

Original Article

Notch pathway mediates podocyte injury and extracellular matrix synthesis in diabetic nephropathy

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Received December 5, 2016; Accepted December 31, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: Background: It remains elusive that Notch pathway mediates podocyte injury and stimulates accumulation of extracellular matrix (ECM) in diabetic nephropathy (DN). This study is aimed at examining the effect of Notch pathway inhibited by γ -secretase inhibitor on podocyte injury and ECM synthesis in DN. Methods: We examined the expression of Notch1, Notch intracellular domain 1 (NICD1), nephrin, podocin, transforming growth factor- β 1 (TGF- β 1), type IV collagen and laminin in high glucose (HG)-induced podocytes and the kidney of diabetic mice using immunofluorescence, immunohistochemistry, Western blot and real-time PCR. The levels of TGF- β 1, type IV collagen and laminin were determined in the culture medium of the podocytes by enzyme-linked immunosorbent assay. Results: The Notch1, NICD1, TGF- β 1, type IV collagen and laminin expression increased and the nephrin and podocin expression decreased in HG-induced podocytes and the kidney of diabetic mice. Inhibition of Notch pathway increased nephrin and podocin expression, decreased TGF- β 1 level and prevented type IV collagen and laminin expression. Conclusions: These findings indicate that Notch pathway mediates podocyte injury and ECM synthesis in DN.

Keywords: Diabetic nephropathy, notch pathway, podocyte injury, extracellular matrix accumulation

Introduction

Notch pathway plays an important role in cells differentiation, acting primarily to determine and regulate cell survival [1-3]. The binding of a ligand and receptor induces a conformational change of the Notch receptor, which allows an extracellular metalloprotease to cleave the receptor and releases the Notch intracellular domain (NICD). Then, NICD travels into the nucleus where it activates the transcription of downstream genes [4].

Diabetic nephropathy (DN) is one of the most common complications of diabetes and has become a major cause of end-stage kidney disease [5, 6]. It is believed that dysfunction of the podocyte, a type of glomerular epithelial cell, plays a pivotal role in the onset of proteinuria and the progression of DN [7]. Previous studies have been revealed that high glucose (HG) in glomerular disease increases the synthesis of extracellular matrix (ECM) components, including collagens and laminin [8]. It is well established that transforming growth factor- β 1 (TGF- β 1) is a potent stimulator of

ECM production in glomerular injury and may be the most important growth factor in determining the extent of renal fibrosis after injury [9].

Niranjan et al. [10] reported that Notch pathway was activated in podocytes in humans with DN and in mice models thereof. We have also found that Notch pathway may mediate HG-induced podocytes apoptosis [11]. Here we hypothesize podocyte injury and production of ECM through Notch pathway in diabetic nephropathy. The activation of Notch pathway maybe change expression of nephrin, podocin, TGF- β 1 and ECM in HG-induced podocytes and the kidney of streptozotocin (STZ)-induced diabetic mice. Inhibition of Notch pathway activation can prevent podocyte injury, ECM accumulation and glomerulosclerosis.

Materials and methods

Cell culture

Conditionally immortalized mouse podocytes purchased from the Cell Resource Center (Pe-

king Union Medical College, Beijing, China). Podocytes were firstly cultured in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 10 U/ml γ -interferon (Peprotech, Rocky Hill, NJ, USA) in 33°C 5% CO₂ atmosphere to induce proliferation, then incubated in RPMI-1640 medium supplemented with 10% FBS and deprived of γ -interferon in 37°C 5% CO₂ atmosphere for 10-14 days to induce quiescence and the differentiated phenotype, as previously described [12]. Podocytes were grown to 80% confluence under growth restrictive conditions and growth-arrested in serum-free RPMI-1640 for 24 h to synchronize the cell growth. After this time period, the media were changed to fresh serum-free media containing normal glucose (NG, 5.5 mmol/l; Sigma, St-Louis, MO, USA) and HG (30 mmol/l; Sigma) at 48 h. We had found activation of Notch pathway in a time-dependent manner and the maximum expression of Notch pathway was at 48 h after stimulation of HG in podocytes [11]. For inhibitor studies, cells of HG plus γ -secretase inhibitor (GSI; Sigma) group need pretreatment with GSI (1 μ mol/l) for 30 min.

