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Hyperglycemia-induced GLP-1R downregulation causes RPE cell apoptosis

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ABSTRACT

Glucagon-like peptide-1 receptor (GLP-1R) is closely associated with the onset of diabetes and its complications. However, its roles in diabetic retinopathy are unknown. Retinal pigment epithelial (RPE) cells are a crucial component of the outer blood–retina barrier and their death is related to the progression of diabetic retinopathy. Thus, we examined the pathophysiological role of GLP-1R in RPE cell apoptosis. We found that GLP-1R expression was lower in the isolated neuroretina and RPE cells of streptozotocin-treated rats than in vehicle-treated rats. High-glucose treatment also decreased GLP-1R expression in a human RPE cell line (ARPE-19 cells). GLP-1R was silenced in ARPE-19 cells, in order to elucidate the pathophysiological roles of GLP-1R. This increased intracellular reactive oxygen species (ROS) generation and activated p53-mediated *Bax* promoter and endoplasmic reticulum (ER) stress signaling. We also found that GLP-1R knockdown-mediated p53 expression was regulated by ER stress. Interestingly, antioxidant treatment and peroxiredoxin 1 (Prx1) overexpression attenuated GLP-1R knockdown-induced ER stress signaling and p53 expression. Finally, to confirm that GLP-1R activation has protective effects, ARPE-19 cells were treated with exendin-4, a synthetic GLP-1R agonist. This attenuated high-glucose-induced ROS generation, ER stress signaling, and p53 expression. Collectively, these results indicated that hyperglycemia decreases GLP-1R expression in RPE cells. Such a decrease generates intracellular ROS, which increases ER stress-mediated p53 expression, and subsequently causes apoptosis by increasing *Bax* promoter activity. Our data suggested that regulation of GLP-1R expression is a promising approach for the treatment of diabetic retinopathy.

1. Introduction

Retinal pigment epithelial (RPE) cells are an integral component of the blood–retina barrier (BRB). RPE cell damage is closely related to breakdown of the BRB, leading to visual impairment. RPE cells are believed to play an important role in the pathogenesis of diabetic retinopathy. The hyperglycemia of diabetes damages the BRB by increasing the generation of intracellular reactive oxygen species (ROS) in RPE cells, which are known to be vulnerable to oxidative stress (Strunnikova et al., 2004; Li et al., 2012a,b). Our previous report also demonstrated that the pathophysiology of RPE is closely related to the onset of diabetic retinopathy (Lim et al., 2012).

Glucagon-like peptide-1 (GLP-1) is a gut-derived incretin hormone that stimulates insulin secretion by pancreatic β-cells (Baggio and Drucker, 2007). Researchers initially identified that circulating GLP-1 binds to its specific receptor, the GLP-1 receptor (GLP-1R), which is a member of the G-protein coupled receptor superfamily. GLP-1R is located on the pancreatic β-cell surface. Activated GLP-1R signaling promotes insulin synthesis and release (Thorens et al., 1993). GLP-1R expression is decreased in pancreatic islets and INS-1 cells in hyperglycemia, which might contribute to impaired GLP-1 action (Xu et al., 2007; Pan et al., 2009). However, GLP-1R is also expressed in various tissues such as the brain, liver, and kidney (Shughrue et al., 1996; Gupta et al., 2010; Li et al., 2012a,b). Several lines of evidence have reported a role for

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GLP-1R in diabetic complications. Mima et al. (2012) reported that glomerular GLP-1R expression decreased with hyperglycemia in patients with diabetic nephropathy. Zhang et al. (2011) observed that treatment with a GLP-1R agonist prevented retinal cell death in streptozotocin (STZ)-induced diabetic rats. However, the role of RPE GLP-1R in the progression of diabetic retinopathy has not been elucidated. Exendin-4, a synthetic GLP-1R agonist, and GLP-1 treatment of RPE cells inhibits the receptor for advanced glycation end products (RAGE) and adhesion molecule expression in a glycated albumin group, suggesting that there may be a relationship between GLP-1R and RPE cells in patients with diabetic retinopathy (Dorecka et al., 2013). Furthermore, in a very recent study, GLP-1R expression was confirmed in RPE cells (Puddu et al., 2013). Thus, we hypothesized that the regulation of GLP-1R plays a critical role in the function of RPE cells, especially in diabetic retinopathy.

Diverse signal molecules may be involved in the pathogenesis of diabetic retinopathy. Endoplasmic reticulum (ER) stress is a physiological or pathological response to eliminate unfolded or misfolded proteins that excessively accumulate within the ER. Activated ER stress regulates several cellular processes through three major branches: protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Malhi and Kaufman, 2011). First, PERK signaling is activated by autophosphorylation, which subsequently phosphorylates eukaryotic inhibition factor alpha (eIF2α). Phosphorylated eIF2α increases translation of activating transcription factor 4 (ATF4), which increases apoptotic gene expression such as that of CCAAT-enhancer-binding protein homologous protein (CHOP). Second, IRE1 signaling is activated by autophosphorylation, which induces unconventional splicing of X-box binding protein-1 (XBP1) mRNA. Third, ATF6 signaling is activated by cleavage of ATF6 by S1P and S2P in the Golgi apparatus. The cleaved subunit (p50ATF6) then functions as a transcription factor.

