Pigment epithelium-derived factor inhibits high glucose-induced JAK/STAT signalling pathway activation in human glomerular mesangial cells

Tuohua Mao, MBBS, MS, *Hongmin Chen,* MBBS, MS, *Lian Hong,* MBBS, MS, *Jing Li,* MBBS, PhD.

ABSTRACT

الأهداف: تفسير آلية دور مكافحة التليف لدى ظهارة عامل الصباغ المشتق على اعتلال الكلية السكري.

الطريقة: تم علاج خلايا مسراق الكبيبة البشرية بجرعة 30 ماموول لكل لتر جلوكوز في أوقات مختلفة (الساعة 6، 12، 24، و48 ساعة). لدراسة أثر ظهارة عامل الصباغ المشتق قمنا بتحريض خلايا مسراق الكبيبة بتركيز جلوكوز عالي (30mmol/L) بوجود تركيزات مختلفة من ظهارة عامل الصباغ المشتق 10, 40، و 100 نانومول/لتر لمدة 24 ساعة. أجريت الدراسة في مختبر الغدد، مستشفى جامعة وهان، وهان الصين خلال الفترة من يوليو 2012م وديسمبر 2012م. تم قياس عامل النمو المتحول و فيبرونكتين باستخدام المقايسة المناعية. كما تم فحص البروتين المخلق عامل النمو المتحول و فيبرونكتين في مزرعة خلايا مسراق الكبيبة والمقايسة المناعية. كما تم قياس محولات إشارة ومستوى الفسفور لكيناز وSTAT1 باستخدام لطخة ويسترن.

النتائج: أن التعرض لخلايا مسراق الكبيبة لجلوكوز 30 نانومول/لتر يسبب نشاط JAK2 و STAT1. كما أنه يزيد من تعبير TGF–ß1 ويرفع من البروتين المخلق. كما أن ارتفاع الجلوكوز ينتج تغيرات محجوبة بواسطة PEDF.

خاتمة: يقلل عامل الصباغ المشتق من تعبير TGF–ß1 و FN من خلال منع فسفرة JAK2 و STAT1 والذي يعتبر طريقة واعدة لعلاج اعتلال الكلية السكري.

Objectives: To further elucidate the mechanism of the anti-fibrogenic role of pigment epithelium-derived factor (PEDF) on diabetic nephropathy.

Methods: Human glomerular mesangial cells (HMCs) were treated with 30mmol/l D-glucose for different time intervals (6, 12, 24, and 48 hrs). To examine the beneficial effect of PEDF, we incubated the HMCs with high glucose (30mmol/L) in the presence of different concentrations of PEDF (10, 40, and 100nmol/l) for 24 hrs. The study took place in the Laboratory of Endocrinology, Renmin Hospital of Wuhan University, Wuhan, China between July 2012 and December 2012. Transforming growth factor-beta1 (TGF-ß1) and fibronectin (FN) mRNA was measured by reverse transcription-polymerase chain reaction (RT-PCR). The protein synthesis of TGF-ß1 and FN in the culture medium of HMC was detected by enzyme-linked immunosorbent assay. The phosphorylation levels of Janus kinase2 (JAK2) and signal transducers and activators of transcription1 (STAT1) were measured using western blotting.

Results: The exposure of HMCs to 30 mmol/L glucose caused the activation of JAK2 and STAT1. It upregulated TGF-ß1 expression and increased protein synthesis of FN. These high glucose-induced changes were suppressed by PEDF.

Conclusion: The PEDF can decrease the expression of TGF-ß1 and FN, possibly by inhibiting the phosphorylation of JAK/STAT, which may offer a promising strategy in the treatment of diabetic nephropathy.

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From the Department of Endocrinology, Renmin Hospital of Wuhan University, Wuhan, China.

