

Protective role and molecular mechanism of action of Nesfatin-1 against high glucose-induced inflammation, oxidative stress and apoptosis in retinal epithelial cells

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Abstract. Diabetic retinopathy (DR) is a major complication of diabetes mellitus that may cause severe visual impairment. It has been reported that the levels of nesfatin-1 in the serum and vitreous humor were negatively correlated with DR; however, its role in DR has not been fully elucidated. Therefore, the present study was performed to investigate the effect of nesfatin-1 on high glucose-treated human retinal epithelial cells (ARPE-19) and explore the underlying mechanism. The effects of nesfatin-1 on cell viability, inflammation, oxidative stress and apoptosis were examined under high glucose conditions. The Cell Counting Kit-8 assay was used to determine cell viability. The levels of inflammatory cytokines were evaluated using ELISA kits. The reactive oxygen species and malondialdehyde content was estimated using commercial assay kits. Flow cytometry was performed to detect apoptotic cells and western blot analysis was employed to evaluate the expression of apoptosis-associated proteins. Moreover, the levels of NF- κ B, NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and high-mobility group protein B1 (HMGB1) were determined via western blot analysis. The results revealed that nesfatin-1 enhanced cell viability and suppressed inflammation, oxidative stress and apoptosis in the presence of high glucose concentration. Moreover, the activation of the NF- κ B/NLRP3 inflammasome signaling and the expression of HMGB1 were inhibited by nesfatin-1. Furthermore, HMGB1 overexpression partially abrogated

the inactivation of the NF- κ B/NLRP3 inflammasome pathway caused by nesfatin-1. Taken together, these findings demonstrated that nesfatin-1 inhibited the activation of the NF- κ B/NLRP3 inflammasome signaling via modulating HMGB1 and exerted a protective effect on ARPE-19 cells against high glucose-induced inflammation, oxidative stress and apoptosis.

Introduction

Diabetes mellitus (DM) includes a group of metabolic conditions that are caused by disrupted carbohydrate metabolism due to islet dysfunction, which manifests as hyperglycemia (1). Chronic hyperglycemia results in the dysfunction of various tissues and organs, including the eyes, kidney, heart, vessels and nerves, with visual damage appearing in the early stages of DM (2). Diabetic retinopathy (DR) is a major classical microvascular complication of DM, resulting in retinal ischemia, altered retinal permeability, neovascularization and macular edema, eventually leading to visual impairment and blindness (3,4). DR is classified into two stages: Non-proliferative diabetic retinopathy and proliferative diabetic retinopathy (PDR). PDR is the most advanced stage of the disease, and new vessels grow in a chaotic manner, causing vitreous hemorrhage and retinal detachment (5). Epidemiological data have revealed that 93 million individuals (35% of diabetic adults aged 20-76 years) suffered from DR in 2010 worldwide, of whom 17 million exhibited PDR and 21 million displayed diabetic macular edema (3). It has been estimated that the number of DR cases will continue to increase by 2050 (6).

Vascular dysfunction, including endothelial cell damage, pericyte death, retinal capillary basement membrane thickening and tight junction alterations has been a major focus of DR research; however, diabetic vascular dysfunction alone cannot explain the loss of retinal function (7). Accumulating evidence has pointed to the key role of inflammation in the progression of DR (8-10). Previous studies have reported that the levels of various inflammatory cytokines, including IL-1 β , IL-6, IL-8, TNF- α and monocyte chemoattractant protein-1, were increased in the vitreous humor of patients with DR

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compared with healthy subjects (11-13). Of note, a recent study demonstrated that the serum and vitreous concentrations of nesfatin-1 were negatively correlated with DR (14).

Nesfatin-1 is a secretory peptide distributed in the hypothalamus and brainstem and is produced by the hydrolysis of nucleobindin-2 at its N-terminal (15). A previous study has suggested that nesfatin-1 may ameliorate the high glucose-induced toxicity in PC12 cells via the regulation of oxidative stress, autophagy and apoptosis (16). Furthermore, it has been demonstrated that nesfatin-1 suppressed NF- κ B-dependent inflammatory responses and attenuated caspase-3-mediated neuronal cell apoptosis following traumatic brain injury in rats (17), indicating the anti-inflammatory and anti-apoptotic effects of nesfatin-1. However, the detailed function of nesfatin-1 in DR remains elusive.

