Relaxin Inhibits High Glucose-Induced Matrix Accumulation in Human Mesangial Cells by Interfering with TGF-β1 Production and Mesangial Cells Phenotypic Transition

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Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD). DN is characterized by glomerular extracellular matrix accumulation, mesangial expansion, basement membrane thickening, and renal interstitial fibrosis. To date, mounting evidence has shown that H2 relaxin possesses powerful antifibrosis properties; however, the mechanisms of H2 relaxin on diabetic nephropathy remain unknown. Here, we aimed to explore whether H2 relaxin can reduce production of extracellular matrix (ECM) secreted by human mesangial cells (HMC). HMC were exposed to 5.5 mM glucose (NG) or 30 mM glucose (HG) with or without H2 relaxin. Fibronectin (FN) and collagen type IV levels in the culture supernatants were examined by solid-phase enzyme-linked immunoadsorbent assay (ELISA). Western blot was used to detect the expression of α-smooth muscle actin (α-SMA) protein. Quantitative polymerase chain reaction (qPCR) method was employed to analyze transforming growth factor (TGF)-β1 mRNA expression. Compared with the normal glucose group, the levels of fibronectin and collagen type were markedly increased after being cultured in high glucose medium. Compared with the high glucose group, remarkable decreases of fibronectin, collagen type IV, α-smooth muscle actin, and TGF-β1 mRNA expression were observed in the H2 relaxin-treated group. The mechanism by which H2 relaxin reduced high glucose-induced overproduction of ECM may be associated with inhibition of TGF-β1 mRNA expression and mesangial cells’ phenotypic transition. H2 relaxin is a potentially effective modality for the treatment of DN.

Key words human mesangial cell (HMC); human H2 relaxin; extracellular matrix (ECM); transforming growth factor (TGF)-β1; diabetic nephropathy (DN)

Diabetic nephropathy (DN) is one of the most common chronic complications of diabetes, and a major cause of end-stage renal disease (ESRD).1 Once the clinical diabetic nephropathy is diagnosed, most of the DN patients will inevitably develop ESRD after several years due to the lack of effective methods to delay its progression.2 Its pathology is characterized by glomerular extracellular matrix accumulation, mesangial expansion, basement membrane thickening, glomerulosclerosis and renal interstitial fibrosis.3 Laminin, fibronectin and type IV collagen are the main components of extracellular matrix, and the excessive extracellular matrix (ECM) plays a key role in the progression of diabetic nephropathy.5

Previous in vitro studies5,6 revealed that high glucose can stimulate the synthesis of ECM including fibronectin and type IV collagen through the activation of the transforming growth factor (TGF)-β dependent Smad signaling pathway, as well as upregulation of its receptor expression. Recently Liu et al.7 reported that both 30 mM glucose (HG) and TGF-β1 can lead to over production of fibronectin. The underlying mechanism was thought to be mesangial cell caveolae has a role in regulating fibronectin production partly through caveolin-1 phosphorylation.

TGF-β is recognized as a major mediator in the development of ECM accumulation and kidney fibrosis in diabetic nephropathy.8 TGF-β not only stimulates the synthesis of fibronectin and type IV collagen, but also decreases the degradation of matrix by elevating the expression of plasminogen activator inhibitor-1.9 Thus, the inhibition of TGF-β and extracellular matrix may be beneficial for the treatment of diabetic nephropathy. Although a number of strategies for the treatment of diabetic nephropathy have been proposed, such as ACE inhibitors, angiotensin II receptor blockers and novel emerging renoprotective molecules, the results are not encouraging at present.10–12

Human H2 relaxin, a member of the relaxin/insulin peptide hormone (H1, H2 and H3 relaxins, INSL3, 4, 5 and 6) structurally similar to that of insulin, has been considered to be a potent antifibrosis drug by many studies.13–16 Like insulin, H2 relaxin is composed by three disulfide-bonded chains, the A and B chains,13 and exerts a number of pleiotropic effects by binding to different kinds of receptors, categorized into relaxin family peptide (RXFP1-7) receptors.14 It is primarily produced by the ovary and/or placenta in pregnancy or the prostate of mammals.15 The original biological functions of relaxin are the relaxation of the pubic symphysis during pregnancy and the influence on the development of the mammary gland.

