Original Article

HMGB1 is activated in type 2 diabetes mellitus patients and in mesangial cells in response to high glucose

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Abstract: Diabetic nephropathy (DN) is one of the most devastating complications of diabetes, leading the cause of end-stage renal disease (ESRD). And investigations into mechanisms underlying renal inflammation may provide new insight into novel therapeutic targets for patients with DN. However, little is known about the promotion of inflammation in DN. In the present study, we examined the promotion by high glucose to High-mobility group box-1 (HMGB1) in patients with type 2 diabetes mellitus or in renal mesangial SV40 MES 13 cells. Results demonstrated that high glucose promoted the pre-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 in patients with T2DM or in SV40 MES 13 cells. And the serum HMGB1 was also upregulated in T2DM patients, correlating with serum IL-6 and TNFα. The in vitro results indicated that HMGB1 mediated the D-glucose-induced pro-inflammatory cytokines in mesangial cells. And the NF-κB signaling pathway involved in the promotion of pro-inflammatory cytokines by D-glucose. In summary, the present study indicated that HMGB1 was significantly promoted by the glucose in vivo or in vitro, in an association with an upregulation of pro-inflammatory cytokines, via activating NF-κB signaling pathway. And the strategy of HMGB1 inhibition reduced the upregulation of pro-inflammatory cytokines in response to high glucose, via inhibiting the NF-κB signaling pathway. It implies the regulatory role of HMGB1 in the inflammatory responses in DN.

Keywords: HMGB1, pro-inflammatory cytokines, Type 2 diabetes mellitus, renal mesangial cells, high glucose

Introduction

Diabetic nephropathy (DN) is one of the most devastating complications of diabetes, and has been increasingly prevalent worldwide [1, 2]. It leads the cause of end-stage renal disease (ESRD) in developed countries and accounts for approximately 16.4% of ESRD cases of in China [3], and accounts for a significant increase in morbidity and mortality in patients with diabetes, underscoring the importance of therapeutic interventions directed at preventing the development and progression of diabetic kidney disease [4]. In particular, diabetic patients, with either type 1 or type 2, with microalbuminuria typically progress to proteinuria and overt DN, in the absence of intervention. The sustained hyperglycemia results in histo-pathological changes to the kidney include increased glomerular basement membrane (GBM) thickness, microaneurysm formation, mesangial nodule formation (Kimmelsteil-Wilson bodies) [5]. However, little is known about the molecular pathogenesis of DN.

DN is historically considered to be a non-inflammatory disease, however, current available management, which includes tight control of blood pressure and blood glucose, and inhibition of the renin-angiotensin-aldosterone axis, provides at best a 30% reduction in the rate of decline of kidney function [6, 7]. And immunological and inflammatory processes are conceived by more and more people as key regulators in the development and progression of renal failure of DN [8, 9]. It is well recognized that proinflammatory NF-B is central in mediating signaling path ways that ultimately result in renal fibrosis and renal failure of DN [10, 11]. Therefore, investigations into the mechanisms underlying renal inflammation may provide new insight into novel therapeutic targets for patients with DN.
Research in a rat model indicate that along with hyperglycaemia, inflammation and RAS activation blame for acute kidney injury during general anaesthesia [12]. And such molecules as the β subunit of protein kinase C (PKCβ) [13], CD40-CD40L signaling [14] were shown to promote vascular inflammation in diabetic ApoE null mice or in type 2 diabetes. Toll-like receptors (TLRs) are pattern-recognition receptors, which are important for pathogen recognition by conserved structural motifs [15]. Upon activation by exogenous or endogenous ligands, all TLRs are able to activate the NF-B pathway, which leads to the synthesis of proinflammatory cytokines and chemokines [16]. Moreover, endogenous ligands to TLR2 and 4 such as fibronectin, and heat shock proteins (HSPs) are confirmed in type 2 diabetes mellitus (T2DM) patients to be promoted by high glucose and ischemia-hypoxia, both of which are main pathophysiological presence of DN [17, 18]. And High-mobility group box-1 (HMGB1) which is also a ligand for TLR2 and 4 has also been upregulated in a rat model of diabetes mellitus [19].