High expression of NF- κ B and NACHT, LRR and PYD domains-containing protein 3 (NLRP3) has been indicated to be closely associated with inflammation in DR (18-20). Enhanced expression level of NLRP3, caspase-1 and IL-1 β has been observed in proliferative membranes of patients with DR compared with healthy subjects and in high glucose-treated human retinal endothelial cells compared with untreated cells, whereas inhibition of NLRP3 significantly alleviated inflammatory responses (21). In addition to NLRP3, high-mobility group protein B1 (HMGB1) has also been indicated to promote inflammation in DR, and inhibition of HMGB1 attenuated the NF- κ B activity in human retinal epithelial cells cultured with high glucose (22). Moreover, HMGB1 has been indicated to bind with the receptor for advanced glycation end products to accelerate rat retinal cell apoptosis (23). Previous studies have demonstrated that HMGB1 promoted the activation of the NLRP3 inflammasome, upregulated the expression level of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 (24,25), whereas nesfatin-1 reduced the activation of the NF- κ B pathway via downregulating the expression of HMGB1 (26). Therefore, the present study was performed to investigate the role of nesfatin-1 in DR and explore the potential association among HMGB1, NF- κ B, NLRP3 and nesfatin-1.

Materials and methods

Cell culture and treatment. The human retinal epithelial cell line ARPE-19 was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences and cultured in DMEM/F12 (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in an incubator with 95% air/5% CO₂. In order to examine the effects of hyperglycemia, ARPE-19 cells were cultured with high concentration of glucose (33 mM; Sigma-Aldrich; Merck KGaA), normal concentration of glucose (5.5 mM) or 27.5 mM mannitol + 5.5 mM glucose as an osmotic control for 48 h. ARPE-19 cells were pretreated with nesfatin-1 (2.5 or 5 ng/ml; Sigma-Aldrich; Merck KGaA) for 1 h, followed by exposure to 33 mM glucose for 48 h (16).

Cell transfection. ARPE-19 cells (2x10⁵ cells/well) were seeded into 6-well plates. Subsequently, cells were transfected with

HMGB1 plasmid (pcDNA3.1-HMGB1; 50 nM) or the empty vector plasmid (pcDNA3.1; 50 nM) using Lipofectamine[®] 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C, according to the manufacturer's instructions. These plasmids were provided by Shanghai GenePharma Co., Ltd. After 48 h, cells were treated with glucose/nesfatin as aforementioned. The transfected cells were harvested and protein expression was examined with western blot analysis.

Cell Counting Kit-8 (CCK-8) assay. Cell viability was determined using the CCK-8 assay. Briefly, ARPE-19 cells were seeded into a 96-well plate at a density of 5,000 cells/well. Following incubation with glucose and/or nesfatin-1 as aforementioned, 10 μ l CCK-8 solution (Nanjing KeyGen Biotech Co., Ltd.) was added into each well for 1 h at 37°C. The absorbance of each well was measured using a microplate reader at 450 nm.

Colony formation assay. ARPE-19 cells were seeded into 6-well plates (500 cells/well) and incubated for 24 h at 37°C to allow for adherence. The medium was changed every 3 days. After 2 weeks of culture, the cells were fixed with methanol for 30 min at room temperature and stained with 0.2% crystal violet at room temperature for 30 min. Images were captured using a light microscope (Olympus Corporation) at x10 magnification.

ELISA. ARPE-19 cell culture medium was collected into a centrifuge tube and centrifuged at 1,000 x g for 10 min at 4°C. Subsequently, the concentration of TNF- α (cat. no. SEKH-0047; Beijing Solarbio Science & Technology Co., Ltd.), IL-1 β (cat. no. F01220) and IL-6 (cat. no. F01310; both from Shanghai Xitang Biotechnology Co., Ltd.) was measured by ELISA kits according to the manufacturer's instructions. The absorbance of each well was measured using a microplate reader at 450 nm. The concentration of nesfatin-1 was determined with a commercially available ELISA kit (cat. no. JL19919-96T; Shanghai Jianglai Biological Technology Co., Ltd.) in accordance with the manufacturer's instructions.

Measurement of oxidative stress-related malondialdehyde (MDA). ARPE-19 cells were collected into a centrifuge tube and centrifuged at 8,000 x g for 10 min at 4°C. Subsequently, the reagents of the MDA assay kit (cat. no. BC0025; Beijing Solarbio Science & Technology Co., Ltd.) were added into the centrifuge tube according to the manufacturer's protocol. The mixture was heated at 100°C for 60 min, cooled on ice and centrifuged at 10,000 x g for 10 min at room temperature. Subsequently, 200 μ l supernatant was added into a 96-well plate. The absorbance of each well was recorded using a microplate reader at 532 nm.

