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Original Article

Erythropoietin prevents reactive oxygen species generation and renal tubular cell apoptosis at high glucose level

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ABSTRACT

Erythropoietin (EPO) can induce a series of cytoprotective effects in many non-hematopoietic tissues through interaction with the erythropoietin receptor (EPOR), but whether EPO can prevent the overproduction of reactive oxygen species (ROS) and apoptosis in diabetes remains unclear. Here, we report that renal tubular cells possess EPOR and that EPO reduces high glucose-induced oxidative stress in renal tubular cells. Further, we found that EPO inhibited high glucose-induced renal tubular cell apoptosis and that this protective effect was dependent on reduction of *Bax/caspase-3* expression as well as elevation of *Bcl-2* expression. Our results suggest that EPO can inhibit high glucose-induced renal tubular cell apoptosis through direct effect on anti-oxidative stress and that EPOR may play a key role in this process.

1. Introduction

Diabetic kidney disease (DKD) is a common microvascular complication of diabetes, and it is one of the main causes for end-stage renal disease. There is increasing evidence that reactive oxygen species (ROS) play a major role in the development of DKD [1]. Excessive ROS production is a direct consequence of hyperglycemia, and it can increase intracellular oxidative stress in diabetes patients [1]. Positive blood glucose control and the application of angiotensin II receptor antagonists can delay the occurrence and development of DKD, partly because they can inhibit the overproduction of ROS [2–4]. Meanwhile, traditional anti-oxidants have been shown to prevent or delay the onset of DKD [5].

Erythropoietin (EPO), secreted primarily by renal cortical fibroblast-like cells, has been widely used for the treatment of anaemia associated with chronic kidney disease and cancer chemotherapy. Recently, in addition to erythroid progenitor cells, EPOR has also been identified in many non-hematopoietic tissues [6,7]. Furthermore, EPO exerts a series of cytoprotective effects in these tissues through interaction with erythropoietin receptor (EPOR), including anti-oxidative stress, inhibition of apoptosis, mitogenesis, and promotion of vascular repair by mobilizing endothelial progenitor cells [7,8]. Johnson et al. confirmed that EPO plays a significant renoprotective role when administered to animal models with acute renal injury and that it has no hematological effects [9]. Further, some studies showed that EPO may have protective effects for chronic kidney disease, but whether EPO can inhibit oxidative stress in diabetes is still unclear.

In this study, we invested whether renal tubular cells possess EPOR, and whether EPO can inhibit oxidative stress and renal tubular cell apoptosis induced by high glucose in vitro.

2. Materials and methods

2.1. Cell cultures

NRK-52E cells (renal tubule epithelium cell line from normal rat) were originally obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagles medium (DMEM)/F12 media (Hyclone, Utah, America) supplemented with 5% fetal bovine serum (containing 2 mM glutamine, 15 mM HEPES, 1.5 g $NaHCO_3$, 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate; pH 7.4). Cells were maintained in serum-free media for 24 h before each experiment. The cultures were incubated at 37 °C with humidified air under 5% CO_2.

2.2. Immunocytochemistry

After removal of the medium, cells were fixed in 4% paraformaldehyde for 30 min at room temperature. After washing twice in phosphate-buffered saline (PBS) for 5 min, cells were permeated with 0.1% Triton for 15 min, probed in sequence with primary antibody against EPOR (Santa Cruz, CA, USA), and

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incubated at 4 °C overnight. Cells were washed twice in PBS for 5 min and incubated in the dark with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat secondary antibody (Santa Cruz) for 1 h at room temperature. The cells showing immunofluorescence were observed and photographed using a fluorescence microscope (Olympus, Tokyo, Japan). Additionally, PBS instead of primary antibody against EPOR was used as the negative control.

2.3. Detection of intracellular reactive oxygen species

Since high osmolarity may increase intracellular ROS levels, we used mannitol with equal osmolarity to high glucose as the osmolarity control group. Confluent cells in 96-well plates were exposed to:

- normal DMEM/F12 medium for 24 h;
- 25 mM mannitol for 24 h;
- 25 mM glucose for 24 h;
- 50 U/mL EPO for 1 h followed by 25 mM glucose for 24 h;
- 100 U/mL EPO for 1 h followed by 25 mM glucose for 24 h.

