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S1PR2 antagonist protects endothelial cells against high glucose-induced mitochondrial apoptosis through the Akt/GSK-3β signaling pathway

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ABSTRACT

Vascular complications are the main cause of morbidity and mortality associated with type 2 diabetes mellitus. An early hallmark of the onset of vascular complications is endothelial dysfunction and apoptosis. We aimed to explore the role of sphingosine-1-phosphatereceptor 2 (S1PR2) in high glucose-induced endothelial cells apoptosis and to elaborate the underlying mechanism. Human umbilical vein endothelial cells (HUVECs) were cultured in a high glucose with or without S1PR2 antagonist. The apoptosis of the cells was measured by flow cytometry and mitochondrial membrane permeability was detected by the fluorescent probe JC-1. The expression of the related protein was determined by western blot. Cell apoptosis and the loss of mitochondrial membrane permeability were induced under high glucose conditions in HUVECs. The expression of mitochondrial apoptosis related protein bax increased and bcl-2 decreased in high glucose-induced HUVECs. The level of cytochrome *c* released into the cytoplasm increased when cells were exposed to high glucose. In addition, the expression of p-AKT and p-GSK3β was reduced when HUVECs were treated with high glucose. However, these effects were reversed in HUVECs when cells treated with S1PR2 antagonist. In conclusion, S1PR2 antagonist protects endothelial cells against high glucose-induced mitochondrial apoptosis through the Akt/GSK-3β signaling pathway.

1. Introduction

Diabetes mellitus (DM) is probably the most common metabolic disease worldwide. The number of patients with DM is at 285 million in 2010 [1]. Vascular complications, including both macrovascular and microvascular diseases, have been identified as the leading cause of morbidity and mortality in patients with DM [2]. Endothelial dysfunction and damage are the early stage of development of vascular complications in DM [3].

Spingosine-1-phosphate (S1P) is a serum-borne bioactive lipid mediator synthesized exclusively by sphingosine kinase (SphK), functioning either as an extracellular ligand or intracellular mediator [4,5]. When functioning as an extracellular ligand, extracellular S1P signals through G protein-coupled receptors (S1PR1–5) [6]. Studies have shown that S1PR1, S1PR2, and S1PR3 receptors are expressed in endothelial cells [7,8]. The balance of expression and activation of these three receptors in endothelial cells is proposed to contribute to maintain physiological functions [9–11]. Furthermore, S1PR2 has been proven to be involved in the development of a variety of inflammatory diseases including atherosclerosis [6,12]. Moreover, it has been demonstrated that S1PR2 was significantly up-regulated under diabetic conditions [13,14], and studies further showed that S1PR2 mediated endothelial dysfunction by decreasing the formation of nitric oxide and increasing the production of reactive oxygen species [14,15]. S1PR2 blockage either ameliorates endothelial dysfunction or decreases the production of

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reactive oxygen species [14,15]. In addition, selective blockade of S1P2 signaling attenuates streptozotocin-induced apoptosis of pancreatic β-cells [16]. However, it remains unclear about the role of S1PR2 in apoptosis of endothelial cells and its underlying mechanism under high glucose conditions.

Glycogen synthase kinase-3 (GSK-3), an evolutionarily conserved kinase with two isoforms (α and β), is a serine/threonine kinase that participates in an array of critical cellular functions, including cell signaling, proliferation and apoptosis [17–20]. GSK-3β is a pro-apoptotic kinase, as its overexpression sensitizes cells to apoptosis, and the pharmacological inhibition of GSK-3β attenuates cytochrome *c* release from the mitochondria [19,21]. A subsequent study found that GSK-3β activation played a critical role in human umbilical vein endothelial cells (HUVECs) apoptosis [22]. In addition, it has been reported that GSK-3β is inactivated mainly through phosphorylation of the enzyme at serine 9 by protein kinase B (AKT) [23].

The current study was undertaken to specifically delineate the role of S1PR2 in high glucose-induced cell apoptosis in HUVECs. We showed that S1PR2 antagonist (JTE-013) resulted in the loss of mitochondrial membrane permeability and the release of cytochrome *c* as well as a significant increase in the relative expression of p-GSK3β and p-AKT. S1PR2 antagonist appears to play a role in protecting endothelial cells against high glucose-induced mitochondrial apoptosis through the Akt/GSK-3β signaling pathway.