Experimental animals

Male CD-1 mice at five weeks of age and 20-25 g of body weight were purchased from the Vital River Laboratory Animal Technology (Beijing, China) and housed in a pathogen-free animal facility with free access to food and water. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University. Mice were injected i.p. with STZ (150 mg/kg; Sigma) dissolved in 0.1 M citrate buffer (pH 4.5) to induce diabetes mellitus (DM). The mice of control group were injected with an equivalent volume of saline (150 mg/kg). Three days after injection, DM was confirmed by a concentration of blood glucose higher than 16.7 mmol/l and urine glucose (+++) or above, which was determined using a glucometer (Johnson, USA). Mice in the DM plus γ -secretase inhibitor (DAPT; Sigma) group were administered intraperitoneally daily with DAPT (10 mg/kg) dissolved in dimethyl sulfoxide (DMSO, Sigma). Mice in the control group and DM group were only administered daily with the same volume of DMSO solution. We had found that the maximum expression of Notch pathway was at 4 week in diabetic mice. At 4 weeks

after treatment, 6 mice from each group were housed individually for 24 h in metabolic cages to collect mouse urine to measure 24-hour urinary albumin (using a Siemens IMMULITE 1000 chemistry analyzer, Munich, Germany). These sera were prepared for the measurement of serum blood glucose. Both kidneys were dissected and weighed. The left kidney was fixed in 4% paraformaldehyde in 0.01 mol/l phosphate-buffered saline for histological examination and immunohistochemical staining. The cortex of right kidney was used for Western blot and real-time PCR analyses.

Immunofluorescence

Podocytes were planted on cover slides in 6-well plates. Cells were fixed with 10% formalin at room temperature for 15 min. After pretreatment of 0.3% Triton X-100 for 20 min at 37°C, cells were blocked with goat serum for 30 min at 37°C. Cells were incubated with rabbit anti-nephrin (1:50, Santa Cruz Biotechnology, CA, USA) and podocin (1:100, Santa Cruz) polyclonal antibody overnight at 4°C. After washing with phosphate buffer saline (PBS) for three times slides were incubated with FITC-conjugated goat anti-rabbit secondary antibody (Santa Cruz) for 2 h at 37°C. Then slides were observed after being rinsed with PBS for three times.

Histology

Paraffin-embedded renal tissue sections (4 μ m) were prepared and stained with periodic acid-Schiff (PAS). Renal cortical sections were observed using light microscopy with a computer image analysis system.

Immunohistochemistry

Paraffin-embedded renal tissue sections (4 μ m) were deparaffinized with xylene and rehydrated in graded ethanol solutions. Endogenous horseradish peroxidase activity was blocked by pretreatment with 3% H₂O₂ for 10 min at room temperature. Antigen recovery was performed using a microwave. To block nonspecific binding, the sections were incubated at 37°C for 30 min in PBS containing 10% goat serum. Finally, the sections were incubated with rabbit polyclonal antibodies against nephrin (1:50, Santa Cruz) and podocin (1:50, Santa Cruz), overnight at 4°C. On the next day, af-

Table 1. Changes in basic parameters in the control, DM and DM+DAPT groups

Group	BG (mmol/L)	UAE (mg/24 h)	KW/BW (mg/g)
Control	7.38±1.12	0.35±0.09	9.37±0.93
DM	28.73±7.65**	4.14±0.76**	17.83±2.74**
DM+DAPT	29.43±7.66**	3.21±0.58#	15.23±1.82#

**P<0.01 vs. control group. #P<0.05 vs. DM group. BG, blood glucose; UAE, urinary albumin excretion; KW, kidney weight; BW, body weight.

ter incubation with the PV-9000 Polymer Detection System (Zhongshan Golden Bridge Biotechnology, Beijing, China), the sections were stained with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin. Negative controls were obtained by replacing the primary antibody with PBS.

