

Original Article

High glucose concentration induces retinal endothelial cell apoptosis by activating p53 signaling pathway

Qian Li^{1,2}, Lei Pang³, Hui Shi², Wei Yang², Xin Liu¹, Guanfang Su¹, Yu Dong²

¹Department of Ophthalmology, The Second Hospital of Jilin University, Changchun, Jilin, China; Departments of
²Ophthalmology, ³Anesthesiology, The First Hospital of Jilin University, Changchun, Jilin, China

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Abstract: Diabetic retinopathy (DR) is a retinal disease in patients with diabetes caused by metabolic disorders of glucose, and often leads to irreversible blindness if not treated properly. Dysfunction of retinal endothelial cells (RECs) contributes to the pathogenesis of DR. In the present study we investigated the apoptotic effect of high glucose concentration. High glucose was used to induce cell injury. LDH assay kit was used to detect the leakage of LDH. Western blot was used to detect the expression of bax, cleaved-caspase-3, bcl-2 and p53 proteins. The transmission electron microscope was used to observe apoptotic morphological changes. In the present study, we found that the LDH leakage ratio was significant increased after exposing to 30 mmol/L glucose for 12 h, and both showed a concentration-department manner and time dependent fashion. Next, obvious apoptotic morphological changes were observed after treating the cells with glucose at 30 mmol/L. Further research indicated that the protein levels of p53, pro-apoptotic cleaved-caspase-3, and bax were significantly upregulated, and the level of bcl-2 was decreased after treating with glucose at 30 mmol/L. The protein levels of bax and cl-caspase-3 were significantly increased and the expression of bcl-2 was increased after pretreated with p53 specific inhibitor pifithrin- α . In conclusion, high glucose concentration induces apoptosis in retinal endothelial cells by activating p53 signaling pathway.

Keywords: Diabetic retinopathy, glucose, retinal endothelial cells, apoptosis, p53 signaling pathway

Introduction

Diabetes occurs in approximately 8.5% of adults aged 18 years and older, and caused 1.6 million deaths in 2014 [1, 2]. Hyperglycemia can cause injury to the peripheral nerves, and the renal and vascular systems [3]. Diabetic retinopathy (DR), a common microvascular complication of diabetes, also is a leading cause of adult blindness [4]. Retinal edema is the major cause of visual impairment in patients with DR, and increased apoptosis occurs in many retinal cells including pigment epithelial cells, pericytes, and endothelial cells [5-7]. Although vitrectomy or laser photocoagulation is used for the treatment of DR, these approaches are not satisfactory [8-11]. Therefore, a novel and effective intervention approach is required to decrease retinal injury in patients with DR.

High glucose concentration plays a key role in retinal cell death [12, 13]. Several lines of evidence have shown that high glucose induces the overproduction of pro-inflammatory cyto-

kines such as TNF- α , and IL-1 β , which act by a positive feedback mechanism to induce retinal cell apoptosis [14, 15]. Apoptosis, a form of programmed cell death, can occur in a wide range of physiological and pathological situations. It is characterized by cell shrinkage, programmed DNA degradation, cytoplasmic cytochrome C release, and activation of caspases [16]. The abnormal release of lactate dehydrogenase (LDH) is an index of plasma membrane damage and cell apoptosis [17]. However, the pro-apoptotic effect of high glucose in retinal endothelial cells (RECs) is still unclear especially through regulate p53 signaling pathway. Hence, the aim of the present study was to investigate the apoptotic effect of high glucose in RECs which are involved in the p53 signaling pathway.

Materials and methods

Materials

RPMI 1640 Medium with glucose 5.6 mmol/L was purchased from Gibco (Grand Island, NY,

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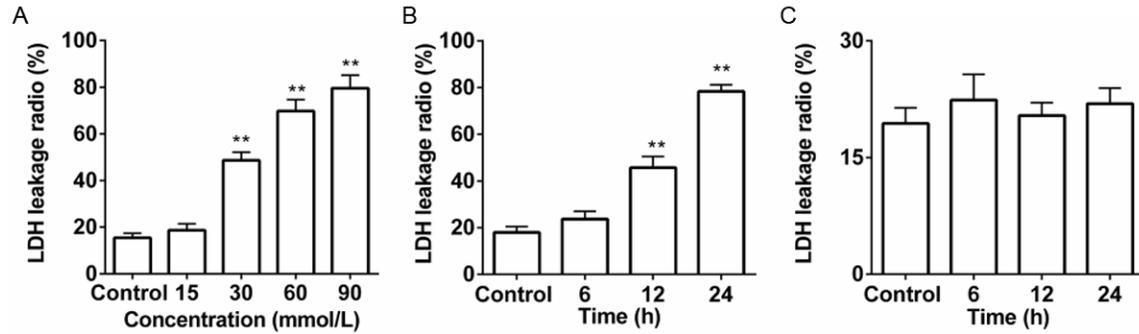


