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High glucose induces activation of NF-κB inflammatory signaling through IκBα sumoylation in rat mesangial cells

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ABSTRACT

The posttranslational modification of proteins by small ubiquitin-like modifiers (SUMOs) has emerged as an important regulatory mechanism for the alteration of protein activity, stability, and cellular localization. The latest research demonstrates that sumoylation is extensively involved in the regulation of the nuclear factor κB (NF-κB) pathway, which plays a critical role in the regulation of inflammation and contributes to fibrosis in diabetic nephropathy (DN). However, the role of sumoylation in the regulation of NF-κB signaling in DN is still unclear. In the present study, we cultured rat glomerular mesangial cells (GMCs) stimulated by high glucose and divided GMCs into six groups: normal glucose group (5.6 mmol/L), high glucose groups (10, 20, and 30 mmol/L), mannitol group (i.e., osmotic control group), and MG132 intervention group (30 mmol/L glucose with MG132, a proteasome inhibitor). The expression of SUMO1, SUMO2/3, IκBα, NF-κBp65, and monocyte chemotactic protein 1 (MCP-1) was measured by Western blot, reverse-transcription polymerase chain reaction, and indirect immunofluorescence laser scanning confocal microscopy. The interaction between SUMO1, SUMO2/3, and IκBα was observed by co-immunoprecipitation. The results showed that the expression of SUMO1 and SUMO2/3 was dose- and time-dependently enhanced by high glucose ($p < 0.05$). However, the expression of IκBα sumoylation in high glucose was significantly decreased compared with the normal glucose group ($p < 0.05$). The expression of IκBα was dose- and time-dependently decreased, and NF-κBp65 and MCP-1 were increased under high glucose conditions, which could be mostly reversed by adding MG132 ($p < 0.05$). The present results support the hypothesis that high glucose may activate NF-κB inflammatory signaling through IκBα sumoylation and ubiquitination.

1. Introduction

Diabetic nephropathy (DN) is a common and serious diabetic microvascular complication. Recent studies have shown that inflammation is a key link in the development of DN [1]. Nuclear factor κB (NF-κB) comprises a family of transcription factors that play a central regulatory role in the immune response and the expression of various cytokines, inflammatory cytokines, and adhesion molecules involved in the occurrence of DN [2].

In unstimulated cells, NF-κB is conjugated to IκBα and kept in an inactive state in the cytosol [3]. IκBα can prevent the nuclear accumulation of NF-κB and promote the nuclear export of bound NF-κB, ensuring the cytoplasmic localization of inactive NF-κB dimers. Canonical NF-κB signaling includes the release of active NF-κB in the cytoplasm by activation of the cytoplasmic kinase complex, known as IκB kinase (IKK) [4]. In most cases, activated IKK promotes the phosphorylation of IκBα and its subsequent degradation by the ubiquitin–proteasome pathway (UPP), thereby exposing the NLS of NF-κB and leading to its translocation from the cytoplasm to the nucleus, where it activates the transcription of genes for the immune response to different physiological or pathological stimuli [5,6].

The posttranslational modification (PTM) of proteins through phosphorylation, methylation, acetylation, or ubiquitylation represents a central mechanism through which various biological processes are regulated [7]. Ubiquitin is well known for its function in targeting proteins for degradation by the 26S proteasome, which is important for the removal of abnormal and damaged proteins and many regulated processes, including the cell cycle, DNA repair, signal transduction, and the regulation of histones [8].

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Among these PTMs, small ubiquitin-like modifier (SUMO) is the best-characterized member of a family of ubiquitin-related proteins [9]. Small ubiquitin-like modifiers are structurally related to ubiquitin. Four different SUMO isoforms have been detected in mammals: SUMO1, SUMO2, SUMO3, and SUMO4. Only SUMO1, -2, and -3 can be conjugated to target substrate proteins. SUMO2 and SUMO3 have very similar sequences; therefore, they are sometimes referred to as SUMO2/3 [10]. Similar to ubiquitin, SUMO is covalently linked to its targets using an enzymatic cascade of E1, E2, and E3 enzymes. Additionally, SUMOs are ligated to the same lysine residues within the target proteins of ubiquitin [9]. Sumoylation is regulated by SUMO-specific proteases (SENPs), which cleave the attached SUMO from the target proteins [11]. Sumoylation has recently emerged as an important regulatory mechanism for alterations in protein activity, stability, and cellular localization, suggesting a novel mechanism for the regulation of immune-responsive gene expression [12].