Intracellular ROS production was measured by the oxidation-sensitive DCFH-DA dye (Molecular Probes, Minnesota, USA), which was oxidized to the highly fluorescent 2′7′-dichloro-uorescein (DCF) by H_2O_2 or OH^- within the cells. Briefly, cells were incubated in the dark for 40 min at 37 °C after adding 10 μmol/L of DCFH-DA, and then washed three times with PBS. ROS generation was detected using a florescence-quantifying instrument (FLUOstar, BMG, Germany). All experiments were repeated three times.

2.4. Apoptosis assay

After being treated as mentioned above in 6-well plates, cells were trypsinized, centrifuged, and washed twice with ice-cold PBS. The cells were then resuspended in a 500-μL cell suspension buffer and added to a 5 mL centrifuge tube; then, 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added to the tube. Cells were incubated at room temperature for 15 min and then analyzed by flow cytometry (Beckman, CA, USA). All experiments were repeated three times.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

After being treated as mentioned above, RNA was extracted from the NRK-52E cells using RNA-Solv reagent (Invitrogen, CA, USA). The total RNA extract (2 μg) was used to synthesize the first-strand cDNA serving as the template for the amplification of genes encoding *Bcl-2*, *Bax*, *caspase-3*, and *β-actin*. The respective forward and reverse primer sequences (SBS Gene Tec, China) were as follows: *Bcl-2*, 5′-CCTGGCATCTTCTCCTT-3′ and 5′-ACATCTCCCTGTTGACG-3′; *Bax*, 5′-CAGGGTTTCATCCAGG-3′ and 5′-TAGCAAAGTAGAAGAGGG-3′; *caspase-3*, 5′-GCTGGACTGCGGTATTGAG-3′ and 5′-ACGGGATCTGTTTCTTTGC-3′; and *β-actin*, 5′-AGCCATGTACGTAGCCATCC-3′ and 5′-TCTCAGCTGTGGTGGTGAAG-3′. The lengths of the PCR products were 355, 135, 290, and 227 bp, respectively.

Amplification was performed using the Master Mixkit (Invitrogen) in a total volume of 50 μL, according to the manufacturer's cycling parameters. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and the band intensities were determined using Image Quant Software.

2.6. Western blotting

After being treated as mentioned above, proteins were extracted using 150 μL of RIPA lysis buffer (Beyotime, Jiangsu, China), centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatant was collected. The protein content was measured by using a bicinchoninic acid (BCA) protein assay kit (PierceBio, USA). The proteins were resolved under denaturing conditions on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and electroblotted onto a nitrocellulose membrane. The blotted membrane was incubated in 5% defatted milk in PBS with 0.1% Tween 20 for 1 h at 24 °C, and then incubated overnight at 4 °C with primary antibodies (Santa Cruz). After washing three times, the membrane was incubated with horseradish peroxidase-conjugated anti-goat or anti-rabbit secondary antibodies (Santa Cruz). Antibody binding was detected using enhanced chemiluminescence (ECL) kit (Santa Cruz) according to the manufacturers' instructions. The intensity of the bands was analyzed with Alpha Ease FC image software. Each experiment was repeated three times.

2.7. Statistical analysis

Data were presented as means ± SD, and were first analyzed using one-way analysis of variance (Anova) and then with Student–Newman–Keuls post-hoc test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. The expression of erythropoietin receptor in NRK-52E cells

Using immunocytochemistry and western blotting, we analyzed the expression of EPOR in NRK-52E cells (Figs. 1 and 2). Immunocytochemistry revealed that EPOR is a transmembrane receptor.

3.2. The effect of high glucose on erythropoietin receptor protein

The effect of high glucose on the EPOR protein was analyzed by western blotting. As shown in Fig. 2, compared with the untreated group, high glucose increased the level of EPOR significantly in NRK-52E cells.