Western blot

Total protein extracts from cells and animal tissues. Protein was loaded and separated on SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA). The membrane was blocked with 5% dry milk and incubated overnight at 4°C with rabbit anti-Notch1 (1:200, Abcam, Cambridge, MA, USA), NICD1 (1:200, Abcam), nephrin (1:400, Santa Cruz), podocin (1:1000, Santa Cruz), TGF-β1 (1:1000, Proteintech, Chicago, IL, USA), type IV collagen (1:1000, Proteintech), laminin (1:1000, Proteintech) and β-actin (1:1000, Santa Cruz) polyclonal antibodies. After washing, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Proteintech). Proteins in Western blot were quantified following acquisition and analysis of the image using the software of a UVP Image Station Lab works 4.5. Proteins expression was quantified by comparison with internal-control β-actin.

Real-time PCR

Total RNA was extracted from podocytes with TRIzol reagent (Invitrogen) and extracted from the animal tissues using the SV-total RNA isolation system (Promega, Madison, WI) according to the instructions of the manufacturer and reverse transcribed using oligo (dT) primer in the presence of avian myeloblastosis virus reverse transcriptase to make cDNA. cDNA was amplified on the 7900HT Sequence Detection

System (Applied Biosystems) at default thermal cycling conditions: 2 min at 50°C, 10 min at 95°C for enzyme activation and then 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension. Results were analyzed using the relative standard curve method of analysis/ΔC_t method of analysis. Two oligonucleotide primers were: 18S, forward 5'-CGC CGC TAG AGG TGA AAT TC-3' and reverse 5'-CCA GTC GGC ATC GTT TAT GG-3'; Notch1, forward 5'-GTG GAT GAC CTA GGC AAG TCG-3' and reverse 5'-GTC TCC TCC TTG TTG TTC TGC A-3'; nephrin, forward 5'-TAC CAC CAG CAT TTC CAC G-3' and reverse 5'-GGG CTC GGC TGT ATG TAT T-3'; podocin, forward 5'-GTG TCC AAA GCC ATC CAG TT-3' and reverse 5'-GAC CTT TCC TTC TCG TAA CG-3'; TGF-β1, forward 5'-ACC GCA ACA ACG CAA TCT ATG-3' and reverse 5'-ATT CCG TCT CCT TGG TTC AG-3'; type IV collagen, forward 5'-GTC AAA CTA CTG CTA TCC CTC CGT GTC-3' and reverse 5'-CAT TCT ATA AAT GGA CTG GCT CGG AAT-3'; laminin, forward 5'-CCT GCC AAA TTC CTC GGT AAC-3' and reverse 5'-ACA TCG TAG GCA GAC GGC TG-3'.

Enzyme-linked immunosorbent assay

After the cells were cultured in 6-well plates under the different experimental conditions, the supernatants were collected. The TGF-β1, type IV collagen and laminin protein was quantified using a commercial quantikine enzyme-linked immunosorbent assay kit (ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's descriptions.

Statistical analysis

Data presented as bar graphs are the means ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance by SPSS13.0 software (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant at P<0.05.

Results

Basic parameters and morphological changes

As shown in **Table 1**, the blood glucose (BG) and urinary albumin excretion (UAE) levels were dramatically higher in the DM group com-