The tumor suppressor p53 is a multifunctional protein that regulates various cellular processes. During oxidative stress, p53 expression is increased, translocates to the nucleus, and binds to p53 response elements (p53RE) in the promoter region of its target genes (Liu and Xu, 2011). *Bax* is a p53 target gene that encodes BCL2-associated X protein (Bax), which induces cellular apoptosis (Fan et al., 2008). p53 and Bax expression is closely associated with RPE cell apoptosis (Medearis et al., 2011; Sawada et al., 2014). Kovacs et al. (2011) reported that p53 is involved in early diabetic retinopathy in streptozotocin-induced diabetic rats. Additionally, GLP-1R signaling negatively regulates p53 signaling in PC12 cells (Guo et al., 2009). These findings suggest that GLP-1R, oxidative stress, and p53 signaling mediate RPE cell dysfunction. Therefore, we examined the role of GLP-1R in RPE cells, especially in diabetic retinopathy, and its related signal pathways.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's nutrient mixture F-12, and fetal bovine serum (FBS) were purchased from Life Technologies (Gibco BRL, Grand Island, NY, USA). D-glucose, streptozotocin, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), *N*-acetylcystein (NAC) and L-ascorbic acid (vitamin C) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thapsigargin and PERK inhibitor were purchased from Millipore (Billerica, MA, USA), and GLP-1R antibody (ab39072), p-IRE antibody (ab48187), ATF6 antibody (ab11909), and Exendin-4 (ab120214) were purchased from Abcam (Cambridge, UK). p53 antibody (#9282), p-eIF2α antibody (#9721), and CHOP antibody (#2895) were purchased from Cell Signaling Technology (Beverly, MA, USA). ATF4 antibody (NB100-852) was obtained from Novus (CO, USA). β-Actin antibody (sc-1616) and p-PERK antibody (sc-32577-R) were purchased from Santa Cruz Biotechnology (CA, USA). HA antibody was obtained from Covance (WI, USA). All reagents were of the highest purity commercially available.

2.2. Animal experiments and retina isolation

Hyperglycemia was induced in overnight fasted, 8-week-old male SD rats ($n = 7$) by intraperitoneal injection of streptozotocin (55 mg/kg) dissolved in cold and fresh citrate buffer (0.1 M and pH 4.5). Control rats ($n = 7$) were injected with citrate buffer. After 2 weeks, all rats were sacrificed and eyes were extracted. Neuroretina and RPE were isolated under microscopic dissection according to modified Sawamiphak's method (Sawamiphak et al., 2010). Isolated tissues were homogenized and western blotting was subsequently performed. All animal experiments were performed in accordance with the National Institutes of Health animal research standards. The protocols were approved by the Chonnam National University Laboratory Animal Research Center.

2.3. Cell culture

The human RPE cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). ARPE-19 cells were grown in DMEM/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 in air. Stock cultures of ARPE-19 cells were subcultured once a week (split ratio 1:6). Cells were grown to confluence in 60 mm dishes in DMEM/Ham's F-12 with 15 mM HEPES buffer, 10% FBS, 5.5 mM glucose, 0.35% additional sodium bicarbonate, 2.5 mM L-glutamine, and 1% penicillin/streptomycin at 37 °C. The media was changed every other day. Passaged cells were plated to yield near-confluent cultures at the end of the experiments.

2.4. Western blotting

Western blot analysis was performed according to methods described previously (Kim and Park, 2013). Transferred membranes were probed with various antibodies diluted at the manufacturer recommended ratio. The bands were visualized with a Luminescent image analyzer (ImageQuant LAS 4000, GE Healthcare, UK) using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare, UK).

2.5. mRNA extraction and quantitative RT-PCR

mRNA extraction and quantitative RT-PCR were performed according to methods described previously (Kim and Park, 2013). The primers used were 5′-TCCTTCATCCTGCGAGCATT-3′ (sense), 5′-TGGGCGGCTGTGCTATACA-3′ (antisense) for human *Glp1r* and 5′-AGGCCCAGAGCAAGAGAG-3′ (sense), 5′-TCAACATGATCTGGGTCATC-3′ (antisense) for human β-actin. β-Actin was used as a control to confirm the quantity of the mRNA.

2.6. Immunofluorescence assay and confocal microscope

Cells were washed twice in PBS and fixed for 10 min with 4% paraformaldehyde in PBS. After three washes in PBS, fixed cells were permeabilized with 0.2% Triton X-100. 1% bovine serum albumin (BSA) solution was used for blocking. The cells were incubated with GLP-1R antibody (dilution ratio, 1:100) for 15 h at 4 °C. After three washes in PBS, the cells were incubated with anti-rabbit FITC secondary antibody (Sigma, USA). The cells were then mounted on slides and the nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI) present in the ProLong Gold