Measurement of reactive oxygen species (ROS). The production of intracellular ROS was determined with a ROS assay kit (cat. no. D6470; Beijing Solarbio Science & Technology Co., Ltd.) using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as a fluorescence probe. ARPE-19 cells were seeded into a 6-well plate, and DCFH-DA (10 μ M) was added into each well according to the manufacturer's protocol for 20 min at 37°C.

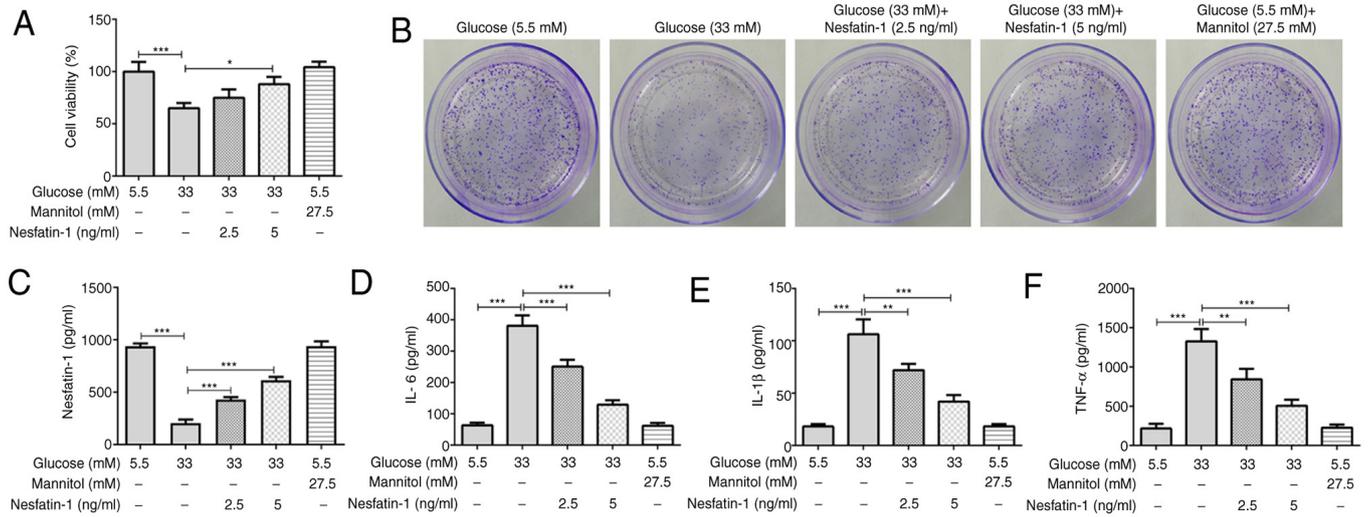


Figure 1. Nesfatin-1 enhances cell viability and decreases inflammatory responses in high glucose-treated ARPE-19 cells. (A) Cell viability in each group was assessed using Cell Counting Kit-8 assay. (B) Cell proliferative ability was evaluated with colony formation assay. (C) The levels of nesfatin-1 were determined using ELISA. (D-F) The concentration of the indicated pro-inflammatory cytokines in the cell culture medium of each group was determined using ELISA. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Subsequently, the cells were washed with DMEM/F12 three times and the absorbance of each well was measured using a fluorescence microplate reader at 488 and 525 nm.

Flow cytometry analysis. To determine apoptosis, 2×10^5 ARPE-19 cells were prepared and washed with PBS twice. Subsequently, the cells were suspended in 500 μ l binding buffer (Nanjing KeyGen Biotech Co., Ltd.), followed by the addition of 5 μ l Annexin V-FITC and 5 μ l PI (Nanjing KeyGen Biotech Co., Ltd.) for 10 min at room temperature in the dark. Subsequently, cell apoptosis was analyzed using a flow cytometer (FACSAria III; BD Biosciences). The data were analyzed using BD Accuri C6 software (version 1.0; Becton, Dickinson and Company).

