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Dexmedetomidine protects high-glucose induced apoptosis in human retinal pigment epithelial cells through inhibition on p75(NTR)

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ABSTRACT

Background: In both human patients and animals, diabetic condition of high blood glucose induced significant apoptotic responses in retinal pigment epithelial (RPE) cells. In this work, we used an in vitro culture model of human ARPE-19 cells to evaluate whether dexmedetomidine (DEX) may protect high D-glucose (DG)-induced diabetic apoptosis.

Methods: ARPE-19 cells were incubated with DG in vitro to induce apoptosis. Cells were also pre-incubated with different concentrations of DEX prior to DG treatment. The apoptotic injury by DG, and possible protection by DEX were evaluated using a TUNEL assay. Western blot assay was used to evaluate DEX-associated signaling pathway proteins, including Casp-3, precursor of the nerve growth factor (proNGF) and p75 neurotrophin receptor (p75(NTR)). Moreover, p75(NTR) was overexpressed in ARPE-19 cells, to assess its mechanistic role in DEX-mediated protection on DG-induced apoptosis.

Results: In ARPE-19 culture, DG induced significant apoptosis, which was protected by pre-incubation of DEX, in a concentration-dependent manner. DG-induce apoptosis was associated with protein upregulation of Casp-3, proNGF and p75(NTR). Among them, Casp-3 and p75(NTR) were inversely reduced by DEX pre-incubation, but not proNGF. In ARPE-19 cells, p75(NTR) overexpression was shown to reverse the protective effect of DEX on DG-induced apoptosis.

Conclusion: DEX was proven to have protective effect on DG-induced RPE apoptosis, possible through inhibition on p75(NTR) and its associated signaling pathways.

1. Introduction

Diabetic retinopathy (DR) is one of the commonly occurring microvascular complications in patients with diabetes, and remains one of the major causes of vision loss and blindness of working-age patients [1–3]. During the development of DR, various retinal tissues underwent significant pathological alterations. Among them, retinal pigment epithelium (RPE), a constituting element of outer blood-retinal barrier (BRB) and an important supporting component of photoreceptor excitability [4,5], was shown to respond to diabetic conditions with irreversible cellular and molecular dysfunctions, including inflammation, reduction of permeability, excessive production of reactive oxygen species (ROS) and apoptosis [5]. Unfortunately, as most of the research on DR has been focused on neuronal and vascular impairments in retina, little is known about the underlying mechanisms contributing to pathological events in RPE.

Dexmedetomidine (DEX) is a potent α2-adrenergic agonist, and commonly used as a sedative reagent in intensive care units (ICUs) [6,7]. During past decades, emerging evidence has demonstrated that DEX may exert protective or therapeutic effects on various types of cellular injuries, especially ischemia/reperfusion (I/R) injury, in both human and animal organs, including lung, heart, liver and brain [8–14]. In retina, it was shown that DEX might also protect or reduce neuro-ocular I/R injury. For instance, in an in vivo rat retina model, DEX was demonstrated to significantly decrease retinal thickness and reduce apoptotic cell population in I/R injury induced by transient elevation of intraocular pressure [12]. Most recently, through biochemical and functional assessments, it was demonstrated that toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF-kB) signaling pathways were involved in the protection of DEX against retinal I/R injury [15]. However, it is unclear whether DEX may also have rescuing effect in diabetes-associated retinal injury, especially in diabetes-injured human retinal pigment epithelial cells.

In the current work, we took advantage of an in vitro RPE diabetic retinopathy model, by culturing human retinal pigment epithelial cell line, ARPE-19 cells and inducing diabetic apoptosis through the

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application of high concentrations of D-glucose (DG) [16–18]. In ARPE-19 culture, pre-incubation of DEX was introduced before DG application, to allow us to investigate the possible protective effect of DEX on DG-induced cellular apoptosis. In addition, we used western blot assay and genetic transfection technology to further explore the downstream signaling pathways associated with DEX-mediated protection on DG-induced apoptosis in ARPE-19 cells.

2. Materials and methods

2.1. Ethic evaluation

In our work, all protocols were reviewed and approved by the Animal Research and Ethic Committee at Jining First People's Hospital in Jining, Shandong province, China. All experimental procedures were performed in accordance with the principles of Declaration of Helsinki, and research regulations in People's Republic of China.

2.2. Cell culture

The human retinal pigment epithelial cell line, ARPE-19 was commercially purchased from the China Center for Type Culture Collection (CCTCC, Beijing, China). Cells were maintained in 24-well plates in Dulbecco's modified Eagle medium / Ham's F-12 nutrient mixture (DMEM/F12, Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China) 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Shanghai, China) in a humidified tissue-culture chamber at 37 °C with 5% CO_2.

2.3. Application of D-glucose and dexmedetomidine in cell culture

It was demonstrated that, treatments of high concentrations of glucose induced significant apoptosis in ARPE-19 cells [16–18]. In this study, we applied 100 mM D-glucose (DG, MilliporeSigma, Shanghai, China) in ARPR-19 culture (24-well plate, 50,000 cells / well) for 24 h at 37 °C to induce apoptosis. Prior to DG application, DRPE-19 cells were pre-incubated with dexmedetomidine (DEX, MilliporeSigma, Shanghai, China) at concentrations (μM) of, 0, 0.01, 0.1, 1, 10, 100, 500, 1000 and 5000 for 6 h at 37 °C.