In the present study, we examined serum HMGB1 levels in patients with T2DM, and then investigated the promotion to HMGB1 in SV40 MES 13 cells by high glucose. Results demonstrated that high glucose promoted the pre-inflammatory cytokines in vivo or in vitro, via upregulating HMGB1 and activating NF-B signaling.

Materials and methods

Patients and tissue specimens

T2DM group: 50 cases of outpatient or inpatient diagnosed with T2DM, from Mar 2010 to Nov 2013, in the Second Hospital of Jilin University, in Changchun, Jilin Province of China. Diagnostic criteria: meeting the 1999 WHO diagnosis of diabetes (fasting plasma glucose ≥ 7.0 mmol/L and/or 2-hour postprandial plasma glucose ≥ 11.1 mmol/L) and the classification criteria, with kidney dysfunction to various degree. Normal control group: 49 cases healthy subjects without family history of diabetes post the health examination in the hospital within the same period. The blood samples were collected from the cubital vein, and the serum was isolated from the whole blood sample post coagulation. This study was approved by the Internal Review Board (IRB) of Second Hospital of Jilin University, and each participant signed the informed consent.

Cell culture and treatment with reagents

Renal mesangial SV40 MES 13 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA) and were cultured in low glucose Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37°C, with 5% CO2. For the high glucose treatment, D-Glucose was added to the culture medium (25 or 50 mM final concentration) for 6 or 24 h. The recombinant HMGB1 (ebioscience, San Diego, CA, USA) was added to the culture (1 μM final concentration) for 6 or 24 h to reconfirm the promotion to pre-inflammatory cytokines. For siRNA experiments, SV40 MES 13 cells were transfected with HMGB1 siRNA (inhibitor) or control scramble RNA (GenePharma Technology, Shanghai, China) using lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions.

RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR)

Total mRNA was extracted from cell samples by using the Trizol reagent (Life Technologies, Grand Island, NY, USA). RT-qPCR assay of TNFα, IL-1β, IL-6 or HMGB1 mRNA level was performed using the SYBR Real-Time PCR kit (Takara, Tokyo, Japan) on the LightCycle 2.0 (Roche, Mannheim, Germany). All mRNA expression levels were normalized to β-actin. ΔΔCt method was utilized for the relative quantification [20].

Protein isolation and Western blot analysis

Proteins in nucleus or in cytosol were prepared with the Nuclear/Cytosol Fractionation Kit (BioVision, San Diego, CA, USA) according to the manufacturer’s guidance, and were supplemented with a protease inhibitor cocktail (Pierce, Rockford, IL, USA). Rabbit polyclone antibodies against mouse HMGB1 (Sigma-Aldrich, St. Louis, MO, USA), p65 (Abcam, Cambridge, UK), β-actin (Sinobiol, Beijing, China), or Lamin A/C (Abcam, Cambridge, UK) were used for the Western blot assay for each protein. Goat anti-rabbit IgG conjugated to
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<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>T2DM</th>
<th>P value</th>
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<tr>
<td>Age</td>
<td>50.48 ± 9.62</td>
<td>55.53 ± 11.16</td>
<td>0.172</td>
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<tr>
<td>Male/female</td>
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<td>27/23</td>
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<td>BMI (kg/m²)</td>
<td>24.20 ± 3.09</td>
<td>26.92 ± 3.16</td>
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<td>eGFR (mL/min/1.73 m²)</td>
<td>115 ± 25</td>
<td>68 ± 28</td>
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<td>FPG (mmol/l)</td>
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<td>9.43±2.12</td>
<td>&lt; 0.001</td>
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<td>HbA1c (%)</td>
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<td>8.65 ± 1.72</td>
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<td>TC (mmol/l)</td>
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<td>5.39 ± 1.21</td>
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<td>TG (mmol/l)</td>
<td>1.03 ± 0.49</td>
<td>2.40 ± 1.07</td>
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<td>HDL-C (mmol/l)</td>
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<td>LDL-C (mmol/l)</td>
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<td>FINS (mU/L)</td>
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<td>1.60 ± 0.31</td>
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<td>HOMA-IR</td>
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<td>1.64 ± 0.20</td>
<td>&lt; 0.001</td>
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<td>IL-6 (ng/ml)</td>
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<td>36.70 ± 7.60</td>
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<td>TNFα (ng/ml)</td>
<td>25.44 ± 6.58</td>
<td>41.29 ± 14.68</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HMGB1 (ng/ml)</td>
<td>4.82 ± 1.53</td>
<td>15.31 ± 5.47</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 1. Clinical and biochemical characteristics of normal and T2DM subjects

Horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA) and ECL detection systems (Amersham Pharmacia Biotech, Amersham, UK) were used for detection.