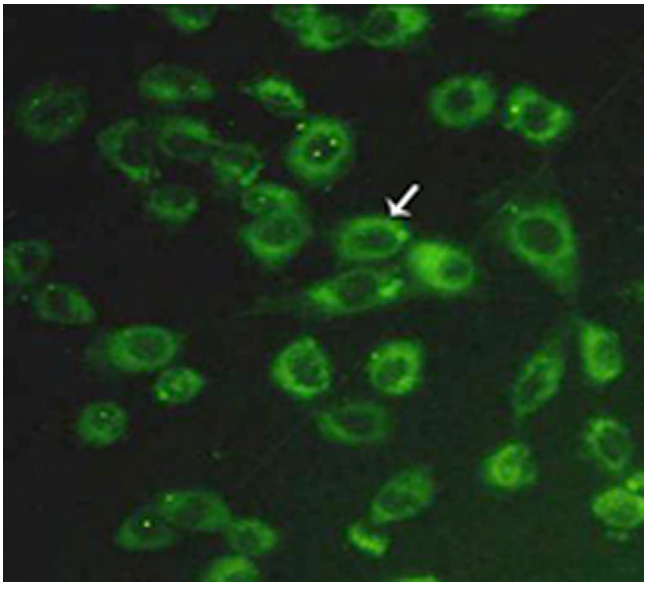

Fig. 1. The expression of erythropoietin receptor in NRK-52E cells treated with normal DMEM/F12 media (immunocytochemistry, × 400).

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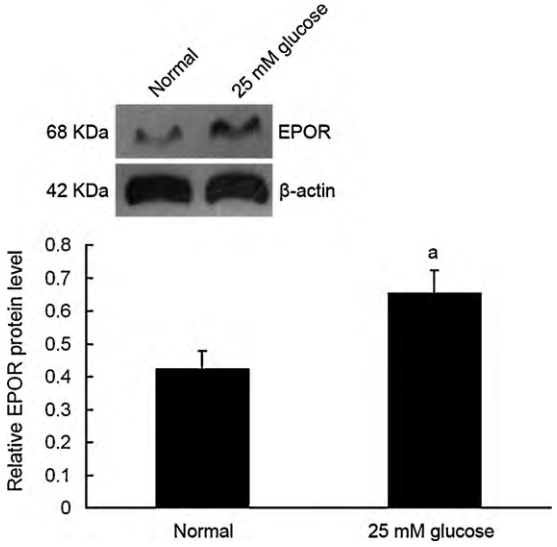

Fig. 2. Western blotting analysis of erythropoietin receptor protein in high glucose-treated NRK-52E cells. Cells were treated with 25 mM glucose (group 2) or with normal DMEM/F12 media (group 1) for 24 h. The cellular proteins were extracted and separated by sulfate-polyacrylamide gel electrophoresis for immunoblotting. β-actin was used as an internal control (values are presented as mean ± SD. a: $P < 0.05$ vs. normal control group).

3.3. Effects of erythropoietin on the high glucose-induced production of intracellular reactive oxygen species

As shown in Fig. 3, the exposure of NRK-52E cells to high glucose levels for 24 h was associated with a significant increase of intracellular ROS generation ($P < 0.05$ vs. normal control group). However, mannitol with the equal osmolarity as high glucose failed to increase the ROS level. Meanwhile, ROS production decreased significantly in EPO pretreated groups, and high-dose EPO (group 5) had a significant inhibitory effect on ROS production.

3.4. Flow cytometric assessment of NRK-52E cell apoptosis

After annexin V and PI double staining, induction of NRK-52E apoptosis by high glucose was confirmed by flow cytometry ($P < 0.05$ vs. normal control group). When NRK-52E cells were pretreated with high glucose and EPO, the rate of both early and late apoptosis decreased remarkably as compared with that of high glucose treatment alone for 24 h (Fig. 4).