Previous studies found that oxidative stress, osmotic pressure, heat shock, ischemia, infection, and other cellular stressors can activate NF-κB signaling via the sumoylation and ubiquitination of IκBα in inflammation and other diseases [13,14]. However, the association between SUMO and NF-κB signaling in DN has not been investigated. The role of sumoylation in the regulation of NF-κB signaling molecules remains to be elucidated. The present study investigated the expression of SUMO1, SUMO2/3, IκBα, NF-κBp65, and MCP-1 and the interaction between SUMO and IκBα in cultured rat glomerular mesangial cells (GMCs) stimulated by high glucose. We sought to explore the role of sumoylation in the regulation of IκBα/NF-κB signaling in DN.

2. Materials and methods

2.1. Cell culture

Rat GMCs (HBZY-1) were purchased from the Preservation Center at Wuhan University and maintained in low-glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Hyclone) at 37 °C and 5% CO_2. The GMCs were used for all of the experiments and randomly divided into the following five groups: normal control group (NC group; with medium that contained 5.6 mmol/L glucose), 10 mmol/L glucose group (HG1 group; with medium that contained 10 mmol/L glucose), 20 mmol/L glucose group (HG2 group; with medium that contained 20 mmol/L glucose), 30 mmol/L glucose group (HG3 group; with medium that contained 30 mmol/L glucose), osmotic pressure group (OP group; with medium that contained 5.6 mmol/L glucose + 24.6 mmol/L mannitol as a control), and MG132 intervention group (MI group; with medium that contained 30 mmol/L glucose + 1 μmol/L MG132, in which MG132 was added to the culture medium to block protein ubiquitination).

Each group was cultured for 6, 12, or 24 h to detect the expression of IKKγ, IκBα, and MCP-1. The cell culture was then extended to 48 and 72 h.

2.2. Protein extraction and Western blot

Total proteins were isolated from GMCs using a total protein extraction kit (Kaiji, Shanghai, China). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblotting was performed using anti-SUMO1 antibody (rabbit; 1:800; Abcam), anti-SUMO2/3 antibody (rabbit; 1:600; Millipore), anti-IκBα antibody (mouse; 1:1000; Cell Signaling Technology), anti-NF-κBp65 antibody (rabbit; 1:1000; Cell Signaling Technology), anti-actin antibody (mouse; 1:1000; Cell Signaling Technology), and anti-GAPDH antibody (mouse; 1:2000; Beyotime).

2.3. RNA extraction and reverse-transcription polymerase chain reaction

Total RNA was extracted from GMCs using an RNA extraction kit (Tiangen Biotech, Beijing, China). Total RNA was reverse-transcribed (RT) using a Takara RNA PCR kit (Baoshengwu, Dalian, China). cDNA was amplified in a gradient thermal cycler (Eppendorf, Germany) using polymerase chain reaction (PCR) Master Mix (Baoshengwu, Dalian, China). The results were determined using an ultraviolet transilluminator and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The primer sequences were the following: SUMO1 (forward, 5′-TAT GGA CAG GAC AGCAG-3′; reverse, 5′-CCA TTCC CAGT TCTT TTG-3′), SUMO2/3 (forward, 5′-GACG AGAA ACC CAA GGA-3′; reverse, 5′-CTG CCGT TCAC AAT AGG-3′), MCP-1 (forward, 5′-AAT GAG TCG GCT GGA GAA-3′; reverse, 5′-GCT TGA GGT GGT TGT GGA-3′), and GAPDH (forward, 5′-CCT CAA GAT TGT CAG CAA T-3′; reverse, 5′-CCA TCC ACA GTC TTC TGA GT-3′).

2.4. Immunofluorescence

Cells were grown on coverslips in six-well plates. After overnight adherence, the cells were treated with media that contained high glucose and MG132 for 24 h. The cells were fixed in 4% paraformaldehyde (Pierce Biotechnology, Rockford, IL, USA) and then blocked with 5% goat serum. The cells were incubated overnight with the primary antibodies (anti-NF-κBp65) and incubated for 40 min with secondary antibody conjugated to the fluorescein isothiocyanate fluorescent dye. 4′,6′-Diamino-2-phenylindole (DAPI) was used to stain the nucleus in the cells. Images were taken with a laser scanning confocal microscope (Leica, Germany).

