High glucose inhibits the survival of HRMCs and its mechanism

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Abstract. – OBJECTIVE: High glucose can promote the apoptosis of glomerular mesangial cells and cause diabetic nephropathy (DN). However, the mechanism remains unclear. In the present study, we investigated the effects of high glucose on the survival of human renal mesangial cells (HRMCs).

MATERIALS AND METHODS: Cells were treated with high glucose (30 mM) or normal glucose (5 mM) for 48 hours. Cell proliferation was determined by trypan blue assay. The relative expression of metalloproteinase-3 (TIMP3) and inflammatory factors detected by real-time polymerase chain reaction (PCR). Protein expression of Smad2/3, p-Smad2/3 and Smad7 in HRMCs were analyzed by Western blot.

RESULTS: Compared with normal glucose, we found that high glucose significantly inhibited cell survival, accompanied by the decrease of tissue metalloproteinase-3 (TIMP3) mRNA expression. Western blot results showed that the expression of p-Smad2/3 was significantly up-regulated, the expression of Smad7 was significantly down-regulated, and inflammatory factors IL-6/IL-8 mRNA expression were increased in the HRMCs cultured with the high glucose. We also found that, compared with the normal glucose, the level of MDA was significantly increased (p<0.01), and the level of SOD was significantly lower (p<0.05) in the HRMCs cultured with the high glucose.

CONCLUSIONS: These findings suggested that high glucose inhibited the survival of HRMCs and may be associated with the downregulation of TIMP3 expression, Smad signaling pathway, inflammation and oxidative stress.

Key Words: High glucose, Survival, HRMCs.

Introduction

Diabetic nephropathy (DN) is the important cause leading to the chronic renal failure1,2. Mesangial cells (MCs) maintain the structure and function of the glomerulus, offer structural stability for capillary loops, and regulate glomerular filtration through their contractility1. High glucose could promote the apoptosis of glomerular mesangial cells and cause the deterioration of DN3,4. However, the mechanism remains unclear. Tissue inhibitor of metalloproteinase-3 (TIMP3), a 24-27 kDa protein belonging to TIMP family, is the most highly expressed TIMP in the kidneys and participates in the regulation of inflammation, cellular migration and proliferation5. Also, TIMP3 is a crucial regulator of inflammatory cytokines6,7. Its expression is dependent on Smad3, p38, and ERK1/2 signaling, and that these signaling pathways cooperate in the regulation of TIMP-3 expression, which may play a role in inflammation, tissue repair, and fibrosis8.

The purpose of this study is to investigate the molecular mechanisms of renal injury in high glucose conditions. Therefore, we explored the effects of high glucose on cell survival and revealed the underlying molecular mechanisms in human renal mesangial cells (HRMCs). This study provides important and persuasive evidence in understanding the mechanism in renal injury and the development of chronic kidney disease (CKD) in high glucose conditions.

Materials and Methods

Cell Culture and Treatment

Human renal mesangial cells (HRMCs) were obtained from American Type Culture Collections (ATCC, Manassas, VA, USA). Cells were cultured in MCM (ScienCell, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% growth factor in 37°C incubator with 5% CO2. Cells were passaged every 4 days and subcultured up to the 9th pas-
Cells were treated with normal glucose (NG, 5 mM) or high glucose (HG, 30 mM) for 48 hours for all the experiments.

**Trypan Blue Assay**

Cell proliferation was determined by trypan blue assay. Cells were plated in 35 mm dishes for 24 hours and then treated with NG or HG for 48 hours, followed by cell counting after cells were stained with trypan blue. Viable cells were counted using a hemocytometer under an inverted light microscope (Nikon ECLIPSE TS100). For each sample, the experiments were performed in triplicates (n=3).

**Western Blot Analysis**

Cells were harvested in lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 10 mM NaF, 0.2 mM Na3VO4] and protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Proteins (30 μg) were fractionated on 8 to 12% SDS-PAGE, blotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Primary antibodies, which included Smad2/3 (Enogene, Nanjing, China), p-Smad2/3 (p-Ser467/425) (AURAGENE BIOTECH, Hunan, China), Smad7 (Proteintech, Wuhan, China) and GAPDH (ZS GQ-BIO, Beijing, China), were used as a loading control. The secondary antibodies were HRP-conjugated anti-rabbit (NA 934 V) and anti-mouse (NA 931 V) antibodies (GE Healthcare Life Sciences, Wellesley, MA). Proteins were visualized by chemiluminescence, according to the manufacturer’s instructions (Thermo, Pittsburgh, PA, USA), followed by exposure to X-ray film. The density of bands was densitometrically quantified using Image J (National Institutes of Health, Bethesda, MD, USA).