Statistical analysis

SPSS17.0 statistical software was utilized for all data normality test, and the data with normal distribution were expressed as mean ± SE, the data without normal distribution (FINS and HOMA-IR) were logarithmically transformed to normality to analyze. ANOVA was used for comparing multiple groups. The coefficient of Pearson’s correlation was used to assess the correlations between serum HMGB1 and serum IL-6 or serum TNFα. X² test was used for comparing count data. P < 0.05 was considered statistically significant.

Results

Clinical and biochemical characteristics

Main metabolic and biochemical characteristics of all subjects are summarized in Table 1. There were 27 males and 23 females with an average age of 55.53 ± 11.16 years in the T2DM group; and 23 males and 26 females were included in the control group (P=0.427), with well matched age of 50.48 ± 9.62 (P=0.172). The detailed value of BMI, FPG, HbA1c, TC, TG, HDL-C, LDL-C, FINS, or HOMA-IR, in the T2DM or control group was shown in Table 1. There was significant difference in each of above-mentioned items, except HDL-C (P < 0.01 or P < 0.001). On the whole, the abnormal lipid metabolism, the deregulated plasma glucose and the high level of insulin resistance discriminated the T2DM subjects from the normal ones (Table 1).

Serum HMGB1 was upregulated in T2DM, correlating with serum IL-6 and TNFα

To investigate the inflammatory reaction to the high glucose in T2DM subjects, we then examined the serum levels of pre-inflammatory cytokines such as TNFα, IL-1β, IL-6 and HMGB1 in T2DM patients and in control subjects. It was demonstrated in Table 1 that the serum IL-6 was averaged 36.70 ± 7.60 pg/ml in T2DM patients, far higher than 18.88 ± 4.51 pg/ml in the control subjects (P < 0.001). And the level of TNFα was also significantly higher in T2DM patients than in control subjects, with 41.29 ± 14.68 vs. 25.44 ± 6.58 pg/ml (P < 0.001). Moreover, HMGB1 was also markedly upregulated in the serum of T2DM patients, the level of which was 15.31 ± 5.47 ng/ml, whereas the serum HMGB1 was only 4.82 ± 1.53 ng/ml in control subjects (P < 0.001).

HMGB1 is identified to induce a signaling cascade that activates NF-B, leading to the synthesis of proinflammatory cytokines [21, 22]. To further investigate the possible regulatory role of HMGB1 in the promotion to IL-6 and TNFα in T2DM, we then analyzed the correlation of the upregulated IL-6 or TNFα with the HMGB1 level in T2DM subjects. As shown in Figure 1, the IL-6 level correlated closely with the serum HMGB1 in these T2DM patients (R²=0.3017, P < 0.001). And such correlation was also confirmed between the serum TNFα and HMGB1 in T2DM subjects, the R² for the two parameters was 0.5544, there was a marked significance between them (P < 0.001). Taken together, HMGB1 was promoted in T2DM, correlating with serum IL-6 and TNFα.

High glucose induced proinflammatory cytokines and HMGB1 in mesangial cells

To confirm the inflammatory reaction to the high glucose in mesangial cells, we then evaluated by RT-qPCR the induction of HMGB1 and
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Figure 1. Univariate correlations of HMGB1 with serum IL-6 or TNFα. A. Correlation between the serum HMGB1 with the serum IL-6; B. Correlation between the serum HMGB1 with the serum TNFα.