2. Materials and methods

2.1. Reagents

D-glucose and mannitol were purchased from Sigma-Aldrich (St Louis, MO, USA). JTE-013 was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Primary antibodies against S1PR2 were purchased from Proteintech (Wuhan, Hubei, China), bax, bcl-2, cytochrome *c*, AKT, pho-AKT((Ser^{473}), GSK-3β, and pho-GSK-3β(Ser^{9}) were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals used were of the highest commercial grade available.

2.2. Cell culture

HUVECs were obtained from American Type Culture Collection (Manassas, VA, USA) grown in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sciencell, Carlsbad, CA, USA). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air and grown to 70%–80% confluence. For the experiments, the HUVECs were treated with 5.6 mM glucose [normal glucose (control group)], 5.6 mM glucose plus with 25 mM [high mannitol (Mnt group, osmotic pressure control)], 30 mM glucose [high glucose (HG group)], or 30 mM glucose and 1 μM JTE-013 (HG + JTE-013 group).

2.3. Western blot analysis

For the western blot analysis, cell aggregates were lysed in a radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) containing protease and phosphatase. The cleared supernatant was collected, and the protein concentration was determined using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Western blotting was performed as previously described [14].

2.4. Apoptosis assay

Annexin V/propidium iodide (PI) (Annexin V-FITC apoptosis detection kit; Beyotime, Shanghai, China) staining was performed to detect apoptosis. The treated cells were collected, washed and then stained with Annexin V/PI for 20 min in the dark at room temperature. The percentage of apoptotic cells was analyzed by flow cytometry (BD Biosciences, CA, USA).

2.5. Analysis of mitochondrial membrane permeability

The fluorescent probe JC-1 (Beyotime, Shanghai, China) was used to measure mitochondrial membrane permeability of HUVECs. The cells were incubated with JC-1 for 30 min at 37 °C, washed with phosphate buffered saline, and observed immediately under a fluorescence microscope. JC-1 aggregate was detected with ex/em = 540/570 nm; JC-1 monomers were detected with ex/em = 485/535 nm. The fluorescence intensity of JC-1 aggregates (red fluorescence) and monomers (green fluorescence) was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The ratio of aggregated JC-1 and monomeric JC-1 represented mitochondrial membrane permeability of HUVECs.

2.6. Statistical analysis

Statistical analysis was performed using SPSS software version 18.0 (SPSS Inc, Chicago, IL, USA). Data were analyzed by one-way analysis of variance. A value of $P < 0.05$ was considered to indicate a statistically significant difference. Data are presented as means ± standard deviation.

3. Results

3.1. S1PR2 antagonist inhibits HG-induced apoptosis in HUVECs

Endothelial apoptosis was quantified using dual-colour flow cytometry with fluorescein-labelled Annexin V and propidium iodide. We found that high glucose induced a significant increase in cell apoptosis in HUVECs, but mannitol treatment did not affect apoptosis of HUVECs. Given that the expression of S1PR2 increased under high glucose condition in our previous study [14], we pretreated S1PR2 antagonist (JTE-013) 1 h before HG treatment to explore the effect of S1PR2 in endothelial cell apoptosis. High glucose-induced apoptosis was significantly inhibited in HUVECs after JTE-013 pretreatment (Fig. 1).

3.2. S1PR2 antagonist inhibits HG-induced loss of mitochondrial membrane potential in HUVECs

JC-1 aggregates (red fluorescence) were prominent in the control group. Exposure of HUVECs to high glucose resulted in reduced red fluorescence and increased green fluorescence, indicating a loss in mitochondrial membrane potential. The pre-treatment of JTE-013 markedly inhibited HG-induced mitochondrial membrane depolarization (Fig. 2).

3.3. S1PR2 antagonist regulates the expression of proteins associated with mitochondrial apoptotic pathways induced by HG

As shown in Fig. 3A, under high glucose condition, protein expression of bax was increased whereas that of bcl-2 was decreased compared to the control group. The level of cytochrome *c* significantly decreased in the mitochondria and increased in the cytoplasm in the HG group as compared to that in the control group (Fig. 3B). However, mannitol had no effect. In contrast, pretreatment with JTE-013 remarkably decreased protein expression of bax and increased protein expression of bcl-2 (Fig. 3C). It also reduced the level of cytochrome *c* released into the cytosol (Fig. 3D).

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