Research Article

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Inflammatory factors secreted from endothelial cells induced by high glucose impair human retinal pigment epithelial cells

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Abstract

Objectives: Diabetic retinopathy (DR) is a retinal disease that arises from impaired glucose tolerance and leads to retinal microvascular leakages. Recent studies have indicated that DR pathogenesis is linked to dysfunctional retinal pigment epithelial (RPE) cells.

Methods: Investigating the potential interplay between endothelial cells (ECs) and RPE cells by treating ECs with high glucose (HG) and evaluating the function of cytokines released from ECs on the growth of RPE cells.

Results: The results revealed that high glucose-stimulated human umbilical vein endothelial cells (HUVECs) activated the NF-κB signaling pathway, increased intracellular levels of reactive oxygen species (ROS) and expression of caspase 3 while also elevating HUVECs delivery of cytokines such as VEGF, TNF-α, IL-6, and IL-1β.

Conclusions: As a result of our study, cytokines released from HG-treated HUVECs impede the growth of ARPE-19 in vitro, highlighting the importance of functional ECs for exploring the underlying mechanisms of vascular-associated retinal dysfunction. Inflammatory factors secreted from endothelial cells induced by high glucose impair human retinal pigment epithelial cells.

Keywords: diabetic retinopathy; ECs injury; inflammatory cytokines; oxidative stress; ARPE-19 dysfunction; the NF-κB pathway

Introduction

Diabetic retinopathy (DR) is a widespread complication of diabetes that is caused by microvascular impairment, which is responsible for renal injury [1]. DR results from glucose intolerance and the consequent damage to endothelial cells (ECs) integrity, leading to retinal microvascular leakage and an increased amount of damaged retinal cells [2–4]. Numerous studies have provided insight into the injury of capillary ECs and its contribution to vascular complications in DR [5, 6]. One characteristic of impaired EC function is the increased production of cytokines and cellular death [7]. In vitro studies have shown that high glucose stimulation of human umbilical vein endothelial cells (HUVECs) mimics EC dysfunction in vivo by enhancing the production of proinflammatory factors and reactive oxygen species (ROS) [8]. Therefore, anti-inflammatory interventions may represent effective treatments for DR.

Human retinal pigment epithelial cells (hRPEs) are a monolayer of epithelial cells located on the outer side of the retina, which serve to separate the vascular and sensory retina and are thus essential for maintaining the function of the retina [9]. Emerging evidence suggests that the inflammatory response is significantly elevated in DR patients, indicating that these factors are associated with the development of DR [10]. Furthermore, high glucose (HG) plays a crucial role in inducing cytokine-mediated inflammation and oxidative stress in endothelial cells, specifically interleukin-1β (IL-1β), which can cause injury to retinal-related cells [8, 11, 12].

HG and IL-1β are key factors contributing to microvascular dysfunction and damage to retinal pigment epithelial cells (RPEs) [13, 14]. In this study, we hypothesize that IL-1β plays a critical role in high glucose-induced retinal injury by regulating apoptosis, oxidative stress, and mitochondrial function in both HUVECs and RPEs. Furthermore, we propose that the underlying mechanism may involve the NF-κB pathway. This study is helpful in providing a new idea for treating human retinal pigment epithelial cells damaged by high glucose-induced endothelial cells secreting inflammatory factors.
**Materials and methods**

**Cell culture**

HUVECs cell line and the ARPE-19 cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Firstly, the HUVECs cell line was cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and the HUVECs were incubated with 5% CO₂ at 37°C. To establish high glucose cultures, additional glucose was mixed into the culture medium at different levels (10, 30, and 50 mM). The HUVECs were then treated with HG for 24, 48, or 72 h. The culture medium of ARPE-19 cells was compounded by DMEM, 10% FBS, and 1% double antibiotics (penicillin/streptomycin).

**MTT assay**

A methylthiazolylidiphenyl-tetrazolium bromide (MTT) assay was used to assess HUVECs viability when treated with high glucose. The HUVECs were cultured in 96-well plates at a density of 5,000 cells per well following the MTT Kit protocol. After 12 h of incubation, 20 μL of MTT solution was added, then HUVECs were incubated for another 4 h. Lastly, abandoned cell culture supernatant, 100 μL of formazan dissolving solution was added, and SpectraMax i3 (Molecular Devices, Sunnyvale, CA) was used to measure each well’s absorbance at 490 nm.