Figure 2. mRNA levels of TNF-α, IL-1β, IL-6 and HMGB1 in mesangial SV40 MES 13 cells, post the treatment with high glucose. Mesangial SV40 MES 13 cells were treated with 5, 25 or 50 mM D-glucose for 6 hours, and then were quantified for the level of TNF-α (A), IL-1β (B), IL-6 (C), or HMGB1 (D) with specific primers for each molecule. Each result was averaged for triple independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ns: no significance.
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Results demonstrated that the stimulation of 25 or 50 mM D-glucose for more than 6 hours promoted significant high mRNA levels of TNF-α, IL-1β, and IL-6 (Figure 2A-C), with a fold change of 1.557 ± 0.1162 or 2.363 ± 0.2714 for TNF-α in the group of 25 or 50 mM D-glucose, in contrast to the group of 5 mM D-glucose (P < 0.05 or P < 0.01); the fold change of IL-1β mRNA was 1.427 ± 0.095 for the group of 5 mM D-glucose. Knockdown of HMGB1 reduces the high glucose-promoted TNF-α, IL-1β, and IL-6 in mesangial SV40 MES 13 cells. (A) Mesangial SV40 MES 13 cells were transfected with 25 or 50 nM HMGB1 inhibitor or scramble RNA, and the HMGB1 level was assayed by real-time quantitative PCR. (B-D) Mesangial SV40 MES 13 cells were treated with 5 or 50 mM D-glucose, or with 1 μg/ml HMGB1, and then were transfected with HMGB1 inhibitor or scramble RNA for 6 hours. Then the mRNA level of TNF-α (B), IL-1β (C), or IL-6 (D) was evaluated by real-time quantitative PCR. Each value was the means ± SD of triple independent results. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no significance.
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mM D-glucose (P < 0.05); and the IL-6 mRNA also increased, with a fold change of 2.552 ± 0.1993 or 3.547 ± 0.2272 for 25 or 50 mM D-glucose (P < 0.01 or P < 0.001). Moreover, the relative HMGB1 mRNA was also upregulated by the high D-glucose, to 1.784 ± 0.182, 2.624 ± 0.177, compared to the control group (P < 0.05 or P < 0.01).

**HMGB1 mediates the D-Glucose-induced pro-inflammatory cytokines in mesangial cells**

To further identify the role of HMGB1 in the induction of pro-inflammatory cytokines by D-Glucose in retinal ARPE-19 cells, we then manipulated the HMGB1 level in mesangial cells using RNAi method, and then re-examined the induction of pro-inflammatory cytokines. Firstly, we knocked down HMGB1 in SV40 MES 13 cells, via transfecting HMGB1-specific inhibitor, with scramble RNA as control. As shown in **Figure 3A**, the transfection with 25 or 50 nM HMGB1 inhibitor significantly reduced the HMGB1 mRNA level in SV40 MES 13 cells, compared to the control scramble RNA (P < 0.05 or P < 0.01). Then we re-examined the mRNA level of TNFα, IL-1β and IL-6 in the SV40 MES 13 cells, post the transfection with 50 nM HMGB1 inhibitor and in the presence of 50 mM D-Glucose. It was shown that the glucose-promoted mRNA level of TNFα (**Figure 3B** column 3 vs. column 2), IL-1β (**Figure 3C** column 3 vs. column 2) or IL-6 (**Figure 3D** column 3 vs. column 2) was significantly reduced by the HMGB1 inhibitor transfection (P < 0.05 for TNFα or IL-6). In addition, to confirm the promotion by HMGB1 to the expression of TNFα, IL-1β and IL-6 in SV40 MES 13 cells, we treated SV40 MES 13 cells with 1 μg/ml exogenous HMGB1. The level of the three cytokines were coincidently upregulated (P < 0.01 or P < 0.001; Column 4 vs. column 1, in **Figure 3B-D**). And the upregulated cytokines were also significantly reduced by the transfection of HMGB1 inhibi-

![Western blot analysis of NF-κB signaling in the high glucose-treated mesangial SV40 MES 13 cells, with or without HMGB1 knockdown. A and B, Western blot assay of HMGB1 in cytosol, of p65 in cytosol or in nucleus of SV40 MES 13 cells, treated with 5, 25 or 50 mM D-Glucose for 24 h; C and D, Western blot assay of the high glucose-induced HMGB1 in cytosol, of p65 in cytosol or in nucleus of SV40 MES 13 cells, with or without HMGB1 knockdown. *P < 0.05, **P < 0.01.](image-url)
NF-κB signaling pathway involves in the promotion by D-Glucose glucose of pro-inflammatory cytokines