Western blot analysis. Total protein from ARPE-19 cells was extracted using a RIPA lysis buffer (Beyotime Institute of Biotechnology). Then, ARPE-19 cells were collected into a centrifuge tube and centrifuged at 10,000 \times g for 10 min at 4°C. The cell lysate was collected into another centrifuge tube and the protein concentration of the lysate was determined using a BCA assay kit (Sigma-Aldrich; Merck KGaA). A total of 20 μ g protein lysate was loaded to 10% SDS-PAGE gels and then transferred to PVDF membranes (EMD Millipore). The membranes were incubated with primary antibodies at 4°C overnight after blocking with 5% non-fat milk for 1 h at room temperature. Primary antibodies against NF- κ B (cat. no. ab32536; 1:1,000), NLRP3 (cat. no. ab263899; 1:1,000), caspase-1 (cat. no. ab179515; 1:1,000), pro-caspase-1 (cat. no. ab207802; 1:1,000), ASC (cat. no. ab151700; 1:1,000), HMGB1 (cat. no. ab18256; 1:1,000), Bcl-2 (cat. no. ab32124; 1:1,000), Bax (cat. no. ab32503; 1:1,000) and GAPDH (cat. no. ab8245; 1:1,000) were all obtained from Abcam. Following incubation with HRP-conjugated goat anti-rabbit IgG (cat. no. ab205718; Abcam; 1:5,000) or goat anti-mouse IgG (cat. no. ab6789; Abcam; 1:5,000) secondary antibodies at 37°C for 1 h, the protein bands of the membranes were detected using Immobilon Western Chemiluminescent

HRP Substrate (EMD Millipore). The relative intensity of target bands was semi-quantified using ImageJ software (version 1.52r; National Institutes of Health) and normalized to the intensity of GAPDH.

Statistical analysis. Data are presented as mean \pm SD. All experiments were performed in triplicate. Statistical analyses were carried out using GraphPad Prism v6 software (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used to compare the differences among groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Nesfatin-1 enhances cell viability and decreases inflammatory responses in high glucose-treated ARPE-19 cells. ARPE-19 cells exposed to high glucose exhibited lower viability compared with that of the normal glucose group. Nesfatin-1 treatment alleviated the decrease in cell viability induced by high glucose, which was dose-dependent. The osmotic group revealed that osmotic pressure exerted no significant effects on cell viability, excluding the effect of osmotic pressure on the high glucose group (Fig. 1A). The results of the colony formation assay indicated that high glucose exposure inhibited the proliferation of ARPE-19 cells compared with the normal glucose group, while nesfatin-1 treatment remarkably increased the cell proliferation relatively to the high glucose group (Fig. 1B). Additionally, a notably reduced nesfatin-1 level was observed following high glucose stimulation, whereas nesfatin-1 treatment significantly enhanced the nesfatin-1 level (Fig. 1C). Moreover, osmotic pressure presented no effect on the levels of IL-6, IL-1 β and TNF- α compared with the normal glucose group, but high glucose significantly induced the secretion of these inflammatory cytokines, whereas nesfatin-1 treatment reduced the levels of inflammatory cytokines compared with the high glucose group (Fig. 1D-F).