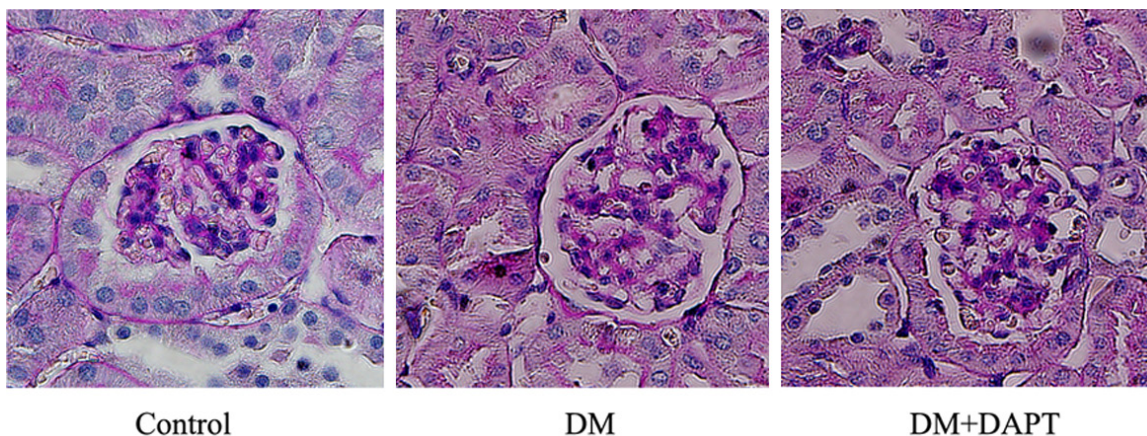


Figure 1. Morphological changes in glomeruli. Renal tissue sections obtained from the mice of the control, DM and DM+DAPT groups were stained with PAS (×400).

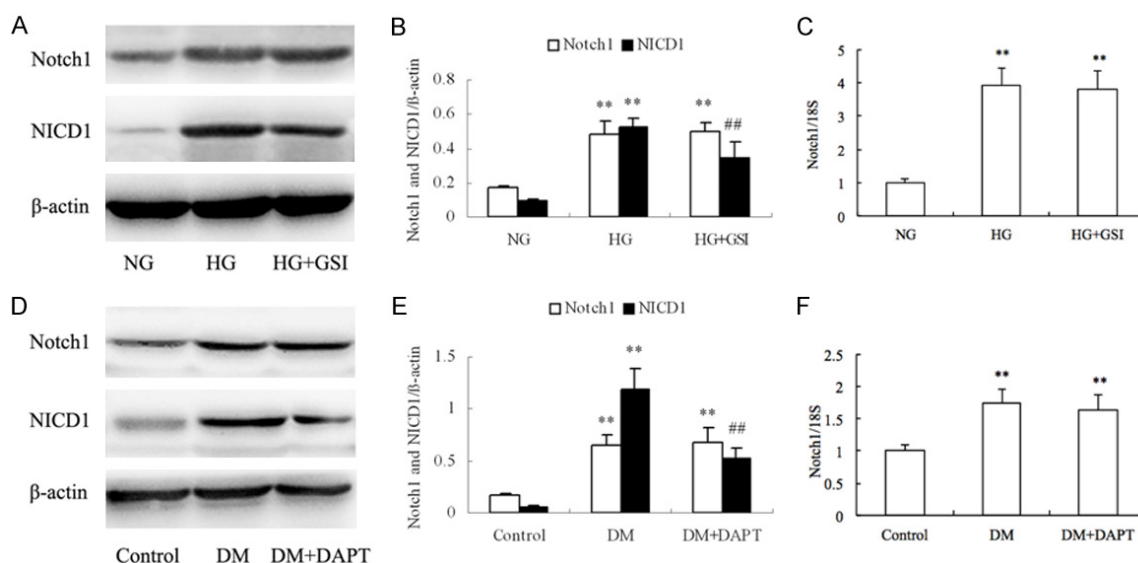


Figure 2. Effect of Notch pathway inhibition on expression of Notch pathway in HG-induced podocytes and the kidney of diabetic mice. The protein expression of Notch1 and NICD1 in HG-induced podocytes was analyzed by Western blot (A). The protein levels of Notch1 and NICD1 in HG-induced podocytes were quantified by densitometry (B). The Notch1 mRNA level in HG-induced podocytes was analyzed by real-time PCR (C). The protein expression of Notch1 and NICD1 in diabetic mice was analyzed by Western blot (D). The protein levels of Notch1 and NICD1 in diabetic mice were quantified by densitometry (E). The Notch1 mRNA level in diabetic mice was analyzed by real-time PCR (F). Protein expression was normalized to β -actin. mRNA expression was normalized to 18S. Data are presented as the means \pm SD. ** $P < 0.01$ vs. NG or control group, ### $P < 0.01$ vs. HG or DM group.

pared to the control group ($P < 0.01$). DAPT did not modulate hyperglycemia because BG concentrations were similar in diabetic mice ($P > 0.05$). However, the amount of UAE in the DM+DAPT group was significantly lower compared to the DM group ($P < 0.05$). The size of the kidney, which was evaluated by the ratio of kidney/body weight (KW/BW), increased in the DM group compared to the control group; tre-

atment with DAPT was associated with a significant reduction in the KW/BW ratio ($P < 0.05$ or $P < 0.01$). The pathological changes of glomeruli were not observed in the control group. However, the basement membrane was thickened and the mesangial matrix was increased in the DM group. DAPT significantly attenuated the pathological changes of glomeruli compared to the DM group (Figure 1).