As research continues, it has been found that H2 relaxin can regulate extracellular matrix remodeling and reduce fibrosis in a number of organs.20 However, the link between H2 relaxin and diabetic nephropathy remains to be elucidated. Thus, the
The aim of this study is to explore the impact of H2 relaxin on the ECM production and TGF-β1 expression in mesangial cells cultured in high glucose medium.

MATERIALS AND METHODS

Materials Human glomerular mesangial cell line (HMC) was purchased from American ATCC cell library. Recombinant Human H2 Relaxin was obtained from Peprotech (lot # 0607424 U.S.A.). Human Transforming Growth Factor-β1 was from PROSPEC (lot# 411PTGFB17 U.S.A.); Dulbecco’s modified Eagle’s medium (DMEM), from Gibco (U.S.A.); Human fibronection (FN) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Boshide Wuhan, China; Human Collagen Type IV (Col IV) ELISA kit was supplied by R&D U.S.A.; α-smooth muscle actin (α-SMA) protein antibody was provided by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). α-smooth muscle actin (α-SMA) protein antibody GAPDH pAb was provided by R&D U.S.A.; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein antibody GAPDH pAb was from (Bioworld, AP0063); polyvinylidene difluoride (PVDF) membranes, Millipore Corporation (U.S.A.); Fatal bovine serum (FBS), Sijiqing Hangzhou, China; Human Collagen Type IV (Col IV) ELISA kit was supplied by R&D U.S.A.; α-smooth muscle actin (α-SMA) protein antibody GAPDH pAb was provided by Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture HMCs were cultured in normal DMEM media (5.5 mM d-glucose, NG) supplemented with 10% newborn bovine serum (NBS), in 37°C, 5% CO₂ atmosphere. High glucose culture media was made by supplementing normal DMEM medium with additional d-glucose for a final concentration at 30 mM (HG). The cell medium was changed every 2–3 d until the cells became confluent, and after that, HMCs were maintained in a serum free medium for 24 h to synchronize the cell growth. Subsequently HMCs were cultured in either 5.5 mM glucose or 30 mM glucose. In HG group, cells were treated with or without H2 relaxin (10 ng/mL). In Western blot experiment, the cells were treated with exogenous 5 ng/mL TGF-β1 in NG group as positive control. The effect of relaxin on the cell proliferation was examined using a cell count kit (CCK8) method.

ELISA Assay After the intervention at 24, 48, and 72 h with 10 ng/mL H2 relaxin, the cell supernatant was removed, and different concentrations of the standard or sample 100 μL was added in 96 well culture plates, the HMCs supernatant was collected, and the concentrations of Col IV, FN were measured according to the manufacturer’s instructions in the ELISA kit. Each specimen was set 3 wells.

Real-Time Quantitative Polymerase Chain Reaction (PCR) TGF-β1 primer sequences were as follows: forward, 5'-CAC AGATCC CCT ATT CAA GAC CA-3', reverse, 5'-CAG TAT CCC ACG GAA ATA ACC TA-3'. Reference gene ACTB (beta actin) primer sequences: forward, 5'-TCC TCT CCT GG GCC ATG GAG T-3', reverse, 5'-CAG GAG GAG CAA TGA TCT TGA T-3'. The total RNA from mesangial cells was extracted using Trizol Reagent one-step method, and a reverse transcription reaction was performed using 1 μg of total RNA from each sample, according to Fermentas Corporation's M-MLV operating instructions. The reverse transcription of cDNA was stored at −20°C. SYBR Green I real-time PCR method was employed to quantify the relative abundance of mRNA in the samples. PCR amplifications were performed in a 20 μL reaction system. TGF-β1 amplification conditions: pre-denaturation cycle 1, 95.0°C for 2 min, 40 cycles: 95°C 15 s, 59°C 20 s, 72°C 20 s. Housekeeping gene ACTB was used as an internal reference, and compared that with a control group, a 2-ΔΔCt method was used to analyze the data: ΔCt=ΔCt as target gene, −ΔCt as internal reference, ΔΔCt=ΔCt as sample, −ΔΔCt as control. The values of 2-ΔΔCt were calculated if the multiples of the target genes expression in the experimental group changed relative to the control group.