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Address correspondence and reprint request to: Dr. Jing Li, Department of Endocrinology, Renmin Hospital of Wuhan University, Jiefang RD 238, Wuhan 430060, China. Tel. +86 (139) 07114440. Fax. +86 (027) 88042292. E-mail: lijing7823@medmail.com.cn

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Among the diabetic microvascular complications, diabetic nephropathy (DN) is the most common complication and the leading cause of end-stage renal disease. Diabetic nephropathy is characterized by the enlargement of the glomerular mesangium due to the accumulation of extracellular matrix (ECM) proteins, synthesized by the mesangial cells. Although the mechanisms of DN are incompletely understood, high glucose is presumed as an initiating factor. Hyperglycemia leads to increased levels of transforming growth factor-β (TGF-β) and increased production of ECM proteins, such as fibronectin (FN).[1-3] This increased production in glomerular mesangial cells has been implicated in the development of DN.[1-3] In recent years, accumulating evidence demonstrates that Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling cascades contribute to DN.[3-7] This pathway is mainly related to renal cell growth, production of the cytokine TGF-ß, as well as the ECM proteins collagen IV and FN.[8] In mammals, the JAK family consists of 4 members: JAK1, JAK2, JAK3, and receptor tyrosine kinase-2. Of all the JAK/STAT pathways, high glucose stimulates TGF-ß and FN production in glomerular mesangial cells in a JAK2 -STAT1 dependent manner.[5] Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the super family of serine protease inhibitors which assumed beneficial effects on diabetic retinopathy by acting as an endogenous antioxidant,[9-14] although it was first purified from the conditioned media of human retinal pigment epithelial cells as a factor that possesses potent neuronal differentiating activity in human retinoblastoma cells. Previous studies have shown that PEDF is a multifunctional protein with demonstrable neurotrophic, antitumorigenic, anti-angiogenic, anti-atherogenic, and anti-vasopermeability activities.[15-20] A few studies have shown that the PEDF is implicated in the pathogenesis of DN.[21-26] We have found that PEDF is an endogenous anti-oxidative and anti-fibrogenic factor in the kidney, it significantly inhibited the overexpression of TGF-ß1 and ECM proteins (fibronectin and collagen IV) induced by the elevated glucose in human glomerular mesangial cells (HMCs). Pigment epithelium-derived factor also impeded high glucose-induced reactive oxygen species (ROS) generation in HMCs.[21] Although PEDF inhibited the expression of TGF-ß1 and FN in the diabetic kidney, the protective effect of PEDF in diabetic kidney and its mechanism of action have not been demonstrated. Based on the anti-fibrogenic activities of PEDF and the important role of activation of JAK and STAT proteins in the synthesis of ECM molecules, it is logical to hypothesize that PEDF protects the renal structure and function from diabetic injury via inhibiting high glucose-induced JAK/STAT signalling pathway activation. The purpose of study is to further elucidate the mechanism of the anti-fibrogenic role of PEDF on DN.

Methods. The human glomerular mesangial cells were purchased from Xiangya Central Laboratory of Central South University, Changsha, China. The Ethics Committee of Renmin Hospital of Wuhan University confirmed that the ethics approval was not needed. The study is according to the principles of Helsinki Declaration.

Cell culture. Human glomerular mesangial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing glucose (5.6 mmol/L) with 15% fetal bovine serum (FBS), 100μg/ml streptomycin, 100U/ml penicillin, 2mmol/l glutamine at 37°C in a humidified 5% CO2 atmosphere. Cells of passages from 3-6 were used in the experiments. After reaching 80% confluence, the cells were quiescent with medium containing no FBS for 12 hr and then exposed to high glucose (30mmol/L) for different time (6 hr, 12 hr, 24 hr and 48 hr) or in the presence of different concentrations (10, 40, and 100nmol/l) of recombinant human PEDF (Peprotech, Princeton, USA).

Determination of the mRNA levels of TGF-ß1 and FN by reverse transcription-polymerase chain reaction (RT-PCR). The total RNA was extracted from cultured HMC cells using Trizol reagent (Invitrogen Inc, Carlsbad, USA) according to the manufacturer's protocol. Then, the complementary DNA was prepared using the RT-PCR kits (Fermentas, Shenzhen, China). The TGF-ß1 PCR was carried out for 35 cycles according to the following procedure: 94°C for 30 sec, 58°C for 30 sec, and 72°C for 50 sec. The FN PCR was carried out for 40 cycles according to the following procedure: 94°C for 30 sec, 60°C for 30 sec, and 72°C for 50 sec. Primers specific for TGF-ß1 (5′-GGTGGAAACCCACAACGAA-3′; 3′-CTAAGGCGAAA GCCCTCAAT-5′), FN (5′-TAGCCCTGTCCAGGAGTTCA-3′; 3′-CTGCAAGCCTTCAATAGTCA-5′), GADPH (5′-ACCACAGTCCATGCCATCAC-3′; 3′-TCCACCACCCTGTTGCTGTA-5′) were used for PCR. The PCR products were subjected to 1.5% agarose gel electrophoresis and analyzed. The RNA expression was quantified by comparison with internal-control GADPH.