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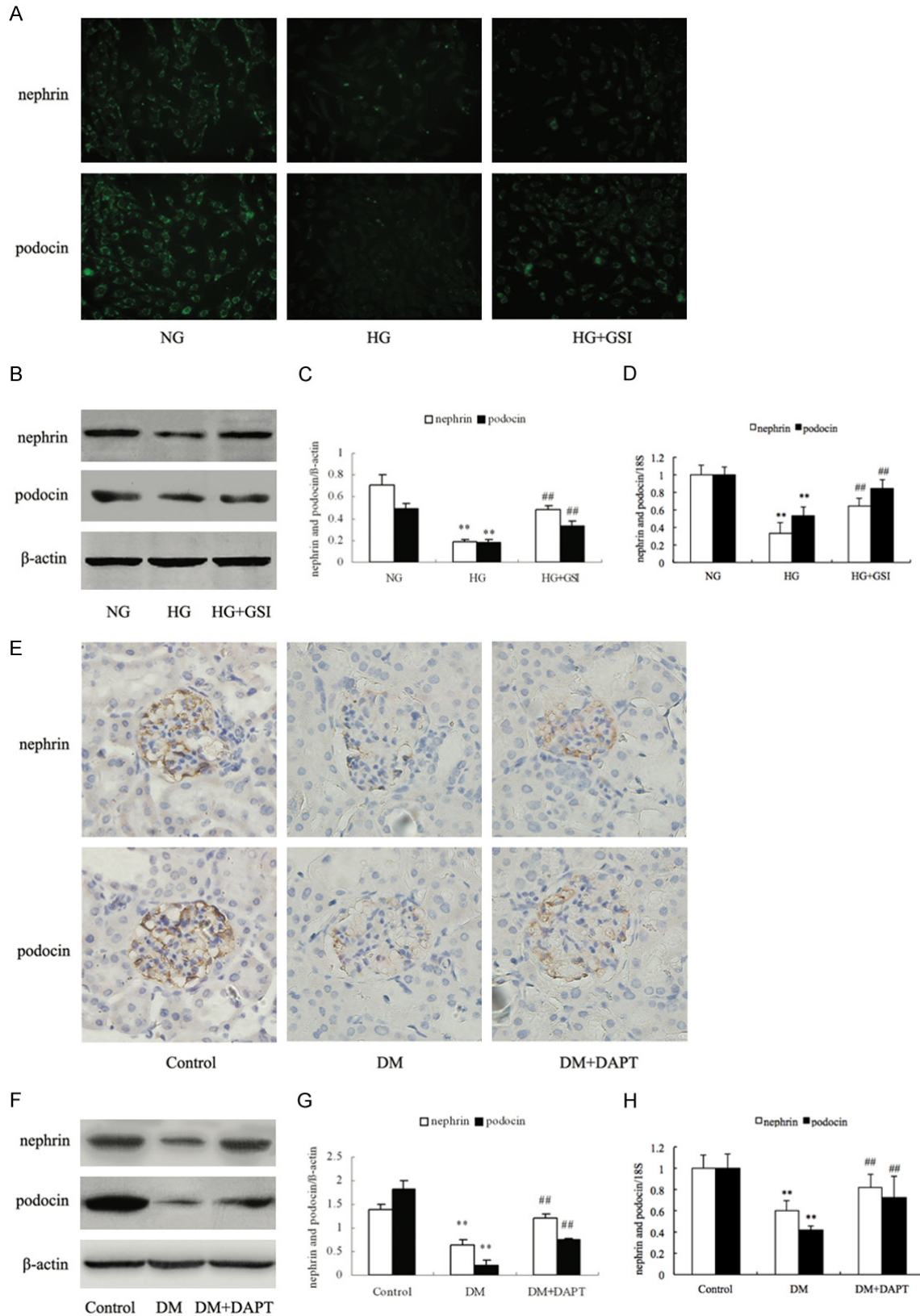