2.5. Immunoprecipitation and immunoblot analysis

Approximately 24 h after being treated with media that contained high glucose and mannitol, the cells were harvested. Ice-cold immunoprecipitation lysis/wash buffer was added using a co-immunoprecipitation kit (Pierce Biotechnology, Rockford, USA) with protease inhibitors (Roche, USA). The cell lysates were clarified by centrifugation at 13,000×*g* for 10 min at 4 °C, and the supernatants were subjected to immunoprecipitation. The supernatants were incubated with monoclonal anti-IκBα antibody (mouse; Cell Signaling Technology) and normal rabbit immunoglobulin G for 12 h at 4 °C. After incubation, protein A/G Sepharose was used for precipitation. The beads were washed with 1× conditioning buffer (Pierce Biotechnology, Rockford, IL, USA). The antigen–antibody complexes were collected, washed, and boiled in 2× non-reducing lane-marker sample buffer (Pierce Biotechnology, Rockford, USA). For the immunoblot analysis, proteins were probed with the appropriate antibodies (anti-SUMO1 and anti-SUMO2/3).

2.6. Statistical analysis

Each experiment was repeated at least twice. The data are expressed as mean ± standard deviation (SD). Differences were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Least Significant Difference *post hoc* test for multiple comparisons. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. High glucose induces SUMO expression in rat glomerular mesangial cells

As shown in Fig. 1, relative SUMO expression (SUMO-to-GAPDH protein ratio) was significantly increased under high glucose conditions compared with the NC group ($p < 0.05$). At a 30 mmol/L glucose concentration, SUMO1 and SUMO2/3 protein levels were highest at 24 h (Fig. 1A). SUMO1 and SUMO2/3 protein levels were significantly enhanced by different concentrations of high glucose at 24 h (Fig. 1C). Although the strongest relative expression of SUMO1 and SUMO2/3 was observed in the 20 mmol/L high glucose group ($p < 0.05$), no significant differences were found between the 20 and 30 mmol/L high glucose groups ($p > 0.05$). A significant difference was found between the 30 mmol/L mannitol (OP) and NC groups. However, SUMO1 and SUMO2/3 protein levels were significantly decreased in the OP group compared with the 20 mmol/L (HG2) and 30 mmol/L (HG3) glucose groups ($p < 0.05$), suggesting that osmotic pressure had little effect on the high glucose-induced increase in SUMO expression. Reverse-transcription PCR showed similar trends with regard to SUMO mRNA expression (Fig. 1B and D).

3.2. SUMO modification of IκBα sumoylation were attenuated by high glucose

We performed an experiment to determine whether SUMO is involved in IκBα sumoylation in GMCs. Cell extracts were prepared and subjected to immunoprecipitation using anti-IκBα antibody, and SUMO1 and SUMO2/3 co-immunoprecipitation was detected by immunoblotting with anti-SUMO1 and SUMO2/3 antibodies, respectively. As shown in Fig. 2A and B, IκBα antibodies immunoprecipitated a polypeptide of about 60 kD that was recognized by the SUMO-specific antibody. The results showed that SUMO1 and SUMO2/3 were co-immunoprecipitated with IκBα, and the SUMO-induced modification of IκBα was detected on endogenously expressed proteins. SUMO and IκBα were able to form a complex in rat GMCs. To determine whether IκBα sumoylation is affected by high glucose, we assessed the effect of 20 mmol/L high glucose and mannitol treatment on IκBα sumoylation. Interestingly, the interaction between IκBα and SUMO1 was decreased by high glucose and high osmotic pressure ($p < 0.05$; Fig. 2C). However, the SUMO2/3-induced modification of IκBα was only affected by high glucose, indicating that high glucose-induced IκBα sumoylation was not an osmotic effect ($p > 0.05$; Fig. 4D).

3.3. High glucose significantly decreased IκBα, and MG132 partially reversed IκBα degradation

The relative expression of IκBα was decreased as the glucose concentrations and time increased ($p < 0.05$). The most significant changes were observed with 30 mmol/L glucose stimulation after 72 h. IκBα was significantly attenuated by different concentrations high glucose, especially in the HG3 group (Fig. 3A). The addition of 30 mmol/L mannitol to normal glucose did not decrease the expression of IκBα ($p > 0.05$), indicating that the high glucose-induced decrease in IκBα was not an osmotic effect. However, the proteasome inhibitor MG132 partially reversed IκBα degradation (Fig. 3B). After MG132 treatment, IκBα protein levels were partially reversed compared with the HG3 group. Moreover, no apparent differences were found between the NC group and NC + MG132 group ($p > 0.05$). These data suggest that high glucose decreased the expression of IκBα through IκBα degradation.