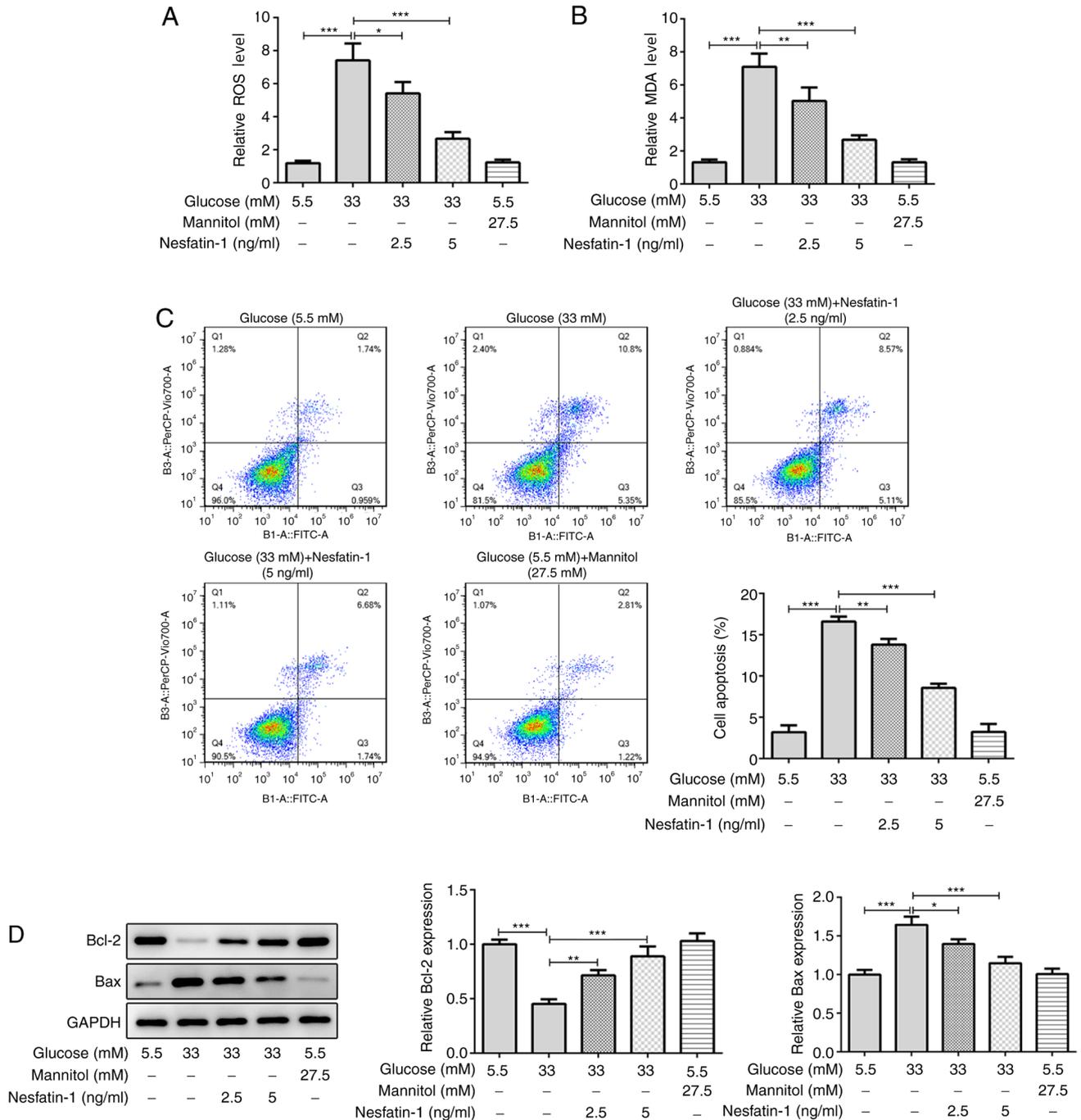


Figure 2. Nesfatin-1 alleviates high glucose-induced oxidative stress and apoptosis in ARPE-19 cells. (A) The ROS and (B) MDA content in each group was determined with commercial assay kits. (C) The apoptotic rate was assessed via flow cytometry. (D) The level of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 was examined via western blot analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ROS, reactive oxygen species; MDA, malondialdehyde.

Nesfatin-1 alleviates high glucose-induced oxidative stress and apoptosis in ARPE-19 cells. A number of studies have demonstrated that high glucose concentration can induce cellular oxidative stress (27,28). It was observed that osmotic pressure exhibited no effect on the levels of ROS and MDA and high glucose markedly increased the levels of ROS and MDA compared with the normal glucose group, whereas nesfatin-1 reduced the cellular ROS and MDA content. Furthermore, the high dose of nesfatin-1 exerted a more potent antioxidant effect compared with the low dose nesfatin-1 (Fig. 2A and B). Consistently, there was no significant difference in cell apoptosis

between the mannitol group and the normal glucose group, but nesfatin-1 reduced high glucose-induced cell apoptosis (Fig. 2C). In addition, osmotic pressure exhibited no effect on the expression of Bcl-2 and Bax compared with the normal glucose group. However, the decreased Bcl-2 and increased Bax level in high glucose-treated ARPE-19 cells also demonstrated that high glucose concentration may increase the cell apoptosis rate, whereas nesfatin-1 treatment reversed the effect of high glucose on the protein level of Bcl-2 and Bax (Fig. 2D). These data provided evidence that nesfatin-1 attenuated high glucose-induced oxidative stress and apoptosis in ARPE-19 cells.