It is reported that the initiation of inflammatory reactions by HMGB1 is mediated by the activation of NF-κB signaling [23]. To clarify the mechanisms by which HMGB1 mediates the high glucose-induced pro-inflammatory cytokines, next, we detected whether NF-κB was activated in high glucose-treated SV40 MES 13 cells. The Western blot analysis indicated that in addition to the HMGB1 promotion, the p65 was upregulated in the cytosol and nucleus in the high glucose-treated SV40 MES 13 cells (P < 0.05 or P < 0.01; Figure 4A, 4B). To further confirm the high glucose-induced pro-inflammatory cytokines was mediated HMGB1 via the NF-κB signaling pathway, cells were knocked down of HMGB1 gene expression by the HMGB1 inhibitor. As shown in Figure 4C and 4D, the HMGB1 inhibitor not only reduced the HMGB1 level in the high glucose-treated SV40 MES 13 cells, but also reduced the p65 level in cytosol and in nucleus (P < 0.01 respectively).

Discussion

High-mobility group box-1 (HMGB1) was initially recognized as a highly conserved regulator to transcription [24], and was later confirmed to be a cytokine which is passively released during cell damage or actively secreted by monocytes and macrophages in response to injury [24-27]. In addition, extracellular HMGB1 is able to induce a signaling cascade that activates NF-κB, leading to the synthesis of pro-inflammatory cytokines [21, 22]. In the present study, we found the inflammatory reaction to the high glucose in T2DM subjects, there was significantly high levels of TNFα, IL-1β and IL-6 in T2DM subjects. Moreover, the HMGB1 was promoted by the high level of glucose in vivo. The serum HMGB1 was upregulated in T2DM, correlating with serum IL-6 and TNFα. And the inflammatory reaction to the high glucose was reconfirmed in mesangial cells. SV40 MES 13 cells, which were treated with high D-glucose expressed a significant high mRNA level of TNF-α, IL-1β and IL-6. In addition, the relative HMGB1 mRNA was also upregulated by the high D-Glucose.

The present study reconfirmed the regulatory role of HMGB1 in the D-glucose-induced pro-inflammatory cytokines in SV40 MES 13 cells. The transfection with HMGB1-specific inhibitor significantly reduced the HMGB1 mRNA level in SV40 MES 13 cells, and inhibited the promotion to TNFα, IL-1β and IL-6 in the SV40 MES 13 cells. In addition, the exogenous HMGB1 also significantly promoted the TNFα, IL-1β and IL-6 in SV40 MES 13 cells, and the upregulated cytokines were also significantly reduced by the transfection of HMGB1 inhibitor. Therefore, the present study confirmed that HMGB1 mediated the glucose-induced pro-inflammatory cytokines in SV40 MES 13 cells. In addition, the NF-κB signaling pathway was also confirmed to involve in the promotion by D-Glucose of pro-inflammatory cytokines. We found that the p65 was upregulated in the cytosol and nucleus in the high glucose-treated SV40 MES 13 cells, and the knockdown of HMGB1 gene expression by the HMGB1 inhibitor reduced the p65 level in cytosol and in nucleus.

Diabetic nephropathy (DN) is now being accepted as a chronic low-grade inflammatory disease, rather than as a metabolic disease. Pro-inflammatory cytokines, such as TNF-α and IL-6 play important roles in the pathogenesis and clinical outcome of DN [28, 29]. On the other side, therapeutic strategies targeting cytokine promotion pose effectiveness against DN. The treatment of anti-inflammatory and antioxidative activities reduces the renal dysfunction and damage that occur in DN [30]. The overexpression of suppressors of cytokine signaling (SOCS) has therapeutic effect in DN [31]. Therefore, it is very important to uncover the activation of inflammatory response in DN, and to investigate the effectiveness of therapeutic agents targeting the activation of inflammatory responses in DN.

In summary, the present study indicated that HMGB1 was significantly promoted by the glucose in vivo or in vitro, in an association with an upregulation of pro-inflammatory cytokines, via activating NF-κB signaling pathway. And the strategy of HMGB1 inhibition reduced the upregulation of pro-inflammatory cytokines in response to high glucose, via inhibiting the NF-κB signaling pathway. It implies the regula-
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Disclosure of conflict of interest

None.

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