Role of sphingosine-1-phosphate receptor 1 and sphingosine-1-phosphate receptor 2 in hyperglycemia-induced endothelial cell dysfunction

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Abstract. The hyperglycemia-induced production of oxidative stress results in endothelial cell dysfunction. Previous studies have demonstrated that sphingosine-1-phosphate (S1P) regulates an array of biological activities in endothelial cells mediated by sphingosine-1-phosphate receptors (S1PRs). However, the role of S1PR-mediated signaling pathways in hyperglycemia-induced endothelial cell dysfunction is currently unknown. In the present study, we aimed to explore the role of S1PRs in endothelial cell dysfunction. For this purpose, hyperglycemia-induced oxidative stress was examined using human umbilical vein endothelial cells (HUVECs) cultured with either normal (5.6 mM) or high (25 mM) levels of glucose. The levels of reactive oxygen species (ROS) and nitric oxide (NO) were determined by flow cytometric (FCM) analysis and nitrate reductase, respectively. Endothelial morphogenesis assay was performed in three-dimensional Matrigel. The mRNA and protein expression levels of S1PRs in the HUVECs were determined by RT-qPCR and western blot analysis, respectively. In addition, ROS, NO and endothelial morphogenesis assays were conducted using the high glucose-treated endothelial cells transfected with adenoviral vector expressing exogenous S1PR1 gene (pAd-S1PR1) or with adenoviral vector expressing S1PR2-specific shRNA (pAd-shRNA-S1PR2). The expression levels of S1PR1 and S1PR2 in the endothelial cells treated with high levels of glucose decreased and increased, respectively. However, the effects of high levels of glucose on S1PR3 were minimal. In addition, high levels of glucose enhanced ROS generation and markedly reduced NO generation and morphogenetic responses. Nevertheless, all the aforementioned changes were completely reversed by transfection with pAd-S1PR1 or pAd-shRNA-S1PR2, which increased S1PR1 and decreased S1PR2 expression, respectively. It can thus be concluded that S1PR1 and S1PR2 play crucial roles in hyperglycemia-induced endothelial cell dysfunction.

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Introduction

Endothelial function is important for systemic homeostasis; endothelial dysfunction is associated with several pathophysiological conditions, including atherosclerosis, hypertension and diabetes (1,2). As regulators of vascular permeability, vascular endothelial cells (ECs) play a critical role in maintaining the homeostasis of the circulatory system (3). When exposed to anomalous conditions (such as hyperglycemia and hyperlipidemia), vascular ECs may sense and respond to the harmful stimuli, a process known as the EC stress response. However, excessive stress response can lead to dysfunction of the ECs and vascular injury, a key step in the early stages of cardiovascular disease (4,5). Available evidence from clinical research suggests that vascular endothelial injury positively correlates with the incidence and mortality rate of cardiovascular disease (6). Oxidative stress is among the leading causes of endothelial dysfunction and vascular endothelial injury (7,8). Therefore, understanding the molecular mechanisms responsible for vascular endothelial cell dysfunction and endothelial injury induced by oxidative stress may be helpful in both the prevention and treatment of cardiovascular disease.

Sphingosine-1-phosphate (S1P) regulates the proliferation, survival, migration, cell-cell adhesion, angiogenesis and differentiation of a variety of cell types, including ECs, smooth muscle cells (SMCs), T cells and B cells (9-11). The majority of these effects are mediated by a family of G protein-coupled receptors (the sphingosine-1-phosphate receptors, S1PR1 to S1PR5) (12). The functions of S1P in ECs are primarily mediated by S1PR1, S1PR2 and S1PR3 (13). S1P exerts opposite effects on ECs through S1PR1 and S1PR2. In a previous study, Lee *et al* (14) reported that S1P regulates the balance between S1PR1 and S1PR2, thus also regulating cremaster vascular permeability in Sprague Dawley rats. The downregulation of

S1PR1 and S1PR2 increased and decreased cremaster vascular venous leakage in the rats, respectively. Estrada *et al* (15) found that both S1PR1 and S1PR2 were essential for S1P-regulated EC senescence. Either downregulating S1PR1 or upregulating S1PR2 promotes EC senescence. It has also been demonstrated that S1PRs are also involved in high glucose-induced oxidative stress (16,17). However, the role of S1PRs in hyperglycemia-induced oxidative stress and EC dysfunction has not yet been fully elucidated. In the present study, we demonstrate that S1PR1 and S1PR2 play a crucial role in hyperglycemia-induced endothelial cell dysfunction.