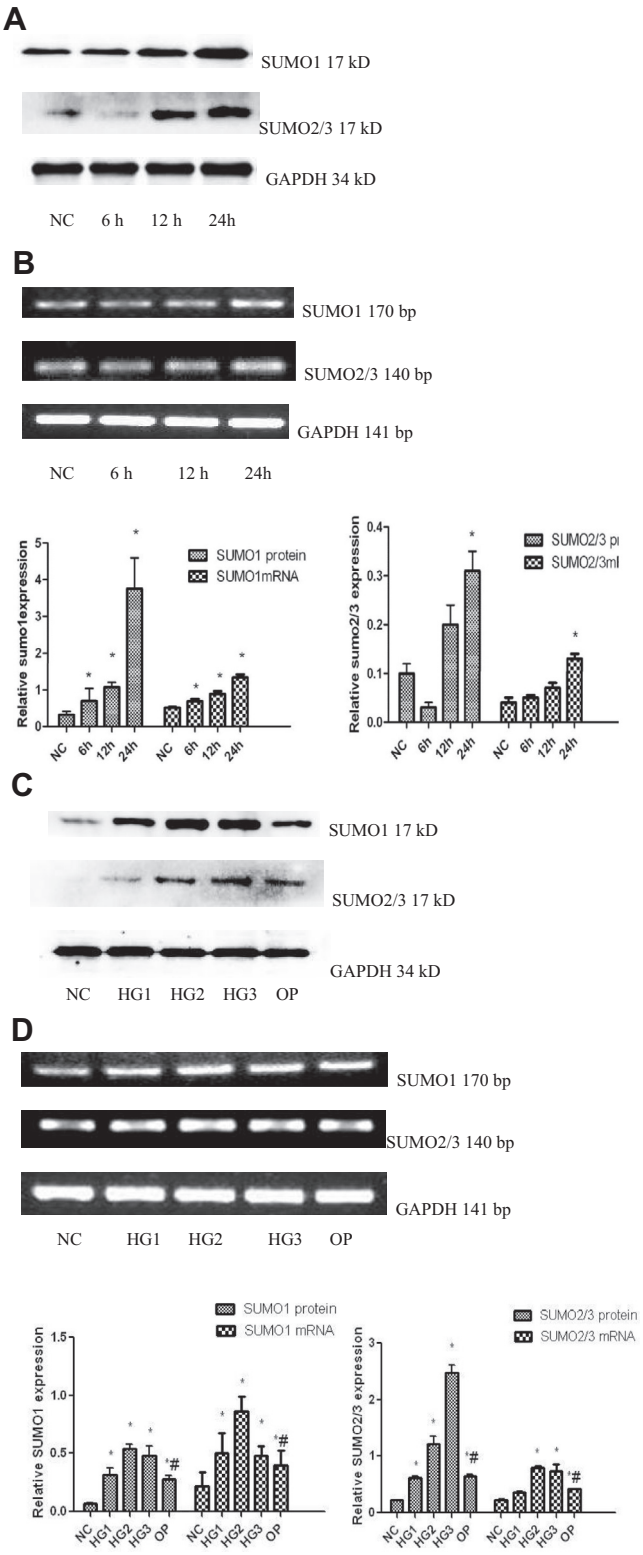

Fig. 1. SUMO protein and mRNA expression after high-glucose challenge for various times and various glucose concentrations determined by Western blot and RT-PCR. (A,B) Cells were treated with 30 mM high glucose for 6, 12, and 24 h, and Western blot and RT-PCR were performed to detect SUMO1 and SUMO2/3 protein levels. (C,D) Cells were treated with the indicated concentrations of glucose or mannitol for 24 h. The gray graph shows the relative statistical values for SUMO1 and SUMO2/3 protein and mRNA expression in each group. The data were normalized to GAPDH and are expressed as mean ± SD. $^{*}p < 0.05$, compared with NC group; $^{\#}p < 0.05$, compared with HG2 and HG3 groups.