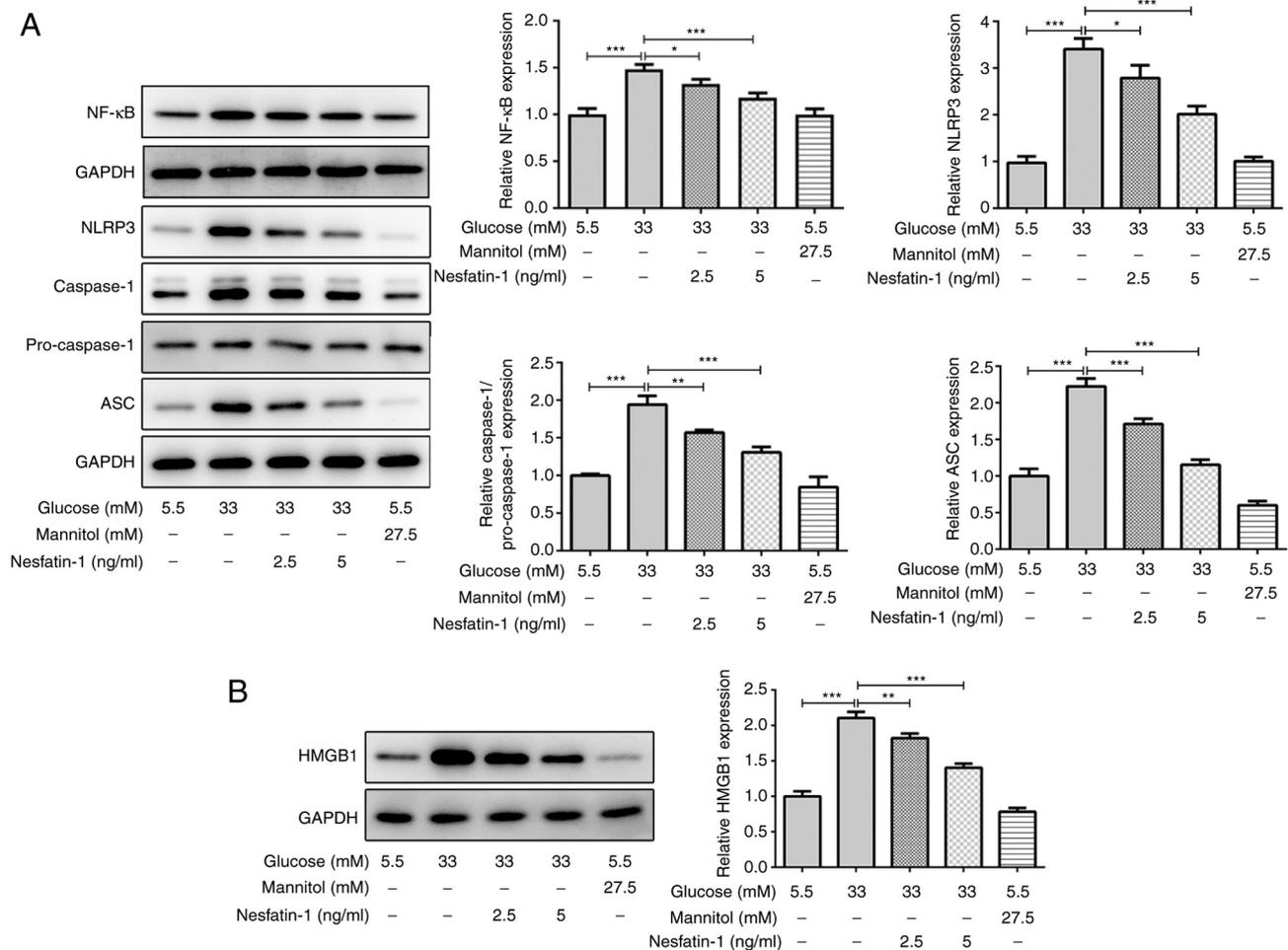


Figure 3. Nesfatin-1 inhibits the activation of NF- κ B/NLRP3 inflammasome signaling and HMGB1 expression in ARPE-19 cells stimulated by high glucose. (A) The protein expression level of NF- κ B and NLRP3 inflammasome proteins, including NLRP3, ASC and caspase-1, and (B) the protein expression level of HMGB1 in each group was determined using western blot analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NLRP3, NACHT, LRR and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; HMGB1, high-mobility group protein B1.

Nesfatin-1 inhibits the activation of NF- κ B/NLRP3 inflammasome signaling and HMGB1 expression in high glucose-stimulated ARPE-19 cells. High glucose concentration stimulates the activation of the NF- κ B/NLRP3 inflammasome pathway, which has been indicated to be associated with the dysfunction of high glucose-treated human retinal endothelial cells (21,29). There was no significant difference in the expression of NF- κ B, NLRP3, caspase-1 and ASC between the mannitol group and the normal glucose group. In high glucose-stimulated ARPE-19 cells, the protein expression levels of NF- κ B, NLRP3, caspase-1 and ASC were increased, whereas the levels of these proteins were markedly reduced by nesfatin-1 treatment (Fig. 3A), indicating that nesfatin-1 inhibited the NF- κ B/NLRP3 inflammasome signaling activation induced by high glucose exposure. Furthermore, osmotic pressure exhibited no significant effect on the expression of HMGB1, but the expression level of HMGB1 was markedly increased in high glucose-treated ARPE-19 cells, and it was reduced following nesfatin-1 treatment (Fig. 3B). Overall, these data suggested that nesfatin-1 suppressed the NF- κ B/NLRP3 inflammasome pathway activation and HMGB1 expression in high glucose-stimulated ARPE-19 cells.