Materials and methods

Reagents. S1P was purchased from Enzo Life Sciences (New York, NY, USA) and was dissolved in 4 mg/ml of fatty acid-free bovine serum albumin (Sigma, St. Louis, MO, USA). M199 medium and fetal bovine serum (FBS) used for cell culture were purchased from HyClone (Logan, UT, USA). TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the RNA, which was used to synthesize cDNA with the First-Strand Synthesis kit (Promega, Madison, WI, USA). The rabbit polyclonal antibodies against S1P1 (Cat. no. R12-3478), S1P2 (Cat. no. R12-2725) and S1P3 (Cat. no. R12-2721) were purchased from Assay Biotechnology (Sunnyvale, CA, USA) and the peroxidase-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) was used for western blot analysis. Both reactive oxygen species (ROS) and nitric oxide (NO) detection kits were purchased from Enzo Life Sciences. Matrigel was purchased from BD Biosciences (Franklin Lakes, NJ, USA) and the BCA protein assay kit was purchased from Merck (Kenilworth, NJ, USA).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were cultured in M199 medium supplemented with 10% fetal bovine serum (FBS), 0.1 mg/ml heparin and 0.05 mg/ml endothelial cell growth supplement (ECGS) at 37˚C in an incubator with 5% CO_2. The culture was passaged according to standard procedures. The HUVECs were cultured until the cells reached 80% confluence. The cells were then trypsinized and re-suspended in fresh medium. For the experiments, the HUVECs were treated with a glucose concentration of 5.6 mM [normal glucose (NG)] or 25 mM [high glucose (HG)].

Cell transfection. The S1PR1 cDNA and S1PR2-specific sh-RNA were connected with Adeno-X Viral DNA, respectively. The recombinant plasmid series were identified using restriction enzymes. Linear pAd-S1PR1 and pAd-shRNA-S1PR2 were transformed into HEK293A cells, respectively. The adenoviral vector was incorporated, and then the adenovirus was reclaimed based on the presence of cytopathic effects. Endothelial cells were transfected directly with pAd-S1PR1 or pAd-shRNA-S1PR2.

Reverse transcrtipion-quantitative (real-time) polymerase chain reaction (RT-qPCR). Total RNA from the HUVECs was prepared using TRIzol reagent. cDNA was synthesized using the First-Strand Synthesis kit according to the manufacturer's instructions. PCR reactions in a final volume of 25 μl containing 1 μl of reverse transcribed cDNA and 10 mM of specific primers were performed at 94˚C (20 sec), 56˚C (30 sec), and 72˚C (30 sec) for 40 cycles using the Mastercycler ep realplex instrument (Eppendorf, Hamburg, Germany). The primers used were as follows: S1PR1 sense, 5'-GCACCAACCCCATCATTTAC-3' and antisense, 5'-TTGTCCCCTTCGTCTCTG-3'; S1PR2 sense, 5'-CAAGTTCCACTCGGCAATGT-3' and antisense, 5'-CAGGAGGCTGAAGACAGAGG-3'; S1PR3 sense, 5'-TCAGGGAGGGCAGTATGTTC-3' and antisense, 5'-GAGTAGAGGGGCAGGATGGT-3'; and β-actin sense, 5'-GAGACCTTCAACACCCCAG-3' and antisense, 5'-TCAGGTCCCGGCCAGCCA-3'.

Western blot analysis. The HUVECs were harvested and suspended in extraction buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 2% Triton X-100, 2 mM EDTA and 0.2% SDS) supplemented with protease inhibitors. The cell suspension was agitated at 4˚C for 30 min followed by centrifugation at 15,000 x g for 20 min. Protein extracts were resolved on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk, washed and incubated overnight with the indicated primary antibodies on a rotary shaker at 4˚C. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibodies for 1 h at room temperature followed by incubation with enhanced chemiluminescence reagent for 1 min. Protein bands were visualized using the imaging instrument (GE Healthcare, Cleveland, OH, USA).

Measurement of intracellular levels of ROS and NO. The HUVECs were washed twice with 25 mM HEPES and stained with 5 μmol/l of the fluorescence dye, DCFH-DA, in the dark at 37˚C for 30 min. After being trypsinized, the cells were collected by centrifugation at 500 x g at room temperature for 8 min and washed with HEPES twice. The intracellular levels of ROS were measured by flow cytometry. For the measurement of NO levels, the HUVEC culture medium was used to estimate the NO levels using the reductase method.