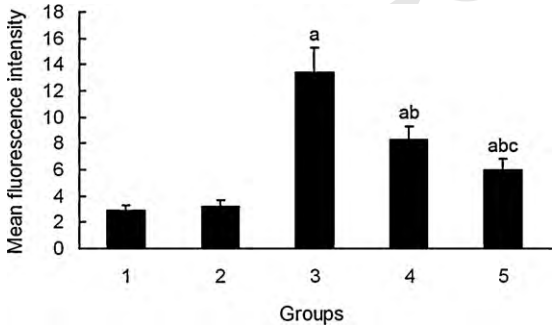

Fig. 3. Effects of erythropoietin on high glucose-induced ROS production in NRK-52E cells. 1, untreated cells; 2, cells treated with 25 mM mannitol; 3, cells treated with 25 mM glucose; 4, cells treated with 25 mM glucose and 50 U/mL erythropoietin; and 5, cells treated with 25 mM glucose and 100 U/mL erythropoietin (values are presented as mean ± SD. a: $P < 0.05$ vs. group 1; b: $P < 0.05$ vs. group 3; c: $P < 0.05$ vs. group 4).

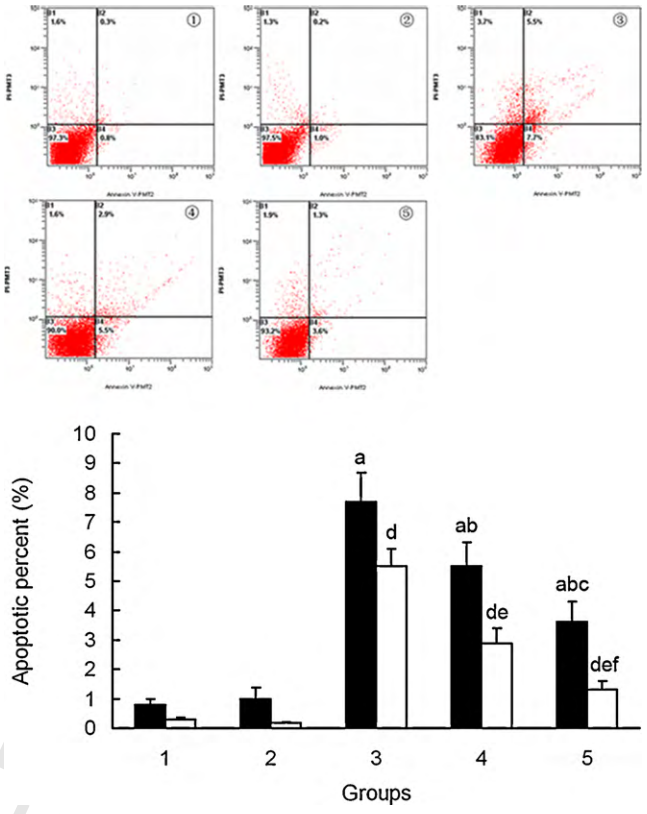

Fig. 4. Effect of erythropoietin on high glucose-induced NRK-52E cell apoptosis. Cells that are A^+ and PI^- are in the early stages of apoptosis and those that are A^+ and PI^+ are in the late stages of apoptosis. 1, untreated cells; 2, cells treated with 25 mM mannitol; 3, cells treated with 25 mM glucose; 4, cells treated with 25 mM glucose and 50 U/mL erythropoietin; and 5, cells treated with 25 mM glucose and 100 U/mL erythropoietin (values are presented as mean ± SD. a, d: $P < 0.05$ vs. group 1; b, e: $P < 0.05$ vs. group 3; c, f: $P < 0.05$ vs. group 4).

3.5. Alterations of Bcl-2, Bax, and caspase-3 mRNA expression in NRK-52E cells

The effects of high glucose with or without EPO on the mRNA expressions of *Bcl-2*, *Bax*, and *caspase-3* were analyzed by RT-PCR. As shown in Figs. 5 and 6, untreated NRK-52E cells expressed low levels of *Bax* and *caspase-3* mRNA and high levels *Bcl-2* mRNA, whereas 25 mM high glucose treatment upregulated *Bax* and *caspase-3* mRNA and downregulated *Bcl-2* mRNA. In contrast, EPO significantly reduced high glucose-induced upregulation of *Bax* and *caspase-3* mRNA expression and increased high glucose-induced downregulation of *Bcl-2* mRNA expression.