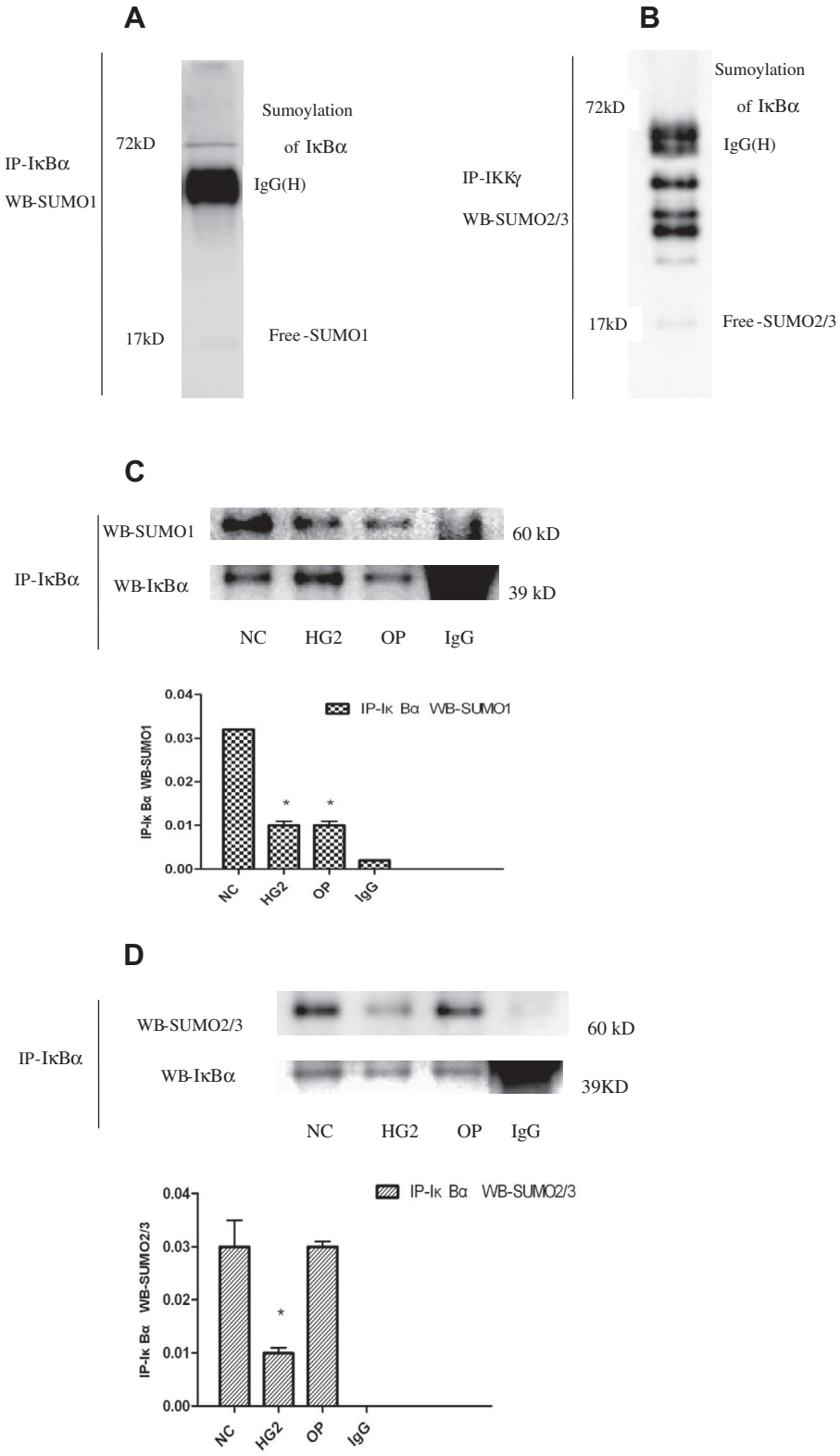

Fig. 2. IκBα is sumoylated by SUMO in mesangial cells. (A, B) IκBα sumoylation was detected by immunoprecipitation (IP) with anti-IκBα antibody or normal mouse IgG antibody as a negative control, followed by Western blot with anti-SUMO1 or anti-SUMO2/3 antibody. IκBα was conjugated with SUMO *in vitro*. IgG-H marks the IgG heavy chain. (C) Cells were treated with 5.6 mM (NC) and 20 mM (HG2) glucose and an equimolar concentration of mannitol (OP) for 24 h. Anti-IκBα immunoprecipitates were subjected to immunoblotting with anti-Smad4 or anti-SUMO antibody to detect IκBα and SUMO-IκBα proteins. IκBα that was sumoylated by SUMO1 was repressed by high glucose and high osmotic pressure. (D) SUMO2/3-induced modification of IκBα was only affected by high glucose. The gray graph confirmed these trends. $^{*}p < 0.05$, compared with NC group.