Figure 3. Effect of Notch pathway inhibition on expression of nephrin and podocin in HG-induced podocytes and the kidney of diabetic mice. The protein expression of nephrin and podocin in HG-induced podocytes was showed by immunofluorescence ($\times 400$) (A). The protein expression of nephrin and podocin in HG-induced podocytes was

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analyzed by Western blot (B). The protein levels of nephrin and podocin in HG-induced podocytes were quantified by densitometry (C). The mRNA levels of nephrin and podocin in HG-induced podocytes were analyzed by real-time PCR (D). The protein expression of nephrin and podocin in diabetic mice was showed by immunohistochemistry ($\times 400$) (E). The protein expression of nephrin and podocin in diabetic mice was analyzed by Western blot (F). The protein levels of nephrin and podocin in diabetic mice were quantified by densitometry (G). The mRNA levels of nephrin and podocin in diabetic mice were analyzed by real-time PCR (H). Protein expression was normalized to β -actin. mRNA expression was normalized to 18S. Data are presented as the means \pm SD. $**P < 0.01$ vs. NG or control group, $##P < 0.01$ vs. HG or DM group.

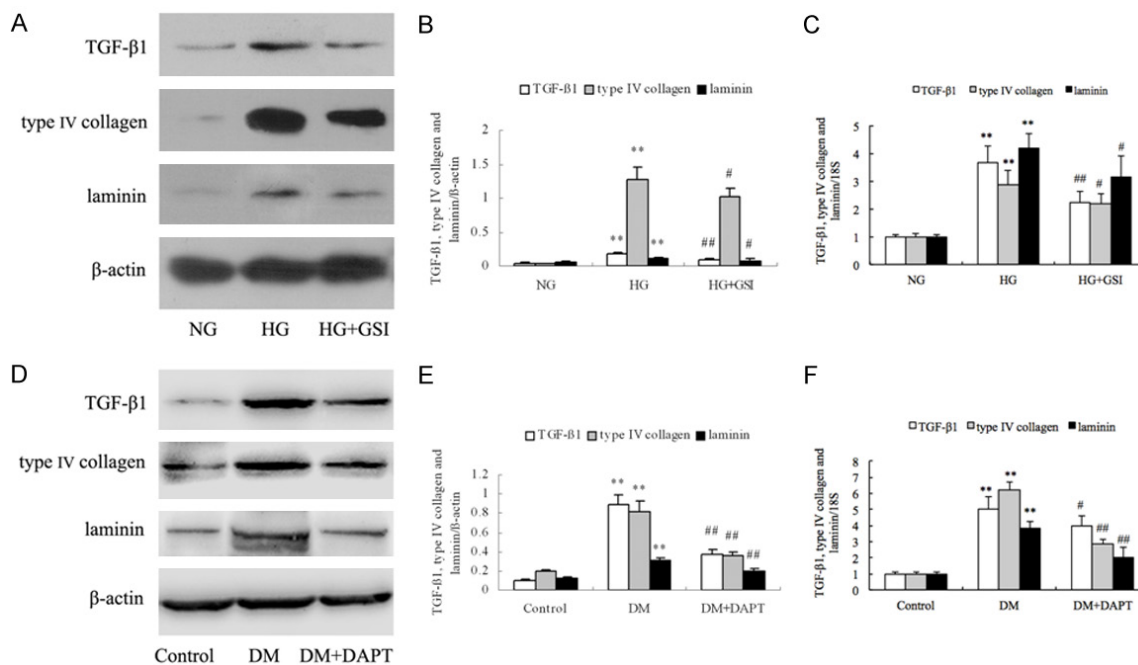


Figure 4. Effect of Notch pathway inhibition on expression of TGF- β 1, type IV collagen and laminin in HG-induced podocytes and the kidney of diabetic mice. The protein expression of TGF- β 1, type IV collagen and laminin in HG-induced podocytes was analyzed by Western blot (A). The protein levels of TGF- β 1, type IV collagen and laminin in HG-induced podocytes were quantified by densitometry (B). The mRNA levels of TGF- β 1, type IV collagen and laminin in HG-induced podocytes were analyzed by real-time PCR (C). The protein expression of TGF- β 1, type IV collagen and laminin in diabetic mice was analyzed by Western blot (D). The protein levels of TGF- β 1, type IV collagen and laminin in diabetic mice were quantified by densitometry (E). The mRNA levels of TGF- β 1, type IV collagen and laminin in diabetic mice were analyzed by real-time PCR (F). Protein expression was normalized to β -actin. mRNA expression was normalized to 18S. Data are presented as the means \pm SD. $**P < 0.01$ vs. NG or control group, $#P < 0.05$, $##P < 0.01$ vs. HG or DM group.