Figure 1. High glucose concentration induces LDH release in retinal endothelial cells (RECs). A. Cells were treated with glucose (15, 30, 60 or 90 mmol/L) for 12 h; B. Cells were treated with glucose at 30 mmol/L for 0, 6, 12 or 24 h; C. Cells were treated with mannitol at 24.4 mmol/L for 0, 6, 12 or 24 h. After the indicated treatment, LDH release was detected. Results were from three independent experiments and expressed as mean \pm S.E.M. $^{**}P < 0.01$ vs. control.

USA). Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). p53 specific inhibitor pifithrin- α was obtained from Beyotime (Jiangsu, China). Rabbit polyclonal antibody to bax, bcl-2 and cleaved caspase-3 were provided by Cell Signaling Technology (Danvers, MA, USA). GAPDH antibodies were obtained from Abcam (Cambridge, MA, USA). p53 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Relative second antibodies were from Beyotime (Jiangsu, China). BCA protein assay kit was from Kangchen Biotech (Shanghai, China).

Retinal endothelial cells culture and treatment

Retinal endothelial cell (REC) cell line was originally supplied by the American Type Culture Collection (Manassas VA, USA). The cells are certified as the designated type and have been checked free of contamination. Cells were cultured in the RPMI-1640 medium supplemented with 10% FBS, and 100 U/ml penicillin and streptomycin, and maintained at 37°C in a humidified atmosphere with 5% CO₂. Before the experiments, cells were cultured in the RPMI-1640 medium with 1% FBS for 12 h, followed by the treatment of high glucose.

LDH assay

The RECs ($1-1.5 \times 10^6$ /mL) were exposed to different concentrations of glucose for 12 h, high glucose (30 mmol/L) or mannitol (24.4 mmol/L, an osmotic control) for 6, 12, 24 h. The measurement of LDH leakage was by an LDH activ-

ity assay kit (Beyotime, Jiangsu, China) according to the instruction of the kit. LDH leakage rate was expressed as the percentage of the total LDH activity, according to the equation as follow: % LDH release rate = (LDH activity in medium/total LDH activity) \times %

Transmission electron microscopy

To observe the extent of apoptosis induced by high glucose, the transmission electron microscope (TEM) was used to observe apoptotic morphological changes. The cells were cultured under the indicated treatment; then, cells were collected via centrifugation (800 rpm, 5 min), and washed with PBS and fixed in freshly prepared 1% paraformaldehyde and then with 2% glutaraldehyde for 24 h. Then, the fixed cells were treated with 1% osmium tetroxide for 3 h, dehydrated through an ethanol gradient, and embedded in araldite. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed under a TEM (JEM-101, Jeol Electron Inc, Japan).

Western blot

The cells were washed once in PBS and lysed on ice in lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors (150 μ L). The protein (40 μ g) was loaded, separated by 10% SDS-PAGE, and blotted onto PVDF membrane (0.22 μ m, GE Healthcare, Buckinghamshire, UK). The membrane was incubated with blocking buffer for 5 h at room temperature and then incubated overnight at

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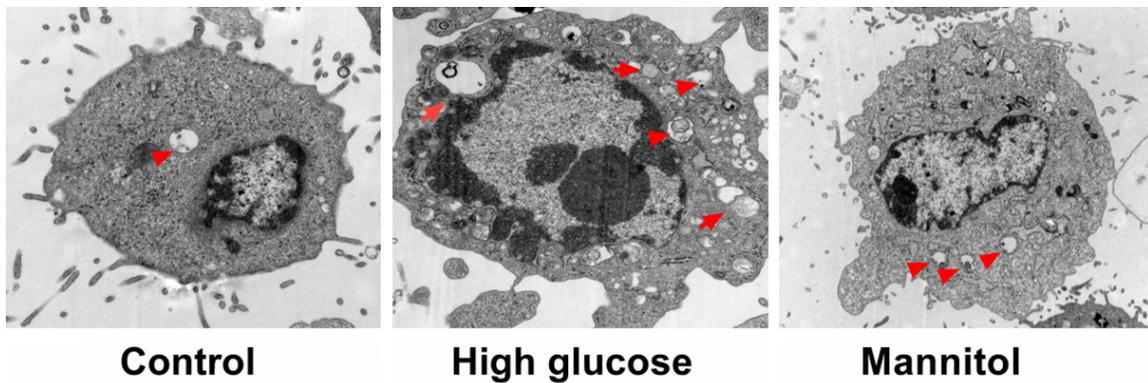


Figure 2. Effect of high glucose concentration on the morphological changes of RECs (20000 \times). Cells were incubated with glucose at 30 mmol/L for 12 h, then apoptotic morphological change was observed by transmission electron microscope.