Measurements of TGF-ß1 and FN by enzyme-linked immunosorbent assay (ELISA). The protein levels of TGF-ß1 and FN in cultured cells were quantified spectrophotometrically at a wavelength of 450nm using the TGF-ß1 ELISA kit (Boster, Wuhan, China) and the FN ELISA kit (USCNLIFE, Wuhan, China) respectively, according to the protocols of the manufacturers.[21] The sensitivity of the TGF-ß1 ELISA kit was 1pg/ml, and the detection range was 15.6-1000 pg/ml. The sensitivity of the FN ELISA kit was 0.039ng/ml, and the detection range was 0.156-10ng/ml.

Western blotting studies of p-JAK2 and p-STAT1 proteins. Human glomerular mesangial cells were lysed in RIPA buffer (1% Na-deoxycholate, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.2). Subsequently, samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Beyotime Institute of Biotechnology, Haimen, China) and blocked by a 60-minute incubation at room temperature (22°C) in Tris Buffered Saline with with 0.05% Tween 20 (TTBS) (pH 7.4) plus 5% skimmed milk powder. The nitrocellulose membrane was incubated overnight at 4°C with antiphosphotyrosine-specific JAK2 and STAT1 antibodies (Cell Signaling Technology, Beverly, MA) or anti-beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA) for protein loading control. Subsequently, the nitrocellulose membranes were washed triple for 10 min each with TTBS and incubated for various times with goat anti-rabbit IgG horseradish peroxidase conjugate. After extensive washing, the bound antibody was identified by enhanced chemiluminescence (ECL kit, Beyotime Institute of Biotechnology, Haimen, China) according to manufacturer's instructions.

Statistical analysis. Data were calculated and expressed as means ± SD. Statistical analysis used wasStudent's t test and Analysis of Variance with post hoc test. Statistical difference was considered significant at a $p \leq 0.05$. The statistical software of SPSS Version 16.0 was used.

Results. ***The effect of high glucose on the mRNA and protein levels of TGF-ß1 and FN.*** Using RT-PCR analysis, we investigated the time course of TGF-ß1 and FN mRNA expressions in cultured HMCs treated with 30mmol/l D-glucose. As shown in **Figure 1**, high glucose significantly upregulated TGF-ß1 mRNA expression from 12-48 hr (p=0.000), and it increased FN mRNA expression from 24-48 hr (p=0.000). We measured the protein expressions of TGF-ß1 and FN using ELISA. As shown in **Table 1**, high glucose significantly upregulated TGF-ß1 and FN protein expressions from 24-48 hr (p=0.000).