Notch pathway inhibition increases nephrin and podocin expression in HG-induced podocytes and the kidney of diabetic mice

Western blot and real-time PCR analyses revealed that GSI or DAPT did not inhibit Notch1 protein and mRNA overexpression in HG-induced podocytes and the kidney of diabetic mice ($P > 0.05$) (Figure 2). When podocytes were incubated with HG, HG markedly increased NICD1 protein expression; However, GSI inhibited HG-induced the NICD1 protein level ($P < 0.01$) (Figure 2A and 2B). Compared with the kidney of the control group, NICD1 protein level

significantly increased in the DM group ($P < 0.01$). DAPT decreased the protein overexpression of NICD1 in diabetic mice ($P < 0.01$) (Figure 2D and 2E). Figure 3A showed the results of indirect immunofluorescence studies on podocytes by using polyclonal rabbit antiserum against nephrin and podocin. The protein expression of nephrin and podocin was located in cytoplasm of podocytes and cells stimulated with HG showed low blue fluorescence that was enhanced with GSI treatment. The protein levels of nephrin and podocin were markedly lower in podocytes stimulated with HG than the cells treated with NG and were dramatically

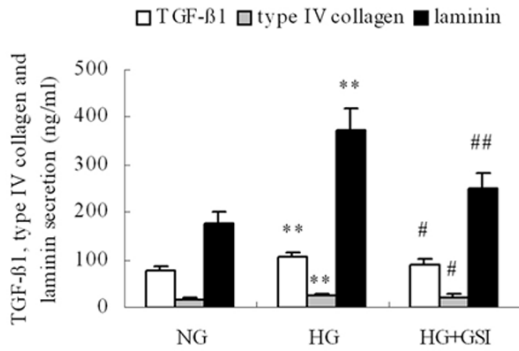


Figure 5. Effect of Notch pathway inhibition on concentration of the TGF-β1, type IV collagen and laminin in the culture medium of HG-induced podocytes by ELISA. Data are presented as the means ± SD. ** $P < 0.01$ vs. NG, # $P < 0.05$, ## $P < 0.01$ vs. HG.

increased in response to GSI ($P < 0.01$) (Figure 3B and 3C). Real-time PCR showed the similar changes of nephrin and podocin mRNA after treatment with GSI ($P < 0.01$) (Figure 3D). Immunohistochemical staining (Figure 3E) revealed that nephrin and podocin protein expression was detected in the glomeruli, which protein expression was decreased in the glomeruli of the DM group compared to the control group; in the DM+DAPT group, the nephrin and podocin protein expression was significantly stronger in the glomeruli than the DM group. Using Western blot (Figure 3F and 3G), the protein levels of nephrin and podocin were markedly lower in the kidney of diabetic mice than that in the control mice and were dramatically increased in response to DAPT ($P < 0.01$). Real-time PCR showed similar changes in nephrin and podocin mRNA in the kidney of diabetic mice after treatment with DAPT ($P < 0.01$) (Figure 3H).

Notch pathway inhibition decreases ECM production in HG-induced podocytes and the kidney of diabetic mice

As shown in Figure 4A-C, incubation with HG resulted in a significant up-regulation in TGF-β1, type IV collagen and laminin protein and mRNA expression than the cells in NG ($P < 0.01$). However, the alternations of TGF-β1, type IV collagen and laminin levels in HG-induced podocytes were reversed by addition of GSI in HG culture medium ($P < 0.05$ or $P < 0.01$). TGF-β1, type IV collagen and laminin protein and mRNA levels in the kidney of diabetic mice increased than that in the control mice