Endothelial morphogenesis assay. The *in vitro* endothelial morphogenesis assay was performed in three-dimensional Matrigel. Briefly, Matrigel was added into 48-well tissue culture plates followed by polymerization for 1 h at 37˚C. The HUVECs were plated ($2x10^4$ cells) onto Matrigel and incubated at 37˚C with or without S1P (100 nM) for 18 h. Cells in 5 fields of triplicates were photographed using the Olympus IX71 inverted fluorescence microscope. Total tubular length was quantified using image analysis software (Olympus, Tokyo, Japan).

Statistical analysis. Data are expressed as the means ± standard error of the means. Significance of the differences between groups was determined using the two-tailed Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of glucose concentrations on the expression of S1PR1, S1PR2 and S1PR3 in HUVECs. To evaluate the effects of glucose on the levels of S1PRs in ECs, the HUVECs were cultured with either a normal concentration of glucose (NG;

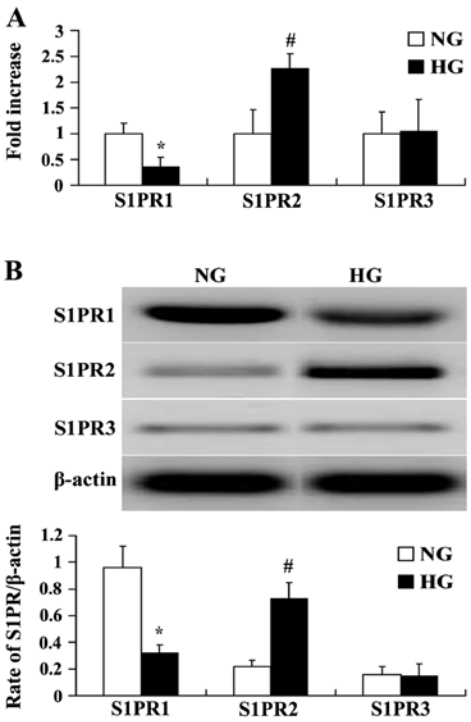

Figure 1. Effects of high glucose (HG) on the expression of sphingosine-1-phosphate receptor (S1PR)1, S1PR2 and S1PR3 in human umbilical vein endothelial cells (HUVECs). (A) The mRNA expression levels of S1PR1, S1PR2 and S1PR3 in HUVECs treated with normal glucose (NG) and HG were measured by RT-qPCR as described in Materials and methods. S1PR1 expression was markedly decreased in the HUVECs treated with HG (*P<0.05, n=3). By contrast, S1PR2 expression in the HUVECs treated with HG was significantly increased (#P<0.05, n=3). However, the differences in S1PR3 mRNA levels were negligible between the HUVECs treated with NG and HG (P>0.05, n=3). (B) The protein expression levels of S1PR1, S1PR2 and S1PR3 in the HUVECs treated with NG and HG were measured by western blot analysis.