Western Blot Analysis After being synchronized, HMC cells were treated with 10 ng/mL H2 relaxin or 5 ng/mL TGF-β1 for 24, 48 and 72 h. Cellular protein was extracted. Forty microgram samples were prepared before being electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation gel. Next, a constant voltage of 80 V for 45 min was used, followed by a constant voltage of 120 V for 1 h. After electrophoresis, the PAGE gel was removed from the glass plate, assembled the transmembrane sandwich, and damp-dried the electroblot for 40 min under a constant voltage of 20 V to transfer the protein to nitrocellulose membrane. The membrane was blocked in confining liquid (a TBST solution containing 5% defatted milk powder) at room temperature for 1–2 h. The primary antibody was diluted by a fresh blocking solution, with 1:5000 dilution.

Fig. 1. Effect of Relaxin on FN Production in Mesangial Cells Cultured in High Glucose

HMCs were incubated in high glucose medium with or without 10 ng/mL relaxin for 24, 48 and 72 h. Type IV collagen protein production was measured by ELISA. Data are expressed as mean±S.D., n=3. *p<0.01 as compared with the NG at the same time point, #p<0.01 as compared with the HG at the same time point.
of the GAPDH protein antibody or 1:750 actin antibody and incubated at 4°C overnight. After the membrane was washed with PBST/TBST for 30 min, the secondary antibodies corresponding to each primary antibody were diluted with a blocking solution (mouse secondary antibody 1:5000; rabbit secondary antibody 1:5000). The membrane was incubated at room temperature for 1 h. Membrane was washed, and then visualized with enhanced chemiluminescent (ECL) reporter system followed by exposure to X-ray film. Intensity of the bands was measured by TINA image software (Raytest, Straubenhardt, Germany).

Statistical Analysis All values are expressed as mean±standard deviation (S.D.). One-way ANOVA followed by a post-hoc Student–Newman–Keuls multiple comparisons test was performed using SPSS Software (V16.0, SPSS, Inc., Chicago, IL, U.S.A.), and a p-value <0.05 was considered to be statistically significant.

RESULTS

High-Glucose-Induced FN and ColIV Expression Are Inhibited by H2 Relaxin There was no significant difference in mesangial cell proliferation between the H2 relaxin treated group and the control group (Table 1). HMCs were cultured for 24, 48 and 72 h under normal or high glucose conditions and the influence of H2 relaxin on the synthesis of FN, ColIV was studied. Compared to HMCs cultured in normal glucose control group, the expression of FN and Col IV increased significantly (p<0.01) in high glucose medium, and after administration of H2 relaxin to these cells at 24, 48 and 72 h, the production of FN and Col IV decreased remarkably as compared to high glucose group (p<0.01, Figs. 1, 2).

High Glucose Induced TGF-β1 mRNA Gene Expression in Mesangial Cells Is Inhibited by H2 Relaxin We then examined if TGF-β1 mRNA expression was influenced by H2 relaxin. As shown in Fig. 3, TGF-β1 mRNA expression increased in the high glucose group cultured for 48 h...
compared to normal glucose group, while the expression was particularly obvious at 72 h. HMCs treated with H2 relaxin in the presence of high glucose showed a significant reduction of TGF-β1 mRNA expression compared to high glucose group at 48 and 72 h, suggesting that H2 relaxin can interfere with HG induced TGF-β1 mRNA production.

Effects of the Addition of H2 Relaxin and TGF-β1 on α-SMA Protein Expression

Exogenous TGF-β1 is well known as an effective stimulus to the synthesis of α-SMA. To examine whether H2 relaxin can induce mesangial cells to change its phenotype, we also added exogenous TGF-β1 as positive control. The upregulation of α-SMA in mesangial cells is considered to be a marker of a switch to a fibrogenic phenotype and the proliferative/secrectory state HMC can lead to an increased matrix turnover. Therefore, we studied the impact of H2 relaxin on the HMC phenotype transition treated with or without H2 relaxin at different time points. The results showed that α-SMA was significantly higher in the high glucose group and in the normal glucose group exposed to exogenous TGF-β1 when compared with the normal glucose group. While after treatment with H2 relaxin for 24, 48, and 72 h, α-SMA protein expression was significantly decreased at all time points (Fig. 4).