($P < 0.01$) (Figure 4D-F). Compared with the DM group, TGF-β1, type IV collagen and laminin protein and mRNA levels significantly decreased in the kidney of diabetic mice treated with DAPT ($P < 0.05$ or $P < 0.01$). The concentration of the TGF-β1, type IV collagen and laminin was examined in the culture medium of the podocytes with ELISA (Figure 5). We found that the podocytes exposed to HG showed higher levels of TGF-β1, type IV collagen and laminin in supernatants than those cultured under NG ($P < 0.01$). Compared to HG-induced overexpression of TGF-β1, type IV collagen and laminin in supernatants, the expression of TGF-β1, type IV collagen and laminin decreased significantly after treatment of GSI ($P < 0.05$ or $P < 0.01$).

Discussion

Notch pathway is an evolutionarily conserved local cell-signaling mechanism that participates in a variety of cellular processes and plays an important role in glomerular development and podocyte injury of DN [10, 13, 14]. In this study, we found that HG induced podocyte injury and increased ECM synthesis via activation of Notch pathway. Notch pathway also took part in podocyte injury and increased ECM synthesis in the kidney of diabetic mice.

The slit-diaphragm (SD) is an important structure between adjacent foot processes, which is a major component of the protein barrier between the circulation and Bowman's space [15]. Nephrin localized to the SD was originally thought to provide the structural link between foot processes, without which it is not possible to maintain the foot process structure [16]. Podocin interacted with nephrin also be identified as important components of the SD [17]. Both of nephrin and podocin appear to be present in a complex within lipid rafts and are associated with the cytoskeleton [18, 19], which are reduced in human podocytes of DN [20]. We found that the levels of nephrin and podocin were markedly lower in podocytes stimulated with HG. Notch pathway inhibition using GSI increased nephrin and podocin expression in podocytes, which showed HG changed nephrin and podocin levels and induced podocytes injury via Notch pathway. Notch pathway was also activated in podocyte in diabetic mice, which was a major molecular mechanism of decreased nephrin and podocin expression [21].

ECM accumulation of glomeruli is considered the most common destructive pathway associated with DN, which is characterized by remodeling of the interstitial ECM, resulting in excessive deposition of ECM including type IV collagen and laminin [22]. It is well established that TGF- β 1 is a potent stimulator of ECM production in DN and may be the most important growth factor in determining the extent of renal fibrosis after injury. It has been shown that TGF- β 1 mediates the production of type IV collagen and laminin in mesangial cells under conditions of HG [23]. Exposure of podocytes to TGF- β 1 also increased the production of the basement membrane components [24]. We found that the exposure of podocytes to HG induced type IV collagen and laminin accumulation by increasing TGF- β 1 mRNA and protein synthesis. Furthermore, the finding that Notch pathway inhibition by GSI markedly prevented HG-induced TGF- β 1 upregulation suggested that HG induced TGF- β 1 expression via Notch pathway. Aoyagi-Ikeda et al. [25] found that Notch pathway induced myofibroblast differentiation through TGF- β 1 that raised SMA expression in alveolar epithelial cells, and increased migratory behavior in pulmonary fibrosis. Blocking Notch pathway activation significantly attenuated liver fibrosis and decreased the expression of TGF- β 1 in hepatic stellate cell line [26]. We also found that TGF- β 1, type IV collagen and laminin protein and mRNA levels in diabetic mice increased than that in the control mice. Inhibition of Notch pathway by DAPT significantly decreased TGF- β 1, type IV collagen and laminin protein and mRNA levels, reduced proteinuria and prevented glomerulosclerosis in diabetic mice.

In summary, our data demonstrated that activation of Notch pathway was found in HG-induced podocytes and the kidney of diabetic mice, which decreased nephrin and podocin, increased TGF- β 1 and the synthesis of ECM components in podocytes. In addition, the blockade of Notch pathway using the chemical inhibitor suppressed podocyte injury and the synthesis of ECM components in podocytes of DN. Since Notch pathway is involved in podocyte injury in DN, targeting Notch pathway may be an effective method to therapy DN.

Acknowledgements

This study was supported by grants from Hebei Natural Science Foundation of China (H20-

14206294) and Department of Education of Hebei Province of China (QN2016014).

Disclosure of conflict of interest

None.

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