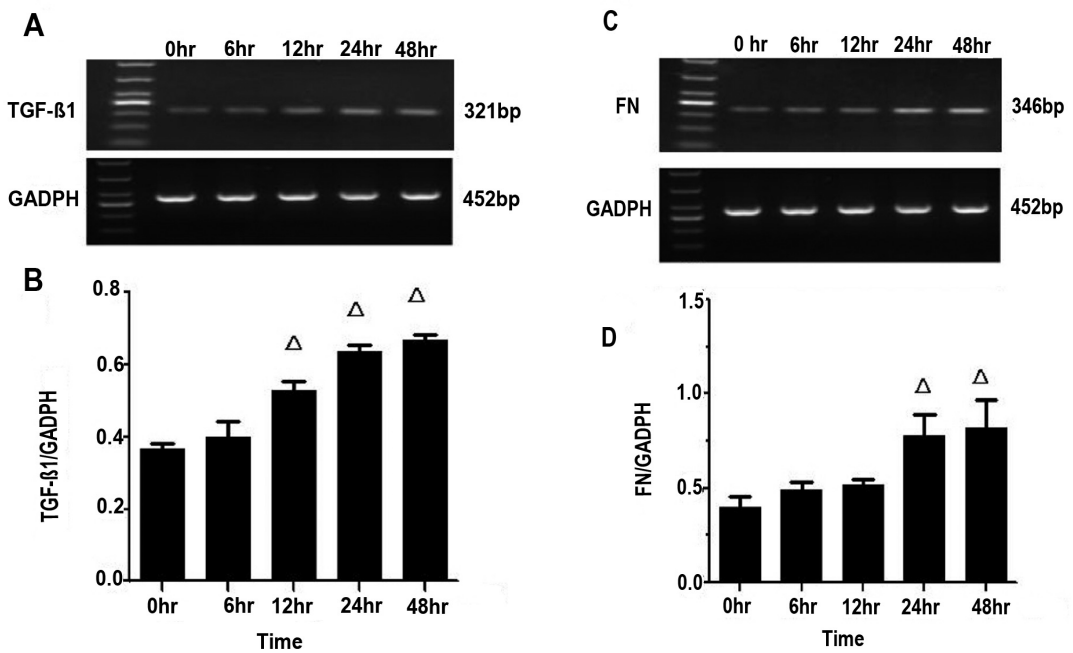

Figure 1 - High glucose (HG) induces transforming growth factor-β1 (TGF-β1) and fibronectin (FN) messenger ribonucleic acid (mRNA) expressions in cultured human glomerular mesangial cells (HMCs). **A & C)** HMCs were incubated for 0hr, 6hr, 12hr, 24hr and 48hr in culture medium containing 30mmol/l D-glucose. The mRNA levels of TGF-β1 and FN were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (n=3-5). **B & D)** Semiquantitative analysis of the mRNA expression. $^{\Delta}p$<0.01 versus 0hr.

The effect of high glucose on the protein levels of p-JAK2 and p-STAT1. Using western blotting analysis, we investigated the time course of p-JAK2 and p-STAT1 protein expressions in cultured HMCs treated with 30mmol/l D-glucose. As shown in **Figure 2**, high glucose significantly upregulated p-JAK2 and p-STAT1 protein expressions from 12-48 hr (p=0.005).

The PEDF decreased high glucose-induced TGF-ß1 and FN secretion in HMC. **Figure 3** and **Table 2** show the effect of PEDF on the TGF-ß1 and FN level. We observed that TGF-ß1 and FN level were significantly upregulated by HG when compared with N group (p=0.000). No significant difference between N group and NG group (p=0.951). The PEDF at concentrations 40-100 nmol/l significantly down-regulated the mRNA and protein levels of TGF-ß1 (p=0.002). Whereas at concentrations 10-100 nmol/l, PEDF decreased the mRNA and protein levels of FN in a dose dependent manner in HMCs (p=0.001)).

Pigment epithelium-derived factor inhibited high glucose-induced overexpression of p-JAK2 and p-STAT1 in HMC. **Figure 4** shows the effect of PEDF on the p-JAK2 and p-STAT1 level. We observed that p-JAK2 and p-STAT1 level were markedly increased by HG when compared to N group (p=0.000). No significant difference between N group and NG group (p=0.436). Pigment epithelium-derived factor significantly inhibit the increase in the p-JAK2 and p-STAT1 level, especially at the 40 nmol/l (p=0.027) and 100 nmol/l concentrations (p=0.000).

Table 1 - High glucose induces transforming growth factor-β1 (TGF-β1) and fibronectin (FN) protein expressions in cultured human glomerular mesangial cells (HMCs).

Time	TGF-ß1 (pg/ml)	FN (ng/ml)
0hr	274.48 ± 10.28	44.13 ± 2.29
6hr	284.07 ± 13.06	47.98 ± 4.81
12hr	294.65 ± 13.11	58.31 ± 6.40
24hr	316.07 ± 27.20*	95.57 ± 10.91*
48hr	324.40 ±16.69*	166.74 ± 16.35*

HMCs were incubated for 0hr, 6hr, 12hr, 24hr and 48hr in culture medium containing 30mmol/l D-glucose.Values are expressed as Mean±SD. *p<0.01 versus 0hr (mean±SD, n=5).