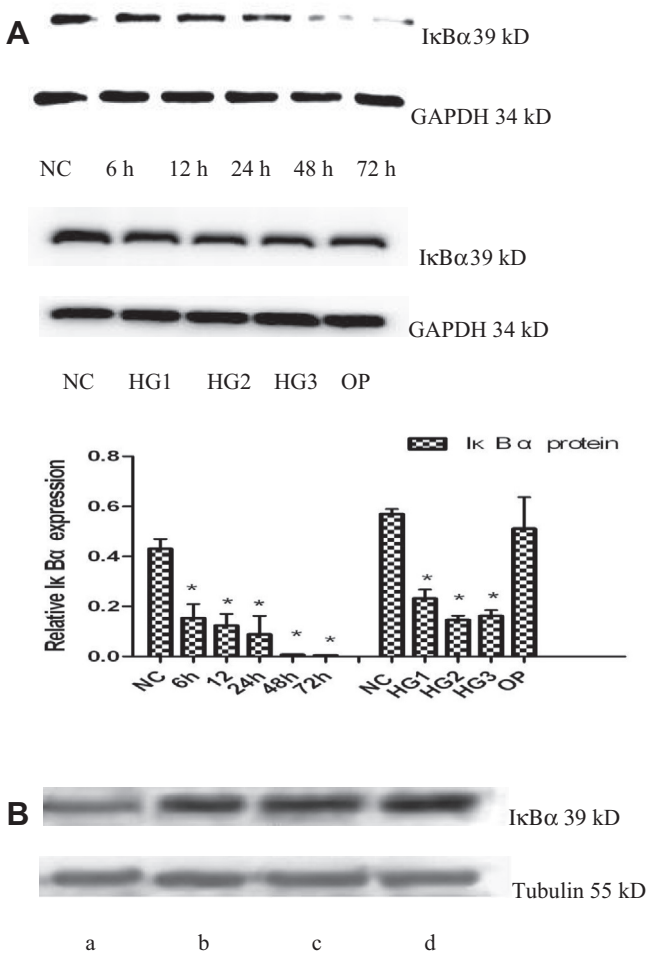

Fig. 3. The expression of IκBα was detected by Western blot. (A) IκBα decreased as the glucose concentration and time increased. The gray graph confirmed these trends. (B) MG132 partially reversed the degradation of IκBα. (a) HG3. (b) HG3 + MG132. (c) NC + MG132. (d) NC. $^{*}p < 0.05$, compared with NC group; $^{\#}p < 0.05$, compared with HG3 + MG132 group.

3.4. High glucose induced the activation of NF-κB and MCP-1

The expression of NF-κBp65 detected by Western blot was concentration-dependently increased in the high glucose group compared with the NC group ($p < 0.01$; Fig. 4A). Immunofluorescence (Fig. 4B) showed that the expression of NF-κBp65 in the nucleus significantly increased in the 20 mmol/L group (HG2) after culturing for 24 h compared with the normal glucose group ($p < 0.01$). However, elevated NF-κBp65 expression in the nucleus was significantly decreased by the addition of MG132 ($p < 0.01$; Fig. 4B). High glucose stimulation resulted in rapid and robust increases in MCP-1, which is a downstream inflammatory NF-κB signaling factor. Reverse-transcription PCR demonstrated that the expression of MCP-1 mRNA was time- and concentration-dependently increased in the high glucose group compared with the NC group ($p < 0.01$; Fig. 4C and D). The addition of 30 mmol/L mannitol to normal glucose did not result in an increase in NF-κBp65 or MCP-1 levels, suggesting that the high glucose-induced increase in NF-κBp65 and MCP-1 expression was not an osmotic effect (Fig. 4A and D).

4. Discussion

The present study investigated the mechanism of activation of the NF-κB family of transcription factors following high-glucose stress. Although hyperglycemia-induced NF-κB was first described more than a decade ago [15], the molecular mechanisms of this phenomenon remain to be elucidated.