**Real-time PCR**

RNA of HUVECs was isolated by Total RNA Isolation Kit and the isolated RNA was transcribed into cDNA by the FastKing RT Kit. M The expression of cytokines was quantified using the SuperReal PreMix Plus (SYBR Green) Kit on a Roche LightCycler® 480 system. Primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) with the following sequences:

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>TGGCAGTGGACGACAGCTAGGG</td>
<td>TTTCACTGTAAGTCCGCCAGGC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GATGCTTTACAGTGCGGCAA</td>
<td>GTCGGGATCTTAAGCTGGA</td>
</tr>
<tr>
<td>VEGFA</td>
<td>CACCAGAGCCAGCACTAGGA</td>
<td>CTCGGAGGAAGGCAGCCAGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>CAGTTGCCCTCTCCGGG</td>
<td>GCCAGTGCTCTTGTGCTG</td>
</tr>
<tr>
<td>eNOS</td>
<td>AACTGGGAGGTGGGGAGCAT</td>
<td>TGAATGAAGCTCGGCAGCTGA</td>
</tr>
<tr>
<td>SOD2</td>
<td>GCCTGCGCAAGCGGAGAGT</td>
<td>ACACAAAGATGACAGGAGGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGTCAACGGATTCTGCGT</td>
<td>GACAAAGCTCCGGTCTCAG</td>
</tr>
</tbody>
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**Western blot analysis**

To extract HUVECs’ total protein, we use Radio Immunoprecipitation Assay (RIPA) buffer (Solarbio, Beijing, China) to lyse cells. The BCA protein assay kit was used to detect HUVECs’ total protein concentration. SDS-PAGE (10%) electrophoresis was taken to quantify the HUVECs protein lysates. The primary antibodies used in this article include anti-human NF-κB p65 antibody (ab288751), anti-human Bax antibody (ab32503), anti-human caspase 1 antibody (ab39901), anti-human caspase 3 antibody (ab32351), anti-human cleaved caspase 3 antibody (ab32042), and anti-human Bcl-2 antibody (ab182838), anti-GAPDH antibody (ab8245) as well as anti-beta actin antibody (ab8226) was used as a loading control.

**The secreted cytokines IL-1β/IL-6/TNF-α were detected by ELISA**

After HG stimulation, the cell suspension and centrifugation are collected to retain the cell supernatant. The levels of HUVECs secreted cytokines IL-1β (ab100562/IL-6/ab178013/TNF-α) were determined using commercial cytokines detection kits for analyzing inflammatory cytokines (Abcam, Cambridge, MA), following the manufacturer’s instructions. Briefly, the treated cell supernatant was added to the detection wells and incubated to allow the factors in the cell supernatant to bind to the well plate. Then, antibodies were added to detect the expression of target proteins in the cell supernatant. Finally, the SpectraMax i3 (Molecular Devices, Sunnyvale, CA) was used to measure each well’s absorbance at 450 nm.

**Malondialdehyde (MDA) and superoxide dismutase (SOD)**

MDA level and SOD activity in HG-stimulated HUVECs were examined using an MDA assay kit (Solarbio, Beijing, China BC0025) and an SOD assay kit (Solarbio, Beijing, China BC0175), respectively. The values of MDA and SOD were detected by a microplate reader (SpectraMax i3, Molecular Devices, USA) at 532/600 nm and 560 nm, respectively. The detailed experimental steps can refer to the protocols of the reagent kit.

**Reactive oxygen species assay**

The DCFH-DA probe was operated to monitor ROS production in high glucose-treated HUVECs. The DCFH-DA fluorescent probe was used to incubate HUVECs to detect ROS (Solarbio, Beijing, China CA1410). After 30 min at 37°C incubation, the green fluorescence intensity of ROS in high glucose-treated HUVECs was detected by a fluorescence microscope and a microplate reader (SpectraMax i3).

**dUTP-biotin nick end labeling (TUNEL)**

Cell climbing sheets were collected for fixation at room temperature for 30 min and were washed with PBS 3 times gently. Next, the climbing sheets were treated with 1% Triton X-100 and penetrate for 3–5 min at room temperature. After the sheets were washed three times again, apoptosis in high glucose-stimulated cells was detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. Following ally, the TdT enzyme reaction solution was prepared in advance; then the cells were labeled in a dark wet box. After a 60 min incubation at 37°C, the washing step was repeated, and the cells were counterstained with DAPI, according to the In Situ Cell Death Detection Kit (Roche, Nutley, NJ, USA). Finally, TUNEL-positive cells were observed under a fluorescence microscope at the excitation and emission wavelength of 543 nm and 571 nm, respectively.
Data analysis

All statistics were analyzed by ANOVA using GraphPad Prism 8.0 software, unpaired Student's t-tests were used to determine statistical significance when comparing two groups, and the results were expressed as $X \pm SD$. A $p < 0.05$ value was considered a significance level and indicated a statistical significance between varied groups.