DISCUSSION

In the present study, we tested the hypothesis that H2 relaxin can reduce the high glucose mediated ECM accumulation by suppressing the TGF-β1 production and mesangial cell phenotypic transition. Our main findings showed that FN and ColIV were significantly higher in HG group than those in NG group in vitro, suggesting that high glucose can stimulate HMC cells to secrete extracellular matrix. After treatment with H2 relaxin, a marked reduction of FN and ColIV was

<table>
<thead>
<tr>
<th>Group</th>
<th>NG 24h</th>
<th>NG 48h</th>
<th>NG 72h</th>
<th>HG 24h</th>
<th>HG 48h</th>
<th>HG 72h</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.69±0.05</td>
<td>1.03±0.03</td>
<td>1.12±0.07</td>
<td>0.78±0.02</td>
<td>0.89±0.06</td>
<td>1.11±0.04</td>
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<tr>
<td>RLX (1 ng/mL)</td>
<td>0.71±0.06</td>
<td>0.99±0.09</td>
<td>1.14±0.08</td>
<td>0.77±0.03</td>
<td>0.91±0.06</td>
<td>1.13±0.03</td>
</tr>
<tr>
<td>RLX (1.95 ng/mL)</td>
<td>0.71±0.03</td>
<td>1.02±0.02</td>
<td>1.10±0.05</td>
<td>0.81±0.03</td>
<td>0.91±0.05</td>
<td>1.05±0.08</td>
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<tr>
<td>RLX (3.9 ng/mL)</td>
<td>0.73±0.04</td>
<td>1.01±0.04</td>
<td>1.18±0.07</td>
<td>0.79±0.04</td>
<td>0.95±0.03</td>
<td>1.12±0.05</td>
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<tr>
<td>RLX (7.8 ng/mL)</td>
<td>0.74±0.04</td>
<td>1.04±0.03</td>
<td>1.17±0.08</td>
<td>0.77±0.03</td>
<td>0.86±0.03</td>
<td>1.14±0.04</td>
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<tr>
<td>RLX (15.625 ng/mL)</td>
<td>0.72±0.03</td>
<td>0.99±0.03</td>
<td>1.15±0.03</td>
<td>0.74±0.07</td>
<td>0.94±0.03</td>
<td>1.14±0.04</td>
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<td>RLX (31.25 ng/mL)</td>
<td>0.68±0.03</td>
<td>1.02±0.04</td>
<td>1.10±0.03</td>
<td>0.76±0.03</td>
<td>0.92±0.04</td>
<td>1.18±0.05</td>
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<tr>
<td>RLX (62.5 ng/mL)</td>
<td>0.66±0.02</td>
<td>1.07±0.05</td>
<td>1.09±0.02</td>
<td>0.78±0.03</td>
<td>0.86±0.06</td>
<td>1.19±0.08</td>
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<td>RLX (125 ng/mL)</td>
<td>0.73±0.02</td>
<td>1.05±0.05</td>
<td>1.19±0.06</td>
<td>0.74±0.04</td>
<td>0.89±0.07</td>
<td>1.10±0.06</td>
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<tr>
<td>RLX (250 ng/mL)</td>
<td>0.66±0.10</td>
<td>1.07±0.03</td>
<td>1.15±0.04</td>
<td>0.82±0.04</td>
<td>0.89±0.04</td>
<td>1.17±0.05</td>
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There was no significant difference (p>0.05) between the relaxin treated groups and control group at the same time point.
observed, suggesting that H2 relaxin can inhibit mesangial cells from producing extracellular matrix in high ambient glucose. Real-time fluorescence quantitative detection revealed that the expression of TGF-β1 mRNA decreased in the H2 relaxin treated group, implying that H2 relaxin can suppress TGF-β1 mRNA in gene level. We also found that both exogenous TGF-β1 and high glucose can lead to the over expression of α-SMA protein in mesangial cells. After the treatment with H2 relaxin, the expression of α-SMA protein decreased significantly, suggesting that H2 relaxin can make a tremendous effect on inhibiting transdifferentiation of glomerular mesangial cells.

