Triton-X 100 0.1% [pH, 8.0]). A 433-bp cDNA fragment of the mouse MMP-9 gene and a 299 bp region of the GAPDH cDNA were amplified simultaneously in 30 cycles for 1 minute at each of the following parameters: 94°C denaturation, 55°C annealing, and 72°C chain extension. Primers for mouse MMP-9 were the following oligonucleotide sequences, which have been described previously [22]: forward: 5'-TTG AGT CCG GCA GAC AAT CC- 3' and reverse 5'-CCT TAT CCA CGC GAA TGA CG-

3'. GAPDH: forward: 5'-ACC CCC AAT GTA TCC GTT GT-3'; reverse: 5'-TAC TCC TTG GAG GCC ATG TA-3'. PCR products were run on 2% agarose gel containing ethidium bromide and visualized by KODAK Gel Logic 100 Imaging System and 1D Image Analysis Software. Values were quantitatively analyzed and normalized to the densities of GAPDH.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Comparison of the multiple groups was performed by one-way ANOVA followed by the Scheffe F test. A value of $P < 0.05$ was considered statistically significant.

Results

Long-term exposure to an HG culture conditions regulates the MMP-9 activity of podocytes

To evaluate the possible origin of enhanced MMP-9 in diabetic nephropathy, we first assessed the effects of long-term (10-day) HG culture on the MMP-9 activity of podocytes. Podocytes incubated with NG medium and MN medium expressed 72-kDa gelatinase (MMP-2) constitutively, but demonstrated relatively little 92-kDa gelatinase (MMP-9) activity and no changes in MMP-9 activity were observed along with the 10 days (Fig. 1A and 1B). Under HG conditions, podocytes expressed 72-kDa gelatinase (MMP-2) constitutively and demonstrated no change in activity during the 10-day study period. However, an HG environment induced prominent changes in MMP-9 activity. Short-term (2-day) incubation in an HG medium markedly enhanced MMP-9 activity, which reached a peak on day 3 ($144.2 \pm 18.7\%$ NG, $P = 0.006$) and then decreased, beginning on day 4, to the basal level on day 5 ($91.6 \pm 16.4\%$ NG, $P = 0.218$). The basal level remained constant through day 10 of the culture period (Fig. 1C).

Long-term exposure to an HG environment modulates supernatant collagen $\alpha 5(\text{IV})$ protein levels of podocytes

We next examined whether the glucose-induced modulation of MMP-9 activity was accompanied by changes in the expression of collagen $\alpha 5(\text{IV})$ in an HG medium incubated for 7 days. As showed in Figure 2A and 2B, in the medium of the NG and MN groups, high basal levels of collagen $\alpha 5(\text{IV})$ protein that remained relatively constant during the 7-day incubation. HG incubation had induced the adverse effect of a decrease in the collagen $\alpha 5(\text{IV})$ protein level by day 2 (Fig 2C). That decrease reached its minimum level on day 3 (41.9

$\pm 25.5\%$ NG, $P = 0.0047$), after which the level of collagen $\alpha 5(\text{IV})$ protein increased to the basal level by day 5 ($90.1 \pm 17.4\%$ NG, $P = 0.78$). That basal level remained unchanged at day 7 of the incubation period.

HG culture regulates the MMP-9 mRNA expression of podocytes

To determine whether an HG environment regulates the expression of MMP-9, the levels of MMP-9 mRNA in cells incubated in an HG medium for 2 days and 5 days were examined. Alterations in MMP-9 mRNA expression correlated with MMP-9 activity (Fig. 3). In an HG medium, the levels of MMP-9 mRNA were significantly upregulated in cells incubated for 2 days ($199.8 \pm 40.2\%$ control, $P = 0.003$) but had decreased by incubation day 5 to the basal level ($90.9 \pm 8.8\%$ control, $P = 0.411$).

An HG environment stimulates the activation of MAPK in podocytes

The MAPK cascade, including ERK1/2, JNK, and p38 MAPK, is integral to a variety of biologic processes relevant to diabetic nephropathy, including cell growth and differentiation and apoptosis. We next sought to identify the signaling of enhanced MMP-9 activity in podocytes. NG and MN medium incubation for 48 hours did not affect the ERK1/2 activation in podocytes (Fig. 4A and 4B). The phosphorylation of ERK1/2 occurred as early as 30 minutes after exposure to an HG medium, reached its peak at 6 hours, remained activation at 12 and 24 hours, and had decreased remarkably at 48 hours (Fig. 4C). In 3 separate experiments, we did not observe the activation of JNK and p38 MAPK by an HG environment (data not shown).