Results

Effect of high glucose on inflammatory responses and cell viability in HUVECs

In our research, we explored the role of high glucose (HG) on HUVECs by examining HUVECs viability and the expression of inflammatory cytokines at the mRNA level. HG induction at a concentration of 30 mM was found to significantly reduce the viability of HUVECs (Figure 1A) and increase the expression of cytokines created by high glucose-stimulated HUVECs (Figure 1B). These outcomes imply that HG can stimulate inflammatory responses in HUVECs and affect their viability. Therefore, a concentration of 30 mM was selected as the stimulation concentration for subsequent research on HUVECs inflammation.

High glucose increases oxidative stress in HUVECs

To estimate the degree of HG-induced damage in HUVECs, we first detected the mRNA expression of genes related to ROS production. As depicted in Figure 2A, the degree of SOD2 in HUVECs was significantly decreased after HG treatment. Furthermore, the production of ROS was detected by the DCFH-DA probe and fluorescence microscopy. As shown in Figure 2B, ROS levels in HUVECs were significantly elevated following treatment with HG (30 mM), and the generation of ROS and MDA in HUVECs was greatly ascended. While SOD activity was suppressed by HG, indicating that HG treatment significantly increased oxidative stress in HUVECs (Figure 2C and D).

High glucose induces apoptosis in HUVECs and promotes the secretion of inflammatory cytokines through the NF-κB pathway

Annexin/PI staining and Western blot were performed to investigate this. The results showed that HG strongly induced HUVECs apoptosis, as evidenced by an increased

![Figure 1](image)

**Figure 1:** Effect of concentration and induction time of high glucose on cell viability and inflammatory responses in HUVECs. (A) Effect of high glucose ranging from 0 to 50 mM on HUVECs viability and induction time, including 24, 48, and 72 h, was compared in this study. (B) The high glucose (30 mM) was used to induce inflammatory cytokines (IL-1β, TNF-α, IL-6) expressions. Data are taken from three individual experiments and expressed as means ± SD (n=3). *p-value <0.05.
number of apoptotic cells (Figure 3A). Moreover, the protein levels of pro-inflammatory cytokines were elevated in HUVECs, accompanied by increased expression of caspase 1. Treatment with HG (30 mM) activated the NF-κB signaling pathway, which in turn accelerated the production of IL-1β and TNF-α in HUVECs (Figure 3B and C).

**IL-1β accelerates HG-induced ROS generation and injury in RPEs**

To explore the role of IL-1β in HG-induced apoptosis, TUNEL staining was performed to examine RPEs apoptosis. The growing number of apoptosis cells in RPEs treated with HG and IL-1β compared to HG alone showed that IL-1β strongly triggered apoptosis in RPEs (Figure 4A). ROS generation in RPEs was strongly enhanced when either IL-1β or HG (30 mM) was added. When IL-1β and HG were added together, the levels of ROS increased significantly, suggesting that IL-1β may aggravate oxidative stress triggered by HG in RPEs (Figure 4B). The protein levels of Bax and cleaved caspase 3 were increased in the HG (30 mM) and IL-1β treated group, along with the reduced expression of Bcl-2. IL-1β enhanced the levels of Bax and cleaved caspase 3 and restored Bcl-2 expression in RPEs, reflecting a marked promotion of HG-induced apoptosis in the RPEs (Figure 4C).
Previous studies have demonstrated that elevated degrees of inflammatory cytokines are related to diabetic retinopathy, and the severity of retinal complications in diabetic patients is positively associated with the levels of inflammatory cytokines [15]. In our research, we investigate the function of IL-1β in high glucose-induced dysfunction of human umbilical vein endothelial cells (HUVECs) and retinal pigment epithelial cells (RPEs). Our findings revealed that high glucose induced the release of IL-1β by HUVECs, subsequently triggering apoptosis, oxidative stress, and inflammatory responses in these cells. Furthermore, we demonstrated that IL-1β contributed to the high glucose-induced apoptosis of RPEs. High glucose levels in cell culture media can lead to dysfunction of HUVECs [16], resulting in increased inflammatory responses and oxidative stress, ultimately leading to dysfunction of RPEs [17, 18]. Our study showed that high glucose activated the NF-κB pathway in endothelial cells, which promoted the expression of inflammatory factors and accumulation of ROS, leading to endothelial cell apoptosis and release of IL-1β. IL-1β, in turn, accelerated the high glucose-induced apoptosis of RPEs, suggesting that IL-1β plays a role in the induction of RPE dysfunction under high glucose conditions. In contrast, Hsu et al. [19] reported that IL-1β can attenuate RPE injury and inflammatory responses. Our study uncovered the synergistic effect of IL-1β in high glucose-induced dysfunction of RPEs.