4 $^{\circ}$ C with the primary polyclonal antibodies against p53 (1:900), bax (1:1200), caspase-3 (1:1000) and bcl-2 (1:1000), followed by incubation with corresponding secondary antibodies. Specific protein bands were visualized with an ECL advanced western blot analysis detection kit (Merck Millipore, Billerica, MA, USA).

Statistical analysis

Statistical analyses was performed by Graphpad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA). All data are presented as means \pm SEM. Steer-Dwass or Mann-Whitney multiple comparison tests were used to compare the differences among groups. A value of $P < 0.05$ was considered statistically significant.

Results

High glucose increases the leakage of LDH in retinal endothelial cells (RECs)

Cytotoxic compounds often impair the integrity of the cell membrane by inducing cell apoptosis or necrosis. LDH serves as a biomarker of cell membrane injury [18, 19]. **Figure 1A** and **1B** indicated that the LDH leakage ratio was significantly increased after exposing to 30 mmol/L glucose for 12 h, and both showed a concentration-dependent manner and time-dependent fashion. However, **Figure 1C** showed that the LDH leakage ratio was not altered after treated with mannitol for 0, 6, 12 or 24 h (24.4 mmol/L, an osmotic control). This result indicated that the leakage of LDH induced by high glucose was not a result of high osmolarity.

High glucose concentration induces cell apoptosis in RECs

Next, we explore whether a high glucose level induces cell apoptosis (programmed cell death). To address this question, we performed transmission electron microscopy to observe cellular morphology in great detail. TEM was able to clearly differentiate between nuclei and organelles, and thus confirm the occurrence of apoptosis in cells [20]. As shown in **Figure 2** that obvious apoptotic morphological changes were observed after treating the cells with glucose at 30 mmol/L for 12 h. This typically included vacuolization in the mitochondria, chromatin condensation, and degranulation in the endoplasmic reticulum. However, when RECs were treated with mannitol at 24.4 mmol/L for 12 h, as an osmotic control, the changes were not shown. This result indicated that cell apoptosis induced by high glucose was not a result of high osmolarity.

High glucose induces expression of cleaved-caspase-3, bax, and bcl-2 in RECs

In order to further explore the apoptotic mechanisms, the protein levels of pro-apoptotic cleaved-caspase-3, bax, and anti-apoptotic Bcl-2 were detected by western blot. As showed in **Figure 3**, the protein expression of Caspase-3, and bax in the high glucose group (30 mmol/L) were significantly upregulated compared with that of the control group ($P < 0.01$), and the level of bcl-2 was decreased after treating with glucose at 30 mmol/L for 12 h in RECs. However, when RECs were treated with manni-

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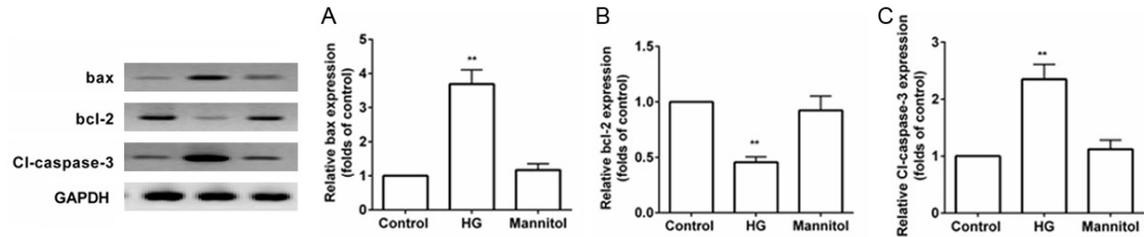


Figure 3. High glucose concentration induces expression of cl-caspase-3, bax and bcl-2 in RECs. Cells were incubated with glucose at 30 mmol/L for 12 h, then each protein level was analyzed by western blot. Levels of protein are shown as fold change from control from 3 independent experiments and expressed as mean \pm S.E.M. ** $P < 0.01$ vs. control.