3.6. Alterations of Bcl-2, Bax, and caspase-3 proteins in NRK-52E cells

The effects of high glucose with or without EPO on *Bcl-2*, *Bax*, and *caspase-3* were analyzed by western blotting. As shown in Figs. 7 and 8, *Bax* and *caspase-3* protein levels increased 2.4- and 2.7-fold by high glucose, respectively, and *Bcl-2* protein levels decreased 2.3-fold. Combined treatment with high glucose and EPO markedly attenuated *Bax* and *caspase-3* protein expression, while treatment with glucose only increased *Bcl-2* protein expression.

4. Discussion

EPO is a 30.4 kDa acidic glycoprotein hormone, primarily synthesized by renal cortical fibroblast-like cells, and to a small degree by the brain, liver, and uterus [10]. During renal tissue

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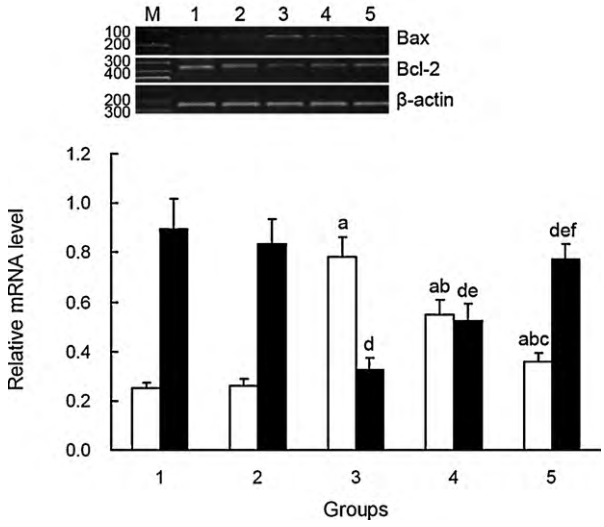

Fig. 5. Effect of high glucose with or without erythropoietin on Bcl-2 and Bax mRNA expression in NRK-52E cells. 1, untreated cells; 2, cells treated with 25 mM mannitol; 3, cells treated with 25 mM glucose; 4, cells treated with 25 mM glucose and 50 U/mL erythropoietin; and 5, cells treated with 25 mM glucose and 100 U/mL erythropoietin (values are presented as mean ± SD. a, d: $P < 0.05$ vs. group 1; b, e: $P < 0.05$ vs. group 3; c, f: $P < 0.05$ vs. group 4).

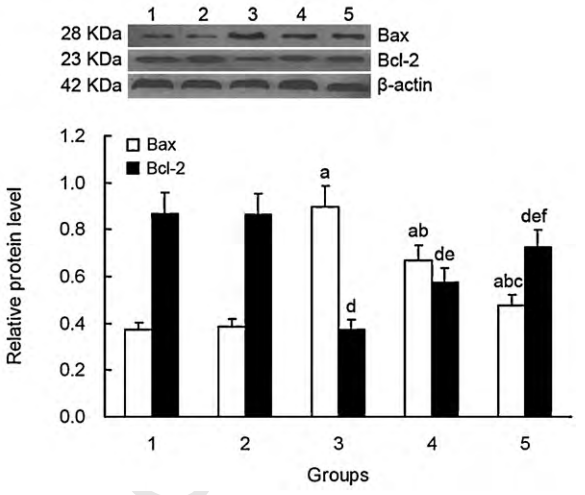

Fig. 7. Effect of high glucose with or without erythropoietin on Bcl-2 and Bax proteins in NRK-52E cells. 1, untreated cells; 2, cells treated with 25 mM mannitol; 3, cells treated with 25 mM glucose; 4, cells treated with 25 mM glucose and 50 U/mL erythropoietin; and 5, cells treated with 25 mM glucose and 100 U/mL erythropoietin (values are presented as mean ± SD. a, d: $P < 0.05$ vs. group 1; b, e: $P < 0.05$ vs. group 3; c, f: $P < 0.05$ vs. group 4).