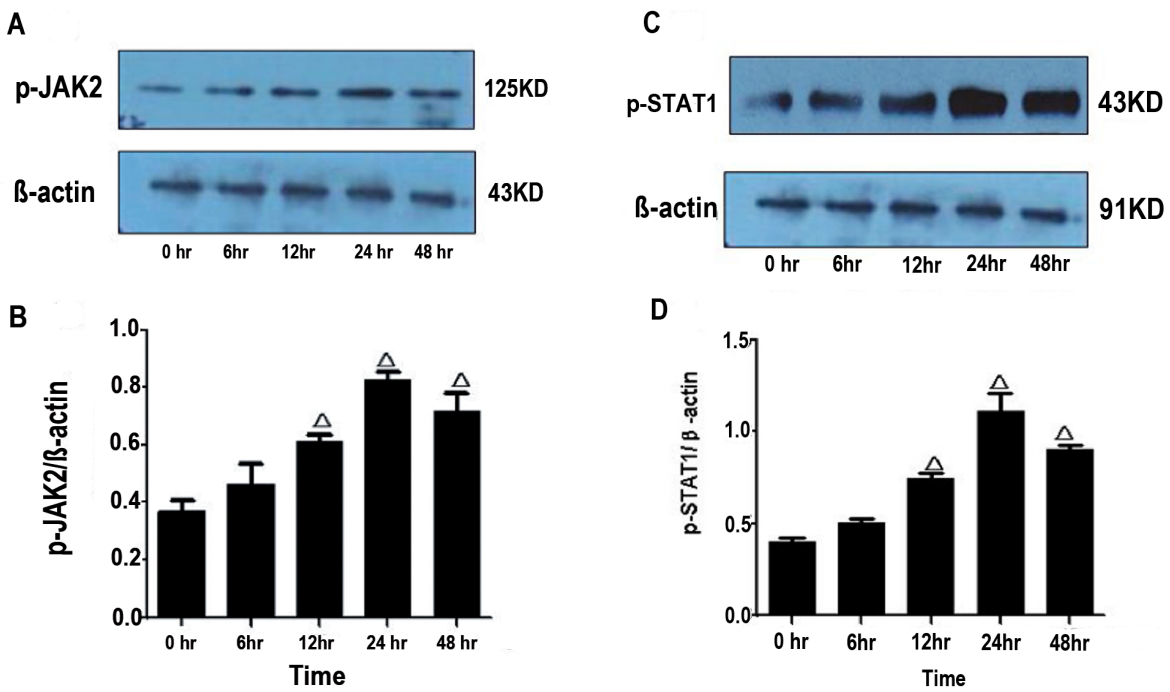

Figure 2 - Time course of the effects of high glucose on the phosphorylation levels of Janus kinase2 (p-JAK2) and signal transducers and activators of transcription1 (p-STAT1) in cultured human glomerular mesangial cells (HMCs). **A & C)** HMCs were incubated for 0h, 6h, 12h, 24h and 48h in culture medium containing 30mmol/l D-glucose. **B & D)** The protein levels of p-JAK2 and p-STAT1 were analyzed by Western blot (n=3-5). $^{\Delta}p$<0.01 versus 0hr.