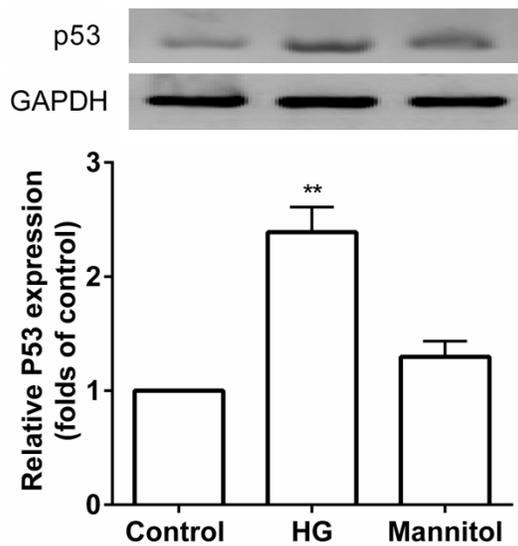


Figure 4. p53 signaling pathway is involved in the apoptotic effect of high glucose concentration in RECs. The cells were subjected to high glucose at 30 μ mol/L or mannitol at 24.4 μ mol/L for 12 h, then, expression of p53 were assayed by western blot. Results were from three independent experiments and data are expressed as mean \pm S.E.M. ** $P < 0.01$ vs. control.

tol at 24.4 mmol/L for 12 h, which is an osmotic control, it did not influence the related proteins. This result indicated that the cell apoptosis induced by high glucose was not a result of high osmolarity.

p53 signaling pathway involve in apoptotic effect of high glucose

As shown in **Figure 4**, p53 expression was increased after stimulation with glucose at 30 μ mol/L for 12 h in RECs. However, exposing cells to mannitol at 24.4 μ mol/L did not affect the level of the above proteins. This suggested

that high glucose causing an increase in the expression of p53 was not a consequence of high osmolarity.

Further research by western blot showed that bax and cl-caspase-3 proteins were significantly decreased and the expression of bcl-2 was increased after pretreated with p53 specific inhibitor pifithrin- α 10 μ mol/L for 1 h. These results indicated that the p53 signaling pathway was involved in the apoptotic effect of high glucose in REC (**Figure 5**).

Finally, we explored whether pretreatment with pifithrin- α , 10 μ mol/L for 1 h, was able to inhibit high glucose-induced cell apoptosis. **Figure 6** showed that the cells in high glucose at 30 mmol/L displayed obvious apoptotic morphological changes, and pre-treatment with specific pathway inhibitors significantly inhibited the apoptotic morphological changes in RECs.

Discussion

Hyperglycemia is the primary factor that contributes to retinal injury in the development of DR [3, 13]. Retinal endothelial cells (RECs) play many important functions in the retina, including phagocytosis of photoreceptor outer segments, isomerization of retinoids, and various metabolic and neurotrophic support functions, and dysfunction of RECs contributes to the pathogenesis of DR [5, 21, 22]. High glucose increases the risk of cell damage and dysfunction in RECs [23]. Much work has demonstrated that RECs are an important cell type affected by diabetic retinopathy, and many others have demonstrated that high glucose increased the levels of apoptosis in REC [24-26]. The abnormal release of LDH is an index of plasma membrane damage and cell apoptosis. Consistent

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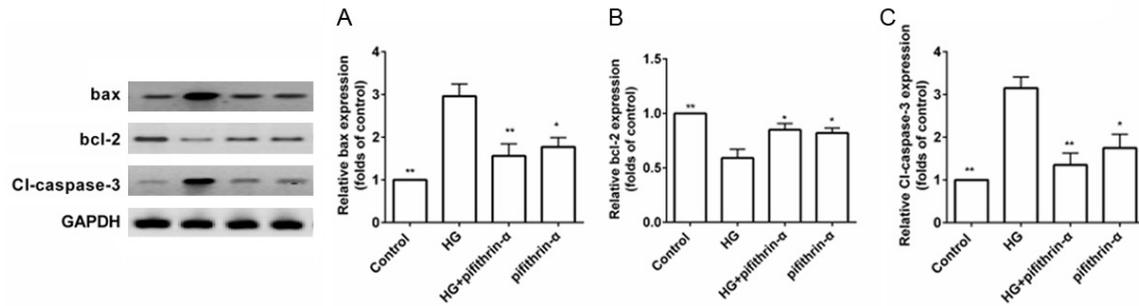


Figure 5. p53 signaling pathway involved in high glucose concentration-induced expression of cleaved-caspase-3, bax and bcl-2 in RECs. Cells were pretreated with pifithrin- α 10 μ mol/L for 1 h and then incubated with glucose at 30 mmol/L for 12 h. Each protein level was analyzed by western blot. Levels of protein are shown as fold change from control from 3 independent experiments and expressed as mean \pm S.E.M.. ** $P < 0.01$ vs. high glucose at 30 mmol/L.