hypoxia, EPO is thought to be primarily released into the circulatory system from the kidneys, where it then binds to the transmembrane receptor EPOR on erythroid progenitor cells [11]. Erythropoiesis is ultimately stimulated by the inhibition of apoptosis of immature erythroblasts through the *Bcl-2* family of anti-apoptotic genes, thereby permitting their progression to mature erythrocytes [11]. As the location of EPOR was previously thought to be limited to erythroid precursor cells, and the role of EPO was restricted to these cells, its additional effects on kidney and other organs have long been neglected. However, in addition to its well-known role in hematopoiesis, the cytoprotective effects of EPO in non-hematopoietic cells have been confirmed. Bianchi et al. identified that EPO can inhibit and remedy diabetic peripheral neuropathy in a diabetic rat model [12]. Bahlmann et al. recently found that weekly subcutaneous administration of low-dose darbepoetin (0.1 μg/kg) to rats following 5/6 nephrectomy significantly attenuated subsequent renal and endothelial damage, preserved renal function, and enhanced survival rate, while having no effect on hematocrit levels [13]. Intriguingly, renal cortical fibroblast-like cells, the primary EPO-producing cells, are in direct contact with the basal aspects of proximal and distal tubular cells [14,15]. Therefore, because of the anatomical relationship between EPO-secreting cells and renal cells, EPO can conveniently play its endocrine and paracrine roles in the kidneys.

There is increasing evidence that excessive ROS production plays an important role in the development of DKD [1,16]. It is now clear that the overproduction of ROS in diabetes is a direct consequence of hyperglycemia and that various types of cells,

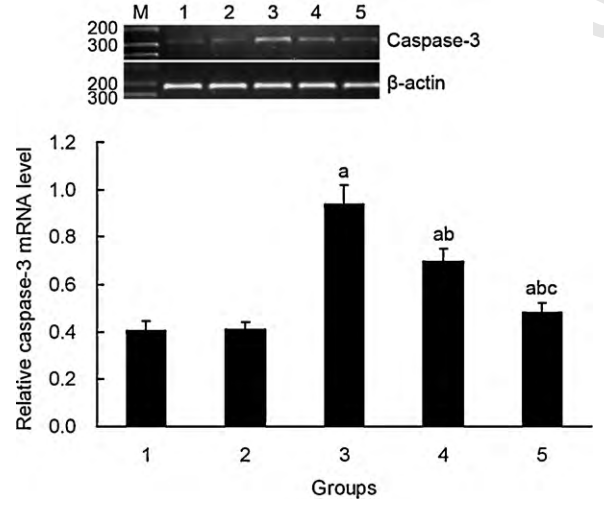

Fig. 6. Effect of high glucose with or without erythropoietin on caspase-3 mRNA expression in NRK-52E cells. 1, untreated cells; 2, cells treated with 25 mM mannitol; 3, cells treated with 25 mM glucose; 4, cells treated with 25 mM glucose and 50 U/mL erythropoietin; and 5, cells treated with 25 mM glucose and 100 U/mL erythropoietin (values are presented as mean ± SD. a, d: $P < 0.05$ vs. group 1; b, e: $P < 0.05$ vs. group 3; c, f: $P < 0.05$ vs. group 4).