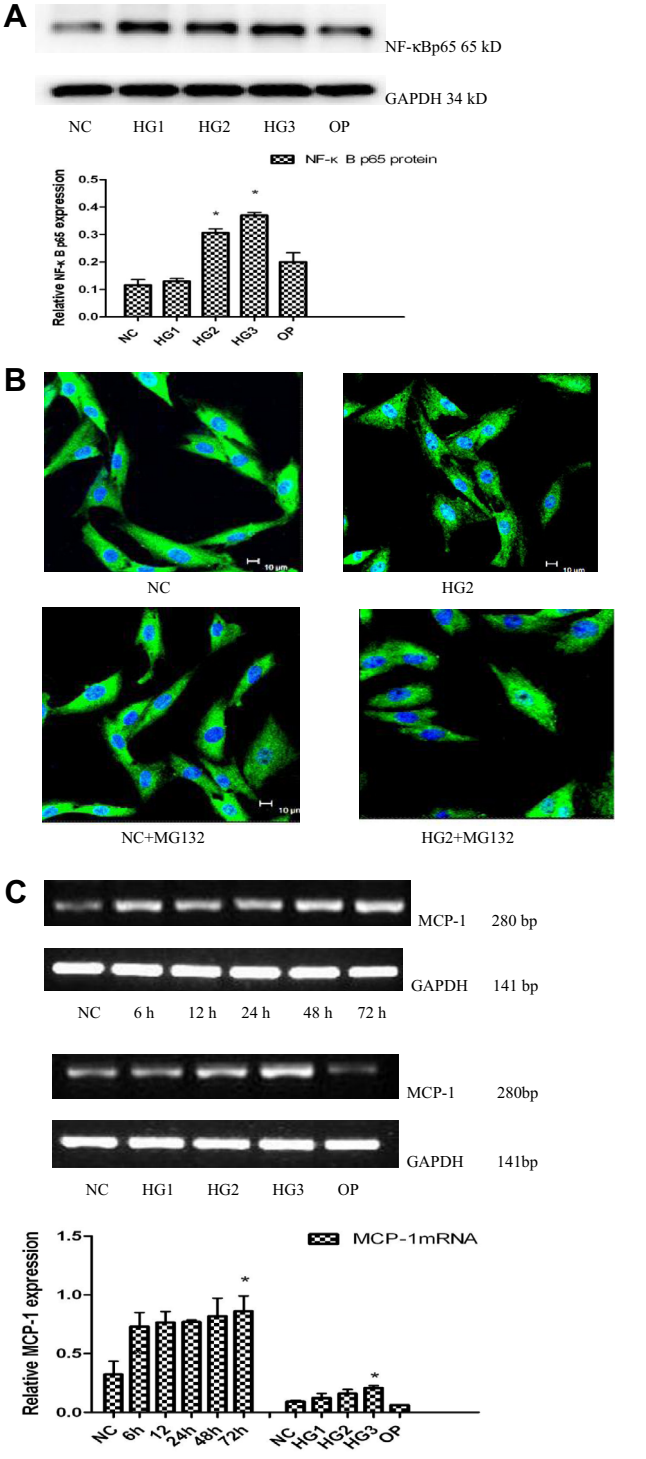

Fig. 4. NF-κBp65 and MCP-1 expression in mesangial cells induced by high glucose. (A) The expression of NF-κBp65 was detected by Western blot. The gray graph confirmed these trends. (B) NF-κBp65 expression detected by immunofluorescence and laser scanning confocal microscopy. NF-κBp65 proteins in the NC group were detected in the cytoplasm as green fluorescence that overlapped with blue fluorescence emitted by the nuclear stain DAPI. After high glucose intervention, NF-κBp65 translocated from the cytoplasm to the nucleus. This trend was strongly reversed in the MG132 intervention group. (C) The mRNA levels of MCP-1 were detected by RT-PCR. The corresponding relative gray value statistics graph of the MCP-1 mRNA level. $^{*}p < 0.05$, compared with NC group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

An increasing number of studies have reported the regulation of sumoylation by stress, suggesting an important role for this modification in the cellular response. The profiles of fractionated SUMO1- and SUMO2/3-modified proteins suggest that some proteins may be preferentially modified by specific SUMO subtypes. SUMO2/3 is thought to be conjugated to protein targets in response to various cellular stress events [16]. We found that high glucose concentration- and time-dependently increased the expression of SUMO1 and SUMO2/3, and osmotic stress had little effect on the expression of SUMO compared with high glucose [17]. The present results suggest that SUMO1 and SUMO2/3 may serve as cellular stress proteins in response to high glucose and be involved in various translational modifications of cell signaling transduction proteins. Through the activation of sumoylation, we speculate that high glucose impacts numerous inflammation and profibrotic signaling pathways, inhibits protein degradation, and participates in the development of diabetic renal diseases.