However, the underlying mechanism by which IL-1β released from HUVECs exacerbates RPEs damage under HG conditions remains largely unexplored. Prolonged exposure to high glucose is known to induce oxidative stress and inflammatory response, leading to cellular injury and even death [20–22]. The ability of IL-1β in RPEs injury induced by HG and its release by HUVECs are not fully disclosed. Previous reports have emphasized the potential of inhibiting oxidative stress and inflammatory response in suppressing RPEs apoptosis and providing retina protection [23]. Concordance with these consequences, our findings demonstrate that HG treatment facilitated the expression of inflammatory cytokines such as IL-1β through the NF-κB pathway, resulting in intracellular accumulation ROS in HUVECs and hREPs, as well as suppressing SOD activity, indicating that HG accelerated oxidative stress in HUVECs. Moreover, the presence of IL-1β induced upregulation of the protein level of Bax and cleavage of caspase 3 in RPEs, while the expression of Bcl-2 was downregulated. Our study provides evidence that IL-1β secreted by HG-induced HUVECs exerts a pro-oxidative effect on RPEs. Inhibition of NF-κB signaling activation has been shown to suppress the expression of Bax and intracellular ROS accumulation, along with IL-1β-induced cell apoptosis [24–26]. IL-1β has been shown to participate in various cellular events by activating NF-κB, which in turn induces inflammatory response and oxidative stress [27, 28].

Overall, high glucose (HG) induces HUVECs to release IL-1β, which could cause ROS build-up in endothelial cells, triggering NF-κB protein to move towards the nucleus and reasonably activating the NF-κB pathway [26]. High glucose can facilitate HUVECs to express related cytokines and caspase-1, which accelerate the cleavage of pro-IL1β [28]. On the other hand, apoptosis of endothelial cells results in a high glucose environment, which reinforces the magnitude and duration of NF-κB activation due to the released IL-1β. Prolonged activation of NF-κB can activate the apoptotic pathway. Massive ROS generation raises oxidative stress, destroys the balance of Bcl-2/Bax, and

![Figure 3](image-url)
induces apoptosis in hRPEs [29]. Thus, HG-induced ROS accumulation and IL-1β activate the NF-κB pathway, which sharpens ROS production and mediates inflammatory response, thus inducing apoptosis in RPEs. IL-1β leads to NF-κB protein transport into the nucleus and promotes downstream responses in RPEs, including ROS build-up and apoptosis (Figure 5). In our exploration, we deliberated the function of cytokines IL-1β in HG-induced RPEs' impairment and the mechanisms underlined in high glucose existence.

Figure 4: IL-1β accelerates HG-induced ROS generation and apoptosis in RPEs. (A) Representative TUNEL staining of the ARPE-19. Original magnification ×400. Scale bar=50 μm. (B) Reactive oxygen species (ROS) levels in ARPE-19 after HG or IL-1β stimulation were examined. Values were presented as mean ± SD, n=3 for each group. (C) Representative images of Western blot and quantification of expression of proteins related to apoptosis, including Bax and cleaved caspase 3 and Bcl-2. Values were presented as mean ± SD, n=3 for each group. *p-value <0.05.
In summary, IL-1β is considered a critical regulator in HG-induced RPEs damage. IL-1β aggravates RPEs’ impairment by promoting ROS production and apoptosis. The IL-1β-NF-κB pathway may be regarded as a potential therapeutic orientation for HG-induced retinal injury.

Conclusions

As a result of our study, cytokines released from HG-treated HUVECs impede the growth of ARPE-19 in vitro, highlighting the importance of functional ECs as a potential orientation for treating vascular-associated retinal dysfunction.

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Data availability: The raw data can be obtained on request from the corresponding author.

References