Previous studies demonstrated that the pivotal pathologic changes in DN are the accumulation of glomerular extracellular matrix and interstitial fibrosis, while the increased extracellular matrix secreted by the mesangial cells plays a central role in the fibrogenesis of diabetic nephropathy. High glucose concentration has a variety of effects on mesangial cells, including cell phenotype transition, increased expression of α-SMA, the activation of protein kinase C and abnormal growth factor synthesis. TGF-β1 plays an essential regulatory role in the ECM metabolism by binding to the receptors on mesangial cells. Not only can it promote mitosis secretion, thus stimulating the synthesis of ECM in mesangial cells, but can also lead to increasingly excessive protein synthesis. Ultimately, TGF-β1 sustained expression can result in glomerulosclerosis and renal interstitial fibrosis.

It is generally recognized that the expression of α-SMA is the active state and hallmark of the mesangial cells transdifferentiation, indicating its myofibroblast phenotypic transition. When in a normal quiescent state, there is none or only very weak expression of α-SMA in mature mesangial cells. However, in pathological conditions, mesangial cells may express the specific phenotypic marker α-SMA, suggesting that mesangial cells are activated to proliferative/secretory state, also known as a phenotypic transition, and thus leading to overproduction of extracellular matrix. Our findings showed that high glucose or TGF-β1 can induce mesangial cells into myofibroblast phenotype transition and stimulate the synthesis of ECM, this process can be attenuated by H2 relaxin intervention.

When relaxin was first identified by Frederick Hisaw in 1926, it was initially known as pelvic ligaments relaxant and hormones that affect the female reproductive tract. Relaxin belongs to the insulin family structurally, the receptor of which is the leucine-rich G-protein-coupled receptor, containing LGR7 and LGR8. Binding to its receptor, H2 relaxin plays a biological role by regulating cAMP content within the target cells.

Mookerjee et al. demonstrated that relaxin signals through a nNOS-NO-cGMP-dependent pathway to inhibit Smad2/TGF-β1 signaling of renal myofibroblast differentiation. Dessauer and Nguyen observed a bifurcated pathway by which relaxin stimulates Gs alpha and P13K/PKCζeta leading to increased cAMP production expression in THP-1 cells. Therefore, we postulated that the results showing relaxin inhibiting the expression TGF-β1 and the production of ECM may occur through the nNOS-NO-cGMP-dependent pathway to inhibit the signaling of Smad2/TGF-β1. However, further studies are needed to confirm this hypothesis.

In Samuel’s study, a therapeutic effect was found in diabetic rats’ cardiomyopathy, including improving the left ventricular diastolic function and myocardial compliance, reducing myocardial collagen content and the expression of tissue inhibitor and metalloproteinase-1.

Combining Dahl salt-sensitive hypertensive with Dahl salt-resistant normotensive rats, researchers in Japan have found that when treated with H2 relaxin after six weeks, DS rats’ systolic blood pressure decreased significantly, glomerular and tubular fibrosis lesions were alleviated and TGF-β signal transduction decreased. In another study, administration of H2 relaxin to rats with the anti-thymocyte serum nephritis model resulted in a decrease of PAS staining positive matrix, SMAD2 phosphorylation and mesangial cell proliferation while α-SMA expression were found. Heeg et al. showed that H2 relaxin can inhibit Smad2 phosphorylation, translocation to nucleus in the TGF-β1 signal pathway, thereby reducing the ECM synthesis and slowing the progression of fibrotic renal diseases. Chow et al. found that H2 relaxin exerts its anti-fibrotic effects in both in-vivo and in-vitro models depending on the heterodimerization of the RXFP-1 and AT2 receptors.

In conclusion, our study showed that H2 relaxin can inhibit high glucose-induced ECM overproduction probably by suppressing the mesangial cells phenotypic transition and inhibiting the overexpression of TGF-β1 mRNA. H2 relaxin therefore might be an effective therapeutic approach for delaying the progression to end-stage renal disease. Coupled with no in-vivo data, further study is needed to testify the renoprotective effect and the molecular mechanism of H2 relaxin in vivo.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