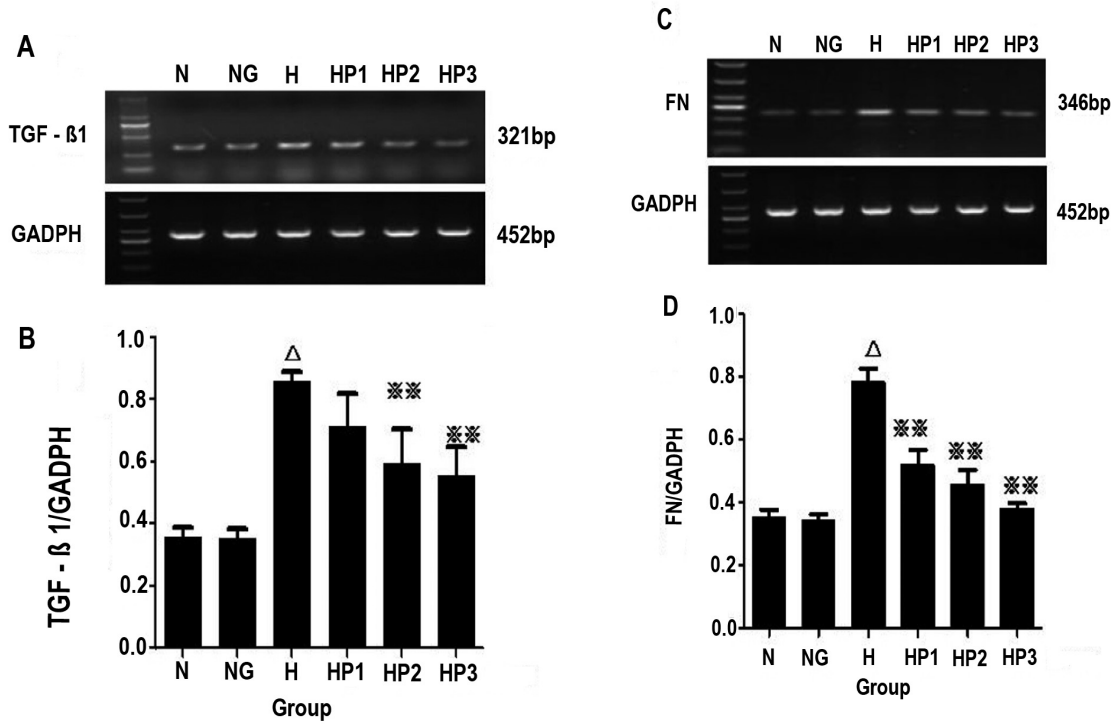

Figure 3 - Effects of pigment epithelium-derived factor (PEDF) on high glucose induced transforming growth factor-beta1 (TGF-ß1) and fibronectin (FN) messenger ribonucleic acid (mRNA) expressions in cultured human glomerular mesangial cells (HMCs). **A & C**) HMCs were randomly divided into 6 groups receiving the following treatments for 24hr: N group - the cells were treated with 5.6mmol/l D-glucose; NG group - the cells were treated with 5.6mmol/l D-glucose+24.4mmol/l mannitol; H group - the cells were treated with 30mmol/l D-glucose; HP1 group - the cells were treated with 30mmol/l D-glucose+10nmol/l PEDF; HP2 group - the cells were treated with 30mmol/l D-glucose+40nmol/l PEDF; and HP3 group - the cells were treated with 30mmol/l D-glucose+100nmol/l PEDF. **B & D**) The mRNA levels of TGF-β1 and FN were analyzed by RT-PCR (n=3-5). Semiquantitative analysis of the mRNA expression. $^{\Delta}p<0.01$ versus N group. $^{※※}p<0.01$ versus H group. GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

Table 2 - Effects of pigment epithelium-derived factor (PEDF) on high glucose induced transforming growth factor-beta1 (TGF-ß1) and fibronectin (FN) protein expressions in cultured human glomerular mesangial cells (HMCs).

Group	TGF-ß1 (pg/ml)	FN (ng/ml)
N	268.32 ± 9.23	54.57 ± 5.28
NG	270.48 ± 12.76	55.38 ± 7.35
H	310.65 ± 8.45*	73.39 ± 2.95*
HP1	302.07 ± 12.89	62.98 ± 2.81†
HP2	290.15 ± 9.13†	55.98 ± 4.98†
HP3	278.48 ± 8.45†	54.54 ± 4.88†

HMCs were randomly divided into 6 groups receiving the following treatments for 24hr: N - the cells were treated with 5.6mmol/l D-glucose; NG - the cells were treated with 5.6mmol/l D-glucose+24.4mmol/l mannitol; H - the cells were treated with 30mmol/l D-glucose; HP1 - the cells were treated with 30mmol/l D-glucose+10nmol/l PEDF; HP2 - the cells were treated with 30mmol/l D-glucose+40nmol/l PEDF; HP3 - the cells were treated with 30mmol/l D-glucose+100nmol/l PEDF. Values are expressed as mean±SD. *$p<0.01$ versus N group. †$p<0.01$ versus H group. (mean±SD, n=5)

Discussion. The major findings in the present study were that high glucose induced increased expressions of cellular levels of TGF-ß1, FN, p-JAK2 and p-STATs1. These effects of hyperglycemia on HMCs were inhibited by PEDF, indicating that PEDF is an endogenous anti-fibrogenic factor in the kidney by inhibiting activation of JAK and STAT proteins in human glomerular mesangial cells under high glucose conditions. Pigment epithelium–derived factor is a 418-amino acid 50-kDa glycoprotein that belongs to the super family of serine protease inhibitors, first identified in cultured retinal pigment epithelial cells. Decreased PEDF levels in the kidney are implicated in DN.[21-27] Moreover, injection of adenovirus PEDF drastically reduced the albuminuria and ameliorated the glomerular hypertrophy in the streptozotocin (STZ)-induced diabetic rat mode.[22] We previously showed that PEDF prevented the high glucose-induced ECM protein accumulation (fibronectin and collagen IV) in