2.4. Apoptosis assay

The method of evaluating ARPE-19 apoptosis was described in our previous publications [19,20]. Briefly, ARPE-19 cells were quickly fixed by 4% paraformaldehyde (PFA, MilliporeSigma, Shanghai, China), and examined by Click-iT® Plus TUNEL assay (Alexa Fluor™ 594 dye, Invitrogen, Shanghai, China) according to the manufacturer's protocol. The nuclei of ARPE-19 cells were identified using the hoechst33342 antibody. Fluorescent images were captured on a Zeiss LSM 510 confocal microscopy (Zeiss, Germany) and analyzed using and ImageJ software (ImageJ, NIH, USA). Percentage of apoptotic ARPE-19 cells were quantified by calculating the numbers of TUNEL / Hoechst double-immunoresponsive cells against Hoechst 33342 immunoresponsive cells.

2.5. Western blot assay

The method of western blot assay was described in our previous publication [19,20]. Briefly, ARPE-19 culture was lysed with a Tris-based RIPA buffer containing 50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1× protease cocktail (MilliporeSigma, Shanghai, China). Then, proteins were separated on 8% SDS-PAGE and then transferred to nitrocellulose membranes, which were blocked by 5% non-fat dry milk and 1% BSA for 2 h. Membranes were incubated with primary antibodies against Caspase-3 (Casp-3, 1:500, MilliporeSigma, Shanghai, China), precursor of Nerve growth factor (proNGF, 1:500, MilliporeSigma, Shanghai, China), p75 neurotrophin receptor (p75(NTR), 1:200, MilliporeSigma, Shanghai, China) and beta-actin (1:1,000, MilliporeSigma, Shanghai, China) overnight at 4 °C. They were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000, New England Biolabs, USA) for 2 h at room temperature. Blots were visualized and dosimetry was analyzed using an enhanced chemiluminescence system (Pierce, USA) according to manufacturer's protocol.

2.6. P75(NTR) overexpression assay

A pcDNA3.1/hygro expression vector with the insertion of human p75(NTR) gene, pc/p75, was commercially purchased from Sangon Biotech (Shanghai, China). An empty pcDNA3.1/hygro was also commercially purchased from Sangon Biotech (Shanghai, China), and applied as control overexpression vector, pc/C. ARPE-19 cells were transfected with pc/p75 or pc/C using Liopfectamine3000 (Invitrogen, Shanghai, China), and selected by hygromycin (0.8 mg/ml, Invitrogen, Shanghai, China) according to manufacturer's protocol. Healthy colonies were passaged for 3 times before transfection efficiency was evaluated by western blot assay. All experiments using the transfected ARPE-19 cells were within 15 passages.

2.7. Statistical analysis

All experiments were repeated at least for three times, and the averaged results were presented as mean ± standard error. Statistical analysis was performed using an unpaired two-tail Student's *t*-test on a windows-based SPSS software (SPSS, USA). Difference was significant if $P < 0.05$.

3. Results

3.1. Dexmedetomidine protects high-glucose induced ARPE-19 apoptosis

Previous studies had shown that, in the in vitro culture of ARPE-19 cells, applications of high concentrations of D-glucose (DG) induced significant apoptosis [16–18]. In this study, we investigated whether Dexmedetomidine (DEX) may rescue this injury. First, ARPE-19 culture was incubated with 100 mM DG for 24 h. Through a TUNEL assay, it showed that the percentage of apoptotic (TUNEL- immunoresponsive) ARPE-19 cells was close to 90%, significantly higher than the percentage of apoptotic ARPE-19 cells (~ 0%) under control condition (no DG) (Fig. 1A, DG vs. Control, * $P < 0.05$).

Then, prior to DG treatment, ARPE-19 cells were pre-incubated with various concentrations of DEX. This time, the TUNEL assay showed that, pre-incubation of moderate to high concentrations of DEX (from 1 to 5000 μM) could considerably ameliorate DG-induced apoptosis (Fig. 1A, DG + DEX vs. DG, * $P < 0.05$).

The imaging results of TUNEL assay were assessed. Under control condition (no DG or DEX), no TUNEL- immunoresponsive ARPE-19 cells were detected (Fig. 1B, Control). Under DG condition (100 mM DG applied in ARPE-19 culture for 24 h), large amount of ARPE-19 cells were TUNEL-immunoresponsive (Fig. 1B, DG). Alternatively, under DG + DEX condition (ARPE-19 culture pre-incubated with 100 μM for 6 h prior to DG treatment), significant reduction of TUNEL-immunoresponsive ARPE-19 cells was detected (Fig. 1B, DG + DEX).

Moreover, ARPE-19 culture was treated with DEX only (No DG) at various concentrations. Measurement on TUNEL assay indicated that, at concentrations between 0.01 and 100 μM, DEX did not cause any significant apoptosis (Fig. 1C). Only at high concentrations, between 500 and 1000 μM, DEX induced apoptosis in 10%~30% of ARPE-19 cells (Fig. 1C, * $P < 0.05$).

Therefore, these results demonstrated DEX protected DG-induced apoptosis in ARPE-19 cells.