**Quantitative RT-PCR Analysis**

Total RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. RNA quantity and quality were measured using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The expressions of mRNAs were quantified by SYBR green (Invitrogen). Real-time qRT-PCR was performed using an ABI PRISM 7300 system (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed in triplicate in different groups. The PCR primer sequences are shown in Table I. Relative expression levels of mRNAs among samples were calculated using the

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**Table I.** PCR primer sequences of genes used in RT reaction and real-time reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primers</th>
<th>Reverse primers</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>AAATTCGGTACATCCTCGACGG</td>
<td>GGAAGGTTCAAGGTGTGTTTCG</td>
</tr>
<tr>
<td>IL-8</td>
<td>GCCAGCTTTCTGATTTCT</td>
<td>GGGTGGAAGGTGGATAG</td>
</tr>
<tr>
<td>TIMP3</td>
<td>GTGCAACTTCGTTGAGAGG</td>
<td>CAGGTAGCAGGACTTGTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GATCAACGGATTTGGTCTG</td>
<td>TGGGATTCCATATTGACA</td>
</tr>
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**Figure 1.** Cell number in HRMC cultured in normal and high glucose conditions. **A**, Cell number in HRMC cultured in normal and high glucose conditions after 48 h. **B**, Cell number in HRMC cultured in normal and high glucose after 72 h. **C**, Cell number in HRMC cultured in normal and high glucose after 96 h. NG: Normal glucose; HG: High glucose. *p<0.05 vs. normal glucose.
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comparative delta CT method (2^{-\Delta\Delta Ct}) after normalization with reference to the expression of GAPDH.

**Oxidative Stress Analysis**

Supernatants were collected for microscale Malondialdehyde (MDA) and Superoxide Dismutase (SOD) detection, using MDA assay kit (Jiancheng, Nanjing, China) and SOD assay kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. The absorbance was measured at 532 nm (MDA) and 550 nm (SOD), using a 96-well microplate reader (SpectraMax M5, Molecular Devices, Carlsbad, CA, USA).

**Statistical Analysis**

All data are presented as the mean ± SD from at least three independent experiments. Statistical comparisons were made between two groups with Student’s t-test. Statistical significance was indicated as p<0.05 or p < 0.01 vs. NG.

**Results**

**Effect of High Glucose on Cell Survival in HRMCs (Figure 1)**

Compared with cells cultured in normal D-glucose concentrations (5 mM), cell numbers were decreased significantly in HRMC cultured in 30 mM D-glucose after 48, 72, and 96 hours. These results suggest that cell survival was decreased at high glucose levels.

**The Expression of TIMP3 mRNA was Decreased in the High Glucose Environment (Figure 2)**

We observed that in the high glucose environment, the expression of TIMP3 mRNA was decreased. This suggests that the damage caused by high glucose levels to HRMC may be by down-regulating TIMP3.

**Effect of High Glucose on Smad Signaling Pathway in HRMCs (Figure 3)**

To investigate whether high glucose activates the Smad signaling pathway, we examined the protein expression changes of genes related to Smad signaling pathway (Smad2/3, p-Smad2/3 and Smad7) in HRMCs treated with high glucose (Figure 3A and 3B). Compared with the control, high glucose significantly increased the phosphorylation levels of Smad2/3 protein, while the expression of Smad7 was significantly downregulated in the HG group (Figure 3C).

**Effect of High Glucose on Inflammatory Factors and Oxidative Stress in HRMCs**

To investigate whether high glucose induces the inflammatory response in HRMCs, we examined the changes in the mRNA expression of the inflammatory factors (IL-6/8). qRT-PCR results showed that the exposure to high glucose increased the mRNA expression levels of inflammatory cytokines IL-6 (Figure 4A) and IL-8 (Figure 4B). To investigate whether high glucose can induce the oxidative stress in HRMC, we measured the level of MDA and activity of SOD. The results show an increase in the MDA level (Figure 4C) and decrease in the activity of SOD (Figure 4D).