Nesfatin-1 prevents the activation of NF- κ B/NLRP3 inflammasome signaling via regulating HMGB1. Subsequently, the mechanism via which nesfatin-1 affected the activation of the NF- κ B/NLRP3 inflammasome pathway was further investigated. ARPE-19 cells were transfected with pcDNA3.1-HMGB1. As demonstrated in Fig. 4A, pcDNA3.1-HMGB1 increased the expression level of HMGB1 compared with the empty vector, suggesting the efficiency of pcDNA3.1-HMGB1 transfection. Of note, under high glucose conditions, nesfatin-1 inhibited NF- κ B/NLRP3 inflammasome signaling, and this effect could be reversed via upregulation of HMGB1 (Fig. 4B), indicating that nesfatin-1 prevented the activation of NF- κ B/NLRP3 inflammasome signaling via the regulation of HMGB1.

Discussion

Nesfatin-1 is considered to have various functions in different systems and metabolic processes, including the endocrine and nervous system, blood glucose concentration, cardiovascular system and lipid metabolism (30,31). It has been reported that the concentration of nesfatin-1 in the serum and vitreous humor was negatively correlated with DR (14). However, the

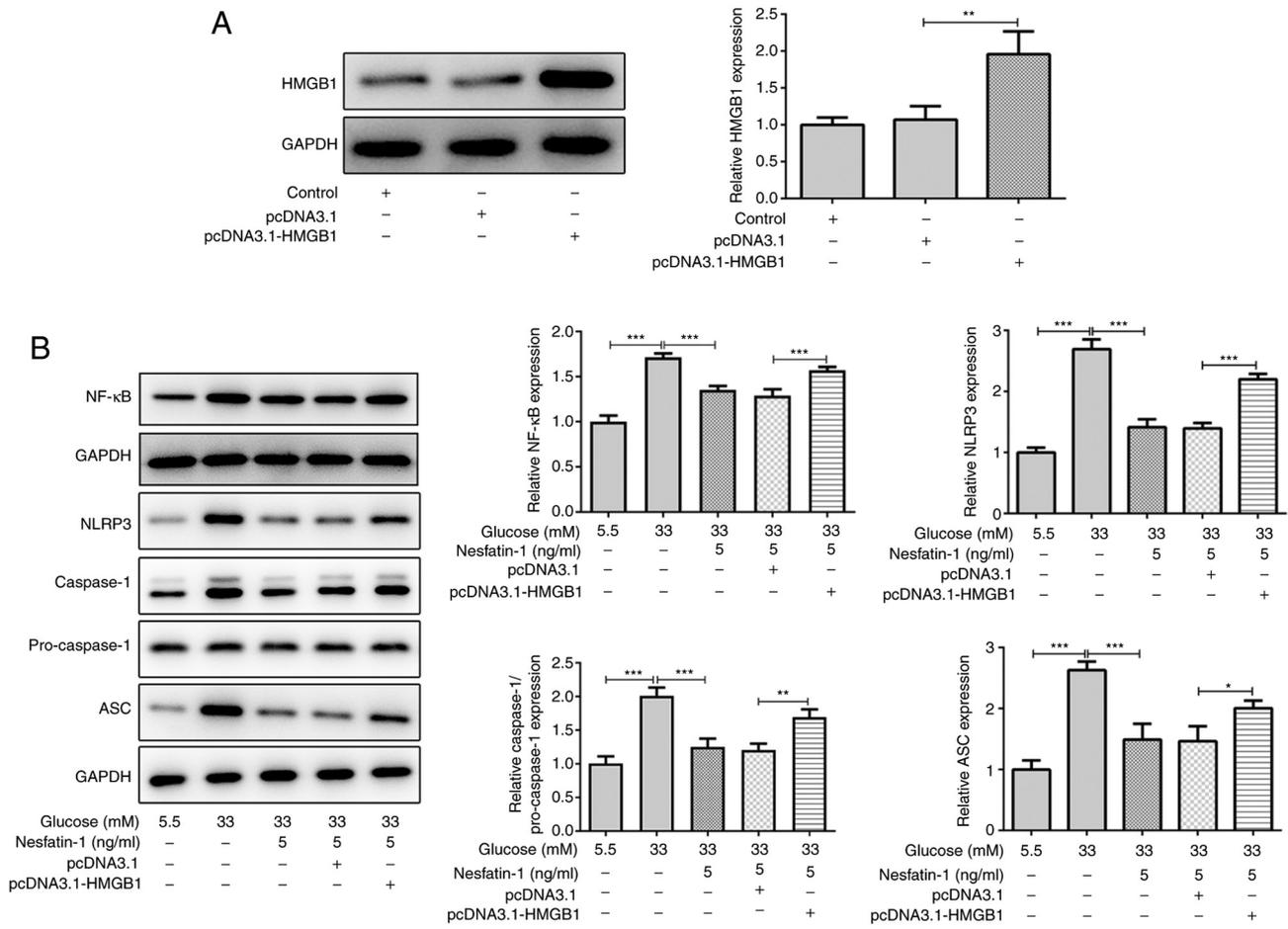


Figure 4. Nesfatin-1 prevents the activation of NF- κ B/NLRP3 inflammasome signaling via regulating HMGB1 in high glucose-treated ARPE-19 cells. (A) The transfection efficiency of HMGB1 overexpression plasmid was validated via western blot analysis. (B) The protein expression level of NF- κ B and NLRP3 inflammasome proteins, including NLRP3, ASC and caspase-1, was evaluated using western blot analysis. * P <0.05; ** P <0.01; *** P <0.001. NLRP3, NACHT, LRR and PYD domains-containing protein 3; ASC, apoptosis-associated speck-like protein containing a CARD; HMGB1, high-mobility group protein B1.

role of nesfatin-1 in DR has not been extensively investigated to date.

Retinal pigment epithelial cells are the main cells involved in DR (32). In the present study, ARPE-19 human retinal epithelial cells were cultured with high glucose *in vitro* to mimic hyperglycemia *in vivo*. It has been previously reported that ROS-induced oxidative stress and low-grade inflammation triggered by chronic hyperglycemia contribute to the progression of DR (33). In the current study, it was observed that high glucose concentration reduced cell viability, induced the expression of inflammatory cytokines and increased the ROS and MDA content following high glucose stimulation. Nesfatin-1 treatment enhanced cell viability and suppressed the levels of TNF- α , IL-1 β , IL-6, ROS and MDA in high glucose-treated cells, suggesting that nesfatin-1 may protect ARPE-19 cells against high glucose-induced inflammation and oxidative stress. In addition, nesfatin-1 decreased cell apoptosis under high glucose conditions.