NF-κB activation is mediated by the poly-ubiquitylation of phosphorylated IκBα proteins, followed by their proteasomal degradation [18,19]. However, whether these mechanisms occur under high glucose conditions is unknown. Our results indicate that IκBα is significantly decreased by high glucose in a dose and time-dependent manner. The proteasome inhibitor MG132 partially reversed IκBα degradation and NF-κB signaling activation, suggesting that high glucose mediates the degradation of IκBα through the UPP. We also demonstrated that the SUMO-induced modification of IκBα was reflected by endogenous expression. Sumoylation is a highly regulated posttranslational modification. Recent work by different laboratories has revealed multiple cross-talk mechanisms between sumoylation and other posttranslational modifications (e.g., ubiquitination, acetylation, and phosphorylation). Previous studies reported that ubiquitin and SUMO compete for the same target lysines on IκBα, namely K21, which is also used for ubiquitin conjugation. As a result, IκBα that is modified by SUMO1 cannot be ubiquitinated and is therefore resistant to proteasome-mediated degradation [20]. Therefore, sumoylation is considered a novel negative regulatory mechanism for the control of transcriptional NF-κB activity. The addition of an NLS to IκBα was shown to restore its sumoylation *in vivo*, suggesting that the nuclear localization of IκBα is necessary for its sumoylation [21]. In the present study, we found that IκBα sumoylation occurs under normal conditions. When stimulated by high glucose or high osmotic pressure, however, IκBα sumoylation was extenuated. Similar studies reported that hypoxia induces IκBα sumoylation by Sumo2/3 [22,23], suggesting that sumoylation prevents IκBα degradation and has a direct impact on NF-κB release. Additionally, the overexpression of SUMO1 inhibits the signaling-induced activation of NF-κB-dependent transcription [24]. We speculate that IκBα sumoylation inhibits the ubiquitination of IκBα, but these mechanism can be extenuated by high glucose stress.

Osmotic stress was shown to have little effect on global sumoylation by SUMO-2/3 compared with heat shock and oxidative stress. At the level of individual targets, osmotic stress induced by sorbitol was shown to increase the sumoylation of the STAT1 transcription factor via Ser-727 phosphorylation [25]. The effects of this increase in sumoylation on STAT1 activity have not yet been elucidated. The underlying mechanisms, although yet unidentified, are expected to be identical to those that occur under high glucose conditions. The present study demonstrated that the SUMO2/3-induced modification of IκBα was only affected by high glucose, with no effect of osmotic pressure. Whether SUMO E3 is involved in the sumoylation of IκBα and whether there a specific SENP removes the SUMO moiety from IκBα have yet to be determined [26]. These additional factors may also change the balance between sumoylated and non-sumoylated pools of IκBα to further regulate NF-κB function [27]. We speculate that high glucose increases the degradation of IκBα by weakening the interaction between SUMO2/3 and IκBα and promoting IκBα ubiquitination.

Excessive activation of the transcription factor NF-κB has been shown to be implicated in the development of DN. Although our experiments indicate that high glucose exposure can impact IκBα sumoylation and activate NF-κB signaling, we suggest that SUMO may be involved in the development of DN. Interventions that involve the SUMO pathway and proteasome inhibitor MG132 may be potential therapeutic targets for the treatment of DN. No convincing evidence has been previously reported to show that sumoylation can prevent the degradation of IκBα induced by high glucose, and additional studies using siRNA or plasmid transfection and animal models are warranted to confirm our *in vitro* results [14].

In conclusion, the present study found that high glucose stimulation dose- and time-dependently enhanced the expression of SUMO1 and SUMO2/3 and significantly decreased IκBα sumoylation. The expression of IκBα was subsequently decreased, and NF-κB signaling was activated. The present results support the hypothesis that high glucose may be involved in the pathogenesis of DN by specifically impacting IκBα sumoylation and activating inflammatory NF-κB signaling. SUMO signaling molecules may be potential therapeutic targets for the treatment of DN.

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