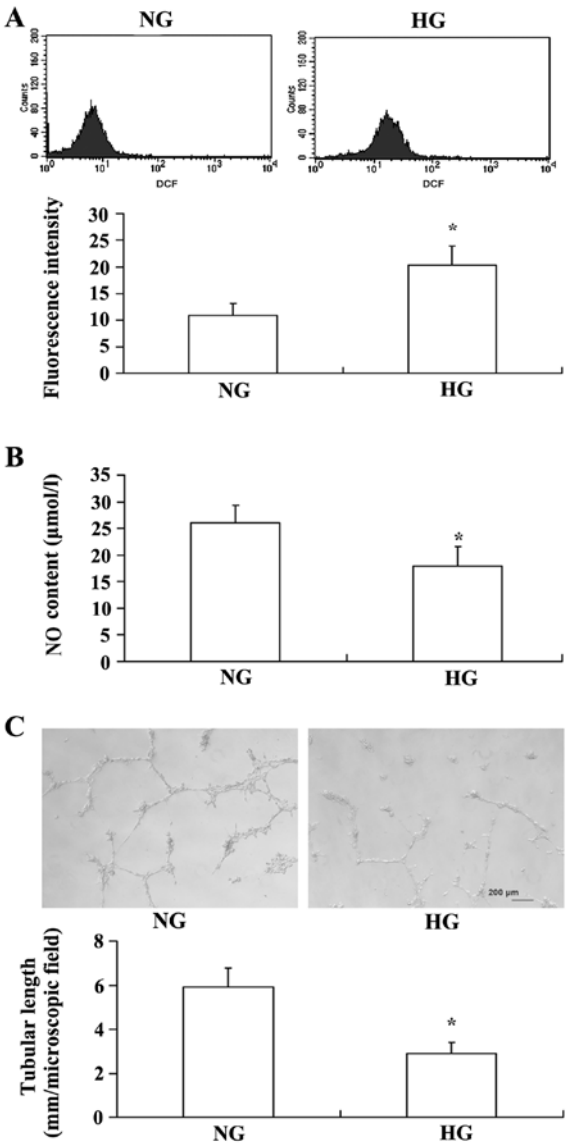

Figure 2. Effects of glucose concentrations on the levels of reactive oxygen species (ROS) and nitric oxide (NO), as well as morphogenesis response in human umbilical vein endothelial cells (HUVECs). HUVECs were cultured in either normal glucose (NG) (5.6 mM) or high glucose (HG) (25 mM) for 48 h and ROS levels, NO content and morphogenesis response were determined. (A) ROS levels in HUVECs treated with HG were markedly elevated compared to those in the NG group (*P<0.05, n=3). (B) NO content in the HUVECs treated with HG was significantly decreased compared to that in the NG group (*P<0.05, n=3). (C) An organized network of tubule-like structures was observed in the HUVECs when treated with NG (tubular length, 5.90±0.88 mm/microscopic field). However, morphogenesis was markedly diminished in the HUVECs when they were treated with HG (tubular length, 2.92±0.49 mm/microscopic field, *P<0.05, n=3).

5.6 mM) or a high concentration of glucose (HG; 25 mM) for 48 h. The mRNA levels of S1PR1, S1PR2 and S1PR3 were estimated by RT-qPCR and are shown in Fig. 1A, and the protein levels of these S1PRs are shown in Fig. 1B. Both the mRNA and protein levels of S1PR1 in the HUVECs decreased significantly (P<0.05) when the cells were treated with HG. By contrast, HG significantly increased the expression of S1PR2 in the HUVECs (P<0.05). However, neither the mRNA level nor the protein level of S1PR3 was affected by treatment with HG (P>0.05). This suggests that HG results in the loss of the balance between S1PR1 and S1PR2, specifically in HUVECs.

Effects of glucose concentrations on ROS and NO levels and morphogenesis in HUVECs. To investigate the effects of HG on oxidative stress and EC function, the ROS levels and NO content in the HUVECs were measured. As shown in Fig. 2A, the levels of ROS in the HG-treated HUVECs were significantly higher than those in the NG-treated group (P<0.05), while the production of NO in the HG-treated ECs (Fig. 2B) was significantly lower than that in the NG group (P<0.05). We then determined the effects of HG on tube formation *in vitro* in the ECs. As shown in Fig. 2C, the HUVECs treated with NG formed an organized network of tubule-like structures (tubular length, 5.90±0.88 mm/microscopic field). However, morphogenesis was markedly diminished in the HUVECs treated with HG (tubular length, 2.92±0.49 mm/microscopic field; P<0.05), indicating the functional impairment of the ECs exposed to HG.

Manipulation of the expression of S1PR1 and S1PR2 in HUVECs. In order to further explore the functional role of S1PR1 and S1PR2, we manipulated the levels of S1PR1 and S1PR2 in the HUVECs. The overexpression of S1PR1 was induced by transient transfection with adenoviral vector expressing exogenous S1PR1 (pAd-S1PR1) and the expression of S1PR2 was knocked down by transfection with adenoviral vector expressing S1PR2specific shRNA (pAd-shRNA-S1PR2). The results from