NLRP3 inflammasome is a protein complex in the innate immune system that recognizes pathogen- and danger-associated molecular patterns, which is composed of NLRP3, ASC and caspase-1 (34). Activation of the NF- κ B/NLRP3 inflammasome signaling serves a key role in the progression of DR (19,20,35-38). In the present study, it was observed that nesfatin-1 reduced

the protein levels of NF- κ B, NLRP3, ASC and caspase-1, and inhibited the activation of the NF- κ B/NLRP3 inflammasome signaling following high glucose stimulation. HMGB1 is a danger-associated molecular pattern receptor that can sense high glucose as a stressor (39). Elevated HMGB1 levels were observed in patients with advanced DR compared with healthy subjects (39). A recent study reported that HMGB1 was revealed to be highly expressed in high glucose-treated human retinal endothelial cells, and it may be associated with the pathogenesis of DR (40). Emerging evidence supports that nesfatin-1 can downregulate the expression of HMGB1 to alleviate lipopolysaccharide-induced acute lung injury (26). The present study demonstrated that high glucose concentration increased the expression level of HMGB1 in ARPE-19 cells, which was reduced by nesfatin-1 treatment. Of note, overexpression of HMGB1 partially reversed the inhibitory effect of nesfatin-1 on NF- κ B/NLRP3 inflammasome signaling activation, suggesting that nesfatin-1 may prevent the NF- κ B/NLRP3 inflammasome pathway activation via inhibiting HMGB1. Consistently, previous studies have reported that HMGB1 increased the expression level of NF- κ B and NLRP3 inflammasome proteins, including NLRP3, ASC, caspase-1 or IL-1 β (25,41,42).

In summary, the present study demonstrated the beneficial effect of nesfatin-1 in high glucose-treated ARPE-19 cells via

inhibiting inflammation, oxidative stress and apoptosis. To the best of our knowledge, the present study was the first to demonstrate that nesfatin-1 prevented the NF- κ B/NLRP3 inflammasome signaling activation via the inhibition of HMGB1. The role of nesfatin-1 in animal experiments, the mechanisms mediating the reduction of HMGB1 by nesfatin-1 in and the effective dose of nesfatin-1 for clinical applications, which are limitations to the present study, should be investigated in future studies.

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Availability of materials and data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS, HZ and ZY searched the literature, designed and performed the experiments. XL and PY analyzed the data and wrote the manuscript. JZ analyzed the data and revised the manuscript. HS and JZ confirm the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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