ERK1/2 inhibition affects MMP-9 activity and collagen $\alpha 5(\text{IV})$ protein levels

Because an HG environment increases ERK activity in podocytes, we tested the effects of ERK inhibition on HG-stimulated MMP-9 activity and collagen $\alpha 5(\text{IV})$ protein levels in medium from cultured podocytes. The cells were pretreated with the specific inhibitors of ERK1/2 kinase, 25 μM PD98059 (Calbiochem, La Jolla, CA, USA), for 30 minutes and were then incubated in an NG, MN, or HG medium, respectively, for 48 hours, after which the media were collected. ERK1/2 inhibition with PD98059 abolished the HG-induced increase in the MMP-9 activity of podocytes (HG $187.6 \pm 2.9\%$ NG, HG+PD98059 $91.6 \pm 10.6\%$ NG; $P = 0.002$ vs HG) (Fig. 5A), and attenuated the HG-induced decrease of collagen

Fig. 4. The effect of culture medium on the activation of ERK1/2 MAPK. A: The normal-glucose medium (NG group) did not affect ERK1/2 MAPK activity by 48hours. B: The mannitol-added medium (MN group) did not affect ERK1/2 MAPK activity by 48hours. C: A serum-free high-glucose (HG group) medium triggered the activation of ERK1/2 as early as 30 minutes; that elevated level was detected in the podocytes for 24 hours. These results are representative of three independent experiments.

$\alpha 5(\text{IV})$ protein (HG $53.8 \pm 10.63\%$ NG, HG+PD98059 $100.97 \pm 29.1\%$ NG; $P=0.002$ vs HG) (Fig. 5B).

HG culture regulates Ets-1 expression in podocytes

Because the activity of MMP-9 varies according to the level of glucose, the control of MMP-9 gene expression by the Ets-1 transcription factor is believed to be very important. We next sought to determine whether mouse podocytes express the Ets-1 transcription factor and can be regulated by an HG environment. Two-day incubation in an HG medium stimulated the expression of Ets-1 protein in podocyte whole-cell lysates ($180.8 \pm 33.1\%$ control, $P=0.001$), but on day 5 of HG incubation, the Ets-1 protein value had decreased to its basal level ($100.6 \pm 11.6\%$ NG, $P=0.351$) (Fig. 6).

ERK inhibition affects the expression and nuclear accumulation of Ets-1 protein and MMP-9 mRNA expression in podocytes

Because the results of this study demonstrate that the incubation of mouse podocytes in an HG medium for 1 hour induced the phosphorylation of ERK kinase, we sought to clarify the role of ERK activity in the nuclear translocation of the Ets-1 transcription factor. As described previously, cell pretreatment with PD98059 abolished the HG-stimulated increases in the expression of the Ets-1 protein (HG $197.4 \pm 11.9\%$ NG, HG+PD98059 $82.6 \pm 28.4\%$ NG; $P=0.002$ vs HG) (Fig. 7A), and the HG-induced nuclear translocation of Ets-1 (Fig. 7B). We also found PD98059 abrogated the HG-stimulated increases in nuclear accumulation of the Ets-1 protein analyzed by Western blotting (HG $205.5 \pm 23.3\%$ NG, HG+PD98059

Fig. 5. The effect of inhibition of ERK1/2 activation by PD98059 on MMP-9 activity and the $\alpha 5(\text{IV})$ collagen protein level in a high-glucose (HG) medium in which podocytes were cultured for 2 days. **A:** The inhibition of ERK1/2 activation by PD98059 significantly suppressed HG-induced MMP-9 activity. **B:** The

inhibition of ERK1/2 activation by PD98059 abolished the decrease in $\alpha 5(\text{IV})$ collagen protein as a result of exposure to a high-glucose (HG) medium. Data were shown with the mean $\pm$ SEM of three independent experiments.

Fig. 6. Western blot analysis of the protein levels of transcription factor Ets-1 in cultured podocytes at 2 or 5 days of incubation. Incubation in a high-glucose (HG) medium for 2 days induced the expression of Ets-1 protein, which had reverted to the basal level by day 5. This is a representative of three independent experiments. Data are shown with the mean $\pm$ SEM.

Fig. 7. The effect of ERK1/2 inhibition on Ets-1 and MMP-9 mRNA levels in podocytes cultured for 2 days. **A:** Inhibition of ERK1/2 activation can repress the high-glucose (HG)-stimulated expression of Ets-1 protein at 2 days of incubation. **B:** HG incubation induced nuclear translocation of Ets-1 in cells assessed by immunohistochemistry. **C:** Inhibition of ERK1/2

activation can reduce the HG-induced nuclear accumulation of Ets-1 analyzed by Western blotting. Cy: cytoplasmic protein, Nu: nuclear protein. **D:** Inhibition of ERK1/2 activation can abrogate HG-upregulated MMP-9 mRNA expression. Data are shown with the mean $\pm$ SEM of three independent experiments.

$133.3 \pm 20.2\%$ NG; $P = 0.001$ vs HG) (Fig. 7C), and the HG-upregulated expression of MMP-9 mRNA (HG $205.9 \pm 50.2\%$ NG, HG+PD98059 $113.2 \pm 24.2\%$ NG; $P = 0.006$ vs HG) (Fig. 7D).