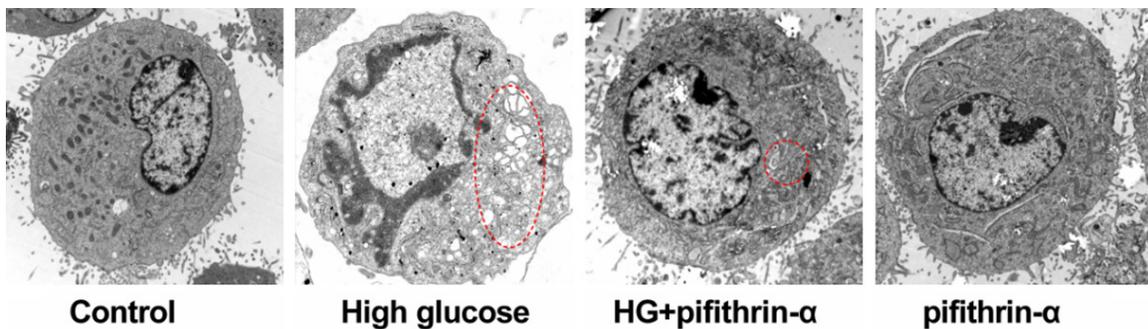


Figure 6. p53 signaling pathway involved in high glucose concentration-induced morphological changes of RECs (20000 \times). The cells were pretreated with pifithrin- α 10 μ mol/L for 1 h and then incubated with glucose at 30 mmol/L for 12 h. Apoptotic morphological change was observed by transmission electron microscope.

with these studies, we found that the LDH leakage ratio was significantly increased after exposure to 30 mmol/L glucose for 12 h, in both a concentration-dependent manner and time-dependent fashion. Next, obvious apoptotic morphological changes were observed after treated the cells with glucose at 30 mmol/L for 12 h. However, those phenomena were not altered after treated with mannitol.

Apoptosis occurs in a wide range of physiological and pathological situations. It is characterized by cell shrinkage, programmed DNA degradation, and activation of caspases. p53 plays a key role in DNA damage-induced apoptosis by affecting its transcriptional target pro-apoptotic bax, or translocates to the mitochondria to interact with anti-apoptotic bcl-2 in a non-transcriptional way [27, 28]. The down-regulation of Bcl-2 and the up-regulation of Bax is able to induce the release of cytochrome-C (cytosol) from mitochondria, and then trigger the activity of caspase-3 to finally cause cell apoptosis [29, 30]. Our data showed that the protein levels of

pro-apoptotic cleaved-caspase-3 and bax were significantly upregulated and the level of bcl-2 was decreased after treatment with glucose at 30 mmol/L. Further study indicated that p53 expression was increased after stimulation with glucose at 30 μ mol/L. However, cells exposure to mannitol at 24.4 μ mol/L did not affect those proteins in RECs. Further research showed that bax and cleaved-caspase-3 proteins were significantly decreased and the expression of bcl-2 was increased after pretreatment with p53 specific inhibitor pifithrin- α . These results indicated that the p53 signaling pathway is involved in the apoptotic effect of high glucose in REC.

In conclusion, high glucose concentration was able to increase cell apoptosis of RECs, characterized by inducing plasma membrane damage as well as increasing the level of bax and caspase-3 and decreasing the expression of bcl-2. The above mechanism occurs mainly via activating the p53 pathway.

Disclosure of conflict of interest

None.

Address correspondence to: Guanfang Su, Department of Ophthalmology, The Second Hospital of Jilin University, 218 Ziqiang Street, Changchun 130021, Jilin, China. Tel: +86 431-88782222; Fax: +86 431-88782222; E-mail: guanfangsu1@163.com; Yu Dong, Department of Ophthalmology, The First Hospital of Jilin University, 71 Xinmin Avenue, Changchun 130021, Jilin, China. Tel: +86-0431-88782312; Fax: +86-0431-88782312; E-mail: yudong1103@163.com

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