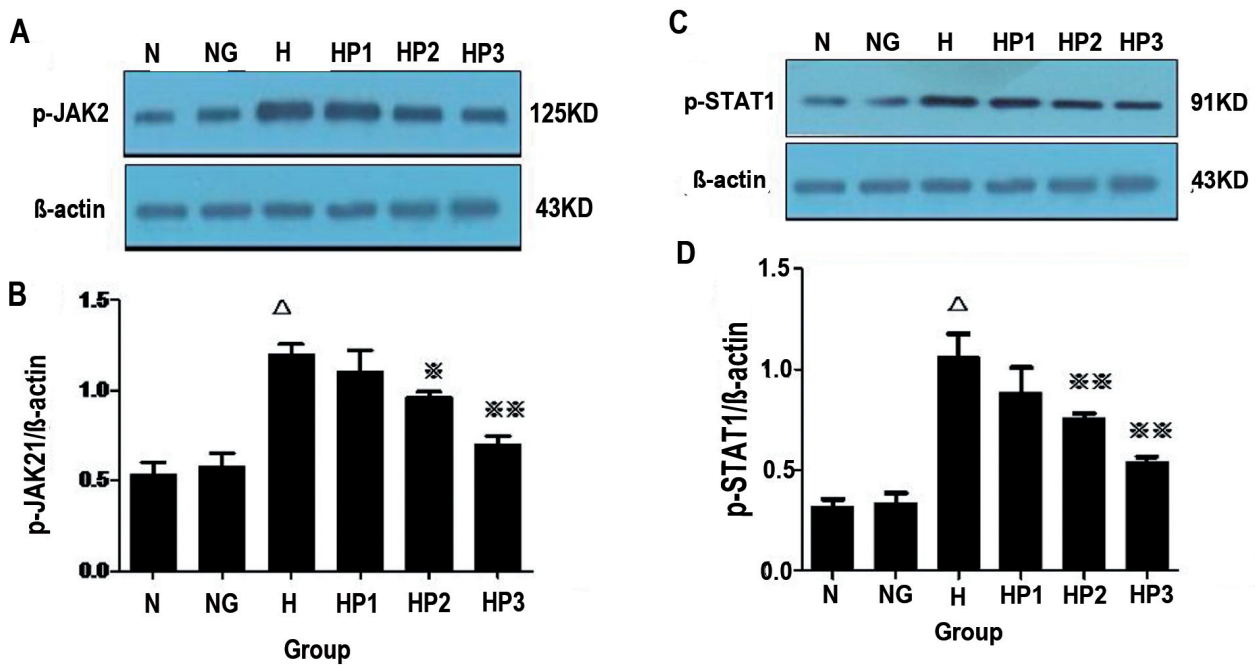

Figure 4 - Effects of pigment epithelium–derived factor (PEDF) on high glucose induced the phosphorylation levels of Janus kinase2 (p-JAK2) and signal transducers and activators of transcription1 (p-STAT1) in cultured human glomerular mesangial cells (HMCs). **A & C)** HMCs were randomly divided into 6 groups receiving the following treatments for 24hr: N group - the cells were treated with 5.6mmol/l D-glucose; NG group - the cells were treated with 5.6mmol/l D-glucose+24.4mmol/l mannitol; H group - the cells were treated with 30mmol/l D-glucose; HP1 group - the cells were treated with 30mmol/l D-glucose+10nmol/l PEDF; HP2 group - the cells were treated with 30mmol/l D-glucose+40nmol/l PEDF; HP3 group - the cells were treated with 30mmol/l D-glucose+100nmol/l PEDF. **B & D)** The protein levels of p-JAK2 and p-STAT1 were analyzed by Western blot (n=3-5). $^{\Delta}p<0.01$ versus N group. $^{※}p<0.05$ versus H group. $^{※※}p<0.01$ versus H group.

HMCs through its anti-oxidative properties.[21] However, a role of PEDF in DN is largely unknown. Therefore, we investigated whether and how PEDF could protect against high glucose induced human mesangial cell damage in vitro.