Discussion

Tight control in the pattern and expression of MMP-2 and MMP-9 is thought to be important in maintaining the integrity of the GBM [23]. We used murine podocytes to study the role of HG concentration in the production of

gelatinase and the degradation of the major ECM components collagen IV. The results of this study, for the first time, demonstrated that an HG environment modulates the expression and activity of MMP-9 in cultured mouse podocytes. Short-term (2-day) exposure to an HG medium induced the production of MMP-9 but not MMP-2, and decrease in secreted $\alpha 5(\text{IV})$ collagen protein in the cultured medium. In the adult kidney, MMP-9 is expressed primarily in podocytes and in collecting duct cells [24-25]. Podocytes have been shown to secrete MMP-9 *in vitro* and *in vivo* under pathologic conditions [26-27]. Recently, elevated concentrations of plasma

MMP-9 were found in patients with type 2 diabetes before the onset of microalbuminuria [28], and an increase in the activity and mean level of urinary MMP-9 was reported in patients with type 1 diabetes [29] and in those with type 2 diabetic nephropathy [30]. Because local MMP-9 proteolytic activities are among the earliest processes in glomerular proteinuria [31], the increase in MMP-9 activity may contribute to the development of albuminuria caused by the structural and functional changes of GBM in early stage and podocytes detachment from GBM in diabetic nephropathy. In addition, we noted that in podocytes incubated in a high-glucose medium for longer than 5 days, the production of MMP-9 and secreted $\alpha 5(\text{IV})$ collagen protein declined and returned to their basal levels. This dynamic changes of MMP-9 activity confirms the theories that in diabetic rats, thickening of the GBM and the subsequent development of glomerulosclerosis are due to the reduction in MMP-9 activity caused by continuous hyperglycemic stimulation in the levels of MMP-9 mRNA and MMP-9 activity in the glomeruli [32, 33], but that the levels of MMP-2 mRNA and MMP-2 activity in glomeruli remain unchanged [32].

Mitogen-activated protein kinase (MAPK) has three subgroups: the extracellular signal-regulated protein kinase (ERK) family, the C-jun N-terminal kinase (JNK), and p38 MAPK, is a major signaling system that transduces extracellular signaling to intracellular responses [34]. In this study, we demonstrated that ERK1/2 MAPK activation is involved in glucose-induced MMP-9 expression and activity in podocytes. Glucose has also been reported to activate p38 MAPK in kidney cells [35], and an increase in MAPKs has been noted in the glomeruli of diabetic rats [36]. That finding suggests that hyperglycemia and MAPKs have a central role in the development of glomerular complications [37]. Recent data have demonstrated that the activation of ERK1/2 is critical for the increased expression of MMP-9 in response to different agonists [38, 39]. However, the mechanism of action by which the glucose-related activation of the MAPK kinase pathway results in the induction of MMP-9 in podocytes remains unclear. In this study, we identified the potential signaling of ERK1/2 as a major factor in the regulation of HG-induced expression and activity of MMP-9 in HG-cultured podocytes. Furthermore, the inhibition of HG-induced MMP-9 by the inhibition of ERK1/2 activation can prevent the level of $\alpha 5(\text{IV})$ collagen protein from decreasing.

The activity of MMPs is tightly regulated at the level

of gene transcription. Various stimuli (growth factors, cytokines, and mechanical stress) induce MMP gene expression. In addition, our study results indicated that glucose-induced changes in MMP-9 activity were reflected by the regulation of MMP-9 gene expression. Several functional transcriptional factors, including Ets-1, nuclear factor- κB , have been shown to regulate MMP-9 gene expression [40–41]. Our previous study together with the others findings have indicated that extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) are involved in the control of gene expression via the upregulation, phosphorylation and activation of the transcription factor Ets-1 [42, 43]. Although an HG environment has been showed to activate the MAPK pathway, very little is known about the regulation of Ets-1 by a high concentration of glucose or HG-induced MMP-9 expression via the pathway that activates Ets-1. We have demonstrated that an HG concentration stimulates the phosphorylation of ERK1/2, which is followed by an increase in the expression and nuclear accumulation of Ets-1. The inhibition of ERK1/2 activation can abolish the HG-induced increase in the levels of Ets-1 as well as MMP-9 mRNA upregulation. Our data suggest that Ets-1 is the nuclear target of the HG-activated ERK1/2 MAPK pathway that promotes MMP-9 gene expression.

In summary, we have demonstrated that levels of MMP-9 activity and secreted $\alpha 5(\text{IV})$ collagen are modulated by HG medium in podocytes. This indicates a novel mechanism by which hyperglycemia could adversely affect the remodeling of the GBM, which can lead to the development of diabetic nephropathy. Furthermore, the ability of glucose to affect the gene expression of MMP-9 results in part from changes in the activation of ERK1/2 MAPK and the transcriptional factor Ets-1. Therefore, it should be considered in the development of appropriate antagonists for the prevention and treatment of diabetic nephropathy.

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