**Discussion**

Diabetic nephropathy is characterized by the apoptosis of mesangial cells, renal tissue basement membrane thickening and mesangial dilatation. Substantial evidence has shown that hyperglycemia or high glucose (HG) induces MC aberrant apoptosis, which has been attributable to the progression of DN. Loss of TIMP3, an extracellular matrix bound protein affecting both inflammation and fibrosis, is a hallmark of DN in human subjects and mouse models. In the present study, we found that the survival of HGMCs was significantly decreased, and the TIMP3 mRNA expression of HGMCs was downregulated after a 48-hour culture with the high concentration of glucose, compared with the normal concentration of glucose. Previous studies have shown that the expression of TIMP3 in diabetes was greatly reduced in the kidney, prompting glomerular cell apoptosis. For example, Lai et al found that TIMP3 expression was downregulated...
in Indian patients with DN, which promoted the apoptosis of mesangial cells and podocyte loss in patients by inhibiting the TGF-β/Smad signaling pathway. Fiorentino et al.\(^{15}\) confirmed that TIMP3 was significantly decreased in patients with DN, leading to the apoptosis of mesangial cells, renal tissue basement membrane thickening, mesangial dilatation, and increased proteinuria in patients through the FoxO1/STAT1 signaling pathway. Basu et al.\(^{16}\) also reported that downregulation of TIMP3 expression promoted the apoptosis of mesangial cells and worsened DN. Menghini et al.\(^{17}\) found that TIMP3 could inhibit apoptosis by inhibiting NH2-terminal and p38 kinase activation, reducing cellular oxidative stress, and inhibiting tumor necrosis factor alpha (TNF-\(\alpha\)) release.

Emerging evidence suggests that Smads signaling pathway plays a critical role in CKD\(^{18}\). Three types of Smads have been identified in biological systems, including receptor-regulated Smads (R-Smads, Smad1, 2, 3, 5, 8), common mediator Smads (CoSmads, Smad4) and suppressive Smads (I-Smads, Smad6, 7)\(^{19,20}\). The Smad signaling system plays a central role in the regulation of transforming growth factor (TGF-\(\beta\))\(^{21}\). As we known, TGF-\(\beta\) is a multifunctional cytokine, which can regulate inflammatory response and promote ECM accumulation\(^{22}\), suggesting that the TGF-\(\beta\)/Smad signaling pathway plays a key role in progressive kidneys’ injuries.

In the present study, we found that Smad7 was down-regulated whereas phosphorylation levels of Smad2/3 (p-Smad2/3) were up-regulated in high glucose-treated cells. These findings suggested that high glucose activated the TGF-\(\beta\)/Smad signaling pathway in HRMC.

In addition, excessive oxidative stress and inflammation have been found to serve as crucial
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Pathogenic factors in the development of DN\textsuperscript{23}. It has been well documented\textsuperscript{24,25} that HG treatment induced the production of oxides and inflammatory cytokines in MCs\textsuperscript{24}. Kang et al\textsuperscript{25} first proposed that high glucose promotes mesangial cell apoptosis through the oxidant-dependent mechanism.

To gain insight into the mechanisms of renal injury, this study aimed at examining oxidative stress and inflammatory responses as outcomes. As we known, MDA level is an index of oxidative stress and is one of the most important end products of lipid peroxidation\textsuperscript{26}. In the antioxidant system, SOD is a powerful antioxidant enzyme that resists the oxidative stress caused by exogenous substances. SOD is considered the cells’ first defense line against ROS\textsuperscript{27}. Consistent with literature, our results showed that high glucose resulted in the increased expression of IL-6 and IL-8 mRNA, induced the higher level of MDA, while SOD activity was significantly decreased. These results indicated that high glucose could induce oxidative stress and inflammatory responses in HRMCs, resulting in renal injury.

**Conclusions**

In summary, the present study found that high glucose might inhibit the survival of HRMCs cells through down-regulation of TIMP3 and activation of Smad signaling pathway, inflammatory responses, and oxidative stress, all contributing to renal injury. These findings provide new evidence to explain the high glucose induced toxicity in renal system and reveals a new potential downstream pathway.

**Funding**

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**Ethical Approval**

The study does not contain any experiment with human subjects conducted by any of the authors. The experimental protocol of this study was approved by the Ethics Committee of the Third Affiliated Hospital, Nanchang University, Nanchang, China.
Conflict of Interest
The authors declare that they have no competing interests.

References