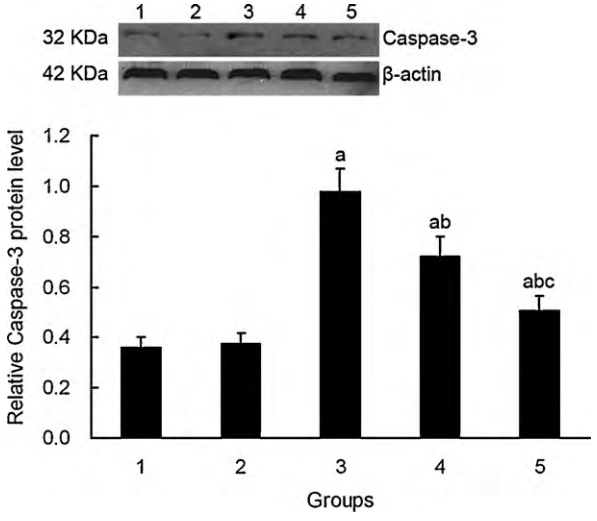

Fig. 8. Effect of high glucose with or without erythropoietin on caspase-3 protein in NRK-52E cells. 1, untreated cells; 2, cells treated with 25 mM mannitol; 3, cells treated with 25 mM glucose; 4, cells treated with 25 mM glucose and 50 U/mL erythropoietin; and 5, cells treated with 25 mM glucose and 100 U/mL erythropoietin (values are presented as mean ± SD. a: $P < 0.05$ vs. group 1; b: $P < 0.05$ vs. group 3; c: $P < 0.05$ vs. group 4).

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including renal tubular cells can produce ROS under hyperglycemic conditions [17]. Excessive ROS production can cause membrane lipid peroxidation, intracellular protein denaturation, and DNA damage, through which ROS directly results in cell damage and finally, the occurrence of diseases [18,19]. Verzola et al. [20] confirmed that ROS is important in high glucose-induced renal tubular cell apoptosis. In addition to activating apoptosis programs, ROS can also regulate the expression of apoptosis-related genes.

The *Bcl-2* family is closely related to apoptosis. *Bcl-2* is an anti-apoptotic gene, while *Bax* is a pro-apoptotic gene, and both participate in the regulation of the mitochondrial apoptotic pathway. When the expression of *Bcl-2* decreases and the expression of *Bax* increases, the mitochondrial membrane undergoes depolarization and cytochrome C and other apoptotic factors are released. These apoptotic factors can lead to *caspase* activation, which is the final step of the apoptotic pathway. This step mediates ROS-induced apoptosis through the mitochondrial pathway [21,22]. In our study, ROS production increased following stimulation with high glucose, as did the mRNA expression of *Bax* and *caspase-3*; however, the mRNA expression of *Bcl-2* decreased. Pretreatment with EPO inhibited the overproduction of ROS, reversed the effects of high glucose on the expression of *Bcl-2*, *Bax*, and *caspase-3*, and prevented high glucose-induced apoptosis of renal tubular cells. These experiments showed that EPO can inhibit the pro-apoptotic effect of high glucose through anti-oxidative stress.

Recently, Li et al. [23,24] confirmed that EPO can cause EPOR dimerization, phosphorylation, and activation of Janus kinase 2 (a tyrosine kinase bound to the β-subunit of the EPOR) after interaction with EPOR. Consequently, a series of signaling pathways are activated, including signal transducer and activator of transcription 5 (STAT5), mitogen-activated protein kinase (MAPK), and the phosphoinositol 3 kinase (PI3K)/Akt signaling pathways, which exert the physiological roles of EPO [23,24]. It would appear that at least some of the renal protective effects of EPO are mediated by the activation of EPOR, since an EPO analogue that does not bind EPOR cannot ameliorate experimental renal injury in some studies [25].

Recently some studies confirmed that EPOR exists in the rat brain, gastric epithelial cells, and Leydig cells [26–28]. Furthermore, EPO stimulates mitotic synthesis in gastric epithelial cells [26] and testosterone synthesis in Leydig cells [27]. Additionally, EPO showed a protective effect on the brain in a cerebral ischemic injury rat model [28]. All of these experiments suggest that EPO can play an important role in many non-hematopoietic cells, and that this process may be mediated by EPOR.

In this study, using immunofluorescence and western blotting, we confirmed that renal tubular cells express EPOR. Moreover, EPOR expression significantly increases after stimulation with high glucose. Because there is no commercially available EPOR-specific inhibitor, we were unable to define the role of EPOR directly in this study. However, combined with previously reported studies, it can be safely inferred that EPOR plays a key role in the cytoprotection of EPO.

In conclusion, EPO, a glycoprotein hormone primarily secreted by renal cortex fibroblast-like cells, can prevent high glucose-induced oxidative stress and apoptosis in renal tubular cells, and this protective effect may be mediated by EPOR. This study identifies new knowledge concerning the function of EPO and may provide a new option for the treatment of DKD.

Competing interests

The authors declare that they have no competing interests.

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