Diabetic glomerular fibrosis is caused by excessive deposition of ECM proteins (collagen I, III and IV and FN) in the mesangial interstitial space. It is believed that TGF-ß is involved in the pathogenesis of early stage DN. Accumulated evidences have shown that overexpression of TGF-ß induced by elevated glucose causes glomerular basement membrane thickening and mesangial matrix expansion via stimulation of matrix protein expression.[28-31] On the other hand, inhibition of TGF-ß expression abolishes above pathological changes. Our study showed that HMC cultured under high glucose conditions produce TGF-ß1 and FN at a significantly faster rate than those cultured under normal glucose conditions. After treatment with PEDF, the secretion of TGF-ß1 and fibronectin were decreased. This result suggests that PEDF prevent DN by the suppression of glomerular TGF-ß1 and FN expression.

The JAK/STAT pathway mediates intracellular signaling in response to many growth factors and cytokines. The JAK enzymes, namely JAK1, JAK2, JAK3 and TYK2, are responsible for the phosphorylation of the STATs (STAT1, STAT2, STAT3, STAT4, STAT5A/B and STAT6), which are latent cytoplasmic transcription factors.[5] Marerro's group has shown that high glucose alters the activation of the JAK/STAT pathway in rat kidney by inducing tyrosine phosphorylation of JAK2 and STAT1 and STAT.[32,34] In cultured mesangial cells, JAK2 activation was shown to mediate collagen IV and fibronectin production, TGF-ß activation, and cell growth due to angiotensin II administration or exposure to high glucose concentrations.[4,32] They also reported that HG stimulated the glomerular phosphorylation of JAK2, STAT1, and STAT3 in vivo and that phosphorylation was reduced in rats treated with the AT1 receptor blocker candesartan and the JAK2-specific inhibitor AG-490.[33] JAK2 and STAT1 were expressed at higher levels in glomeruli samples of patients with DN.[6] These results indicate that the high glucose induced JAK2-STAT1-dependent pathway plays a very important role in the synthesis of ECM protein that occurs during DN. In this study, we examined the

effect of PEDF on the activation of JAK2 and STAT1, in parallel with its effect on TGF-ß1 and FN production in HMC in vitro. We found that PEDF significantly inhibited the increased phosphorylation of JAK2 and STAT1 in HMC cultured under HG conditions. These results suggest that the renal protective effects of PEDF maybe partly through the inhibitory activation of the JAK/STAT signaling pathway.

Enhanced production of ROS by HG has been described as a potential major activator of JAK/STAT signaling and ROS mediate induction of JAK-2 activation in tissues such as cardiac myocytes and vascular smooth muscle cells as well as kidney cells.[6] Recent studies have demonstrated that PEDF may prevent the production of ROS. Ide et al reported that PEDF could inhibit the advanced glycation end products (AGE)-induced inflammatory and thrombogenic gene expressions in human cultured mesangial cells by suppressing NF-ßB activation via inhibition of ROS generation.[23] Pigment epithelium-derived factor inhibited mitochondria-derived ROS generation and decreased vascular endothelial growth factor production in bovine retinal capillary endothelial cells.[34] Yamagishi et al reported that AGEs-bovine serum albumin (BSA) significantly increased intracellular ROS generation in human umbilical vein endothelial cells (HUVECs), which was completely inhibited by PEDF; PEDF or an anti-oxidant N-acetyl-L-cysteine (NAC) significantly restored the decrease in endothelial nitric oxide synthase (eNOS) mRNA levels in AGEs-exposed HUVECs.[35] Pigment epithelium-derived factor decreased ROS generation in AGE-exposed endothelial cells by suppressing triphosphopyridine nucleotide (NADPH) oxidase activity via down-regulation of mRNA levels of $p22^{PHOX}$ and $gp91^{PHOX}$[36] Our previous study demonstrated that PEDF prevented mesangial ECM overproduction and pathological growth factor upregulation in the kidney by eliminating ROS generation.[21] Therefore, the PEDF regulated activation of the JAK/STAT pathway maybe partly via reducing ROS production in HMC.

The limitation of this research is that the mechanism of PEDF inhibiting activation of JAK2 in response to HG was not studied.

In conclusion, PEDF may inhibit HMC synthesis of ECM by antagonizing HG induced activation of the JAK/STAT signaling cascade. Pigment epithelium-derived factor by virtue of its anti-oxidative and anti-fibrogenic properties, may have a therapeutic potential in DN.

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