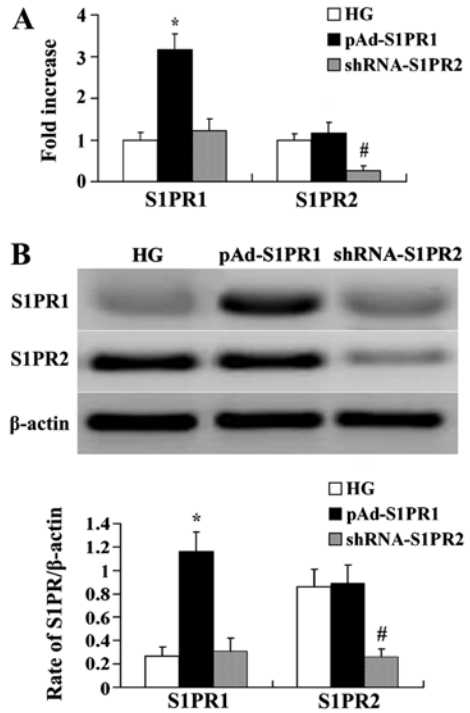

Figure 3. Manipulation of sphingosine-1-phosphate receptor (S1PR)1 and S1PR2 expression in human umbilical vein endothelial cells (HUVECs). HUVECs were transfected with either pAd-S1PR1 or shRNA-S1PR2. (A) The mRNA levels of S1PR1 and S1PR2 in the HUVECs were measured by RT-qPCR and transfection with pAd-S1PR1 significantly increased S1PR1 expression (*P<0.05, n=3). Transfection with shRNA-S1PR2 significantly decreased S1PR2 expression (#P<0.05, n=3). (B) The effects of the overexpression of S1PR1 or the silencing of S1PR2 were further confirmed at the protein level by western blot analysis.

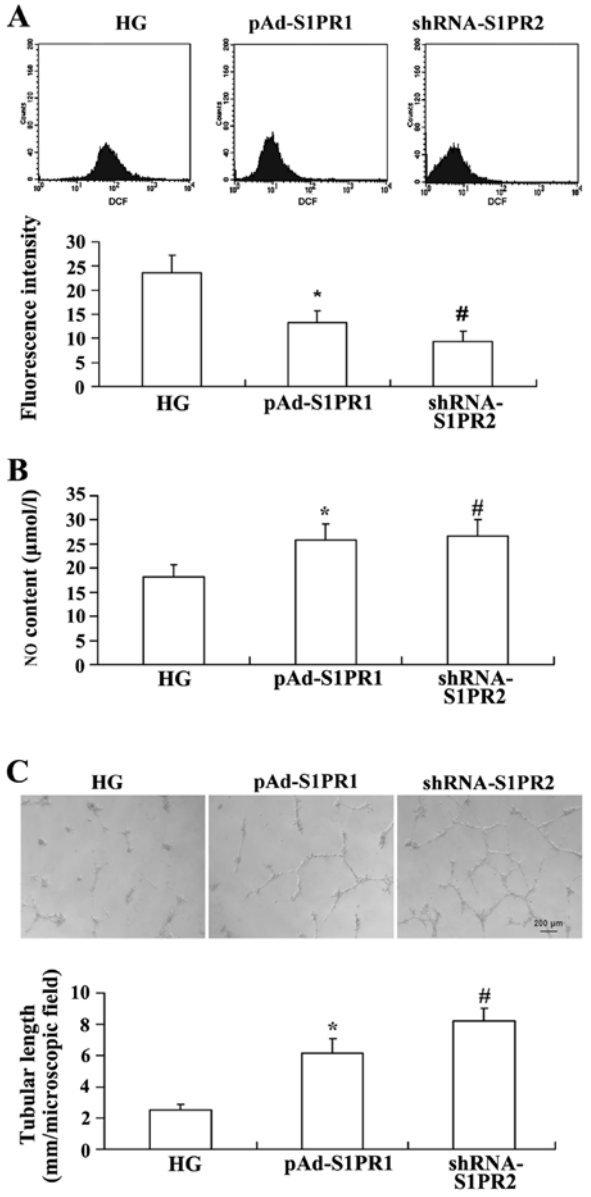

Figure 4. Role of sphingosine-1-phosphate receptor (S1PR)1 and S1PR2 in hyperglycemia-induced oxidative stress and morphogenesis response in human umbilical vein endothelial cells (HUVECs). (A) The levels of reactive oxygen species (ROS) in HUVECs were detected by flow cytometry. Either the overexpression of S1PR1 or the knockdown of S1PR2 significantly reduced the levels of ROS (*,#P<0.05, n=3). (B) The generation of nitric oxide (NO) in HUVECs was detected by the method of nitrate acid reductase. The NO content was significantly increased when S1PR1 was overexpressed or S1PR2 was knocked down (*,#P<0.05, n=3). (C) Morphogenesis response in HUVECs was detected on Matrigel. Either the overexpression of S1RP1 or the knockdown S1RP2 restored the organized network of tubule-like structures in HUVECs (*,#P<0.05, n=3).

RT-qPCR (Fig. 3A) revealed that the mRNA levels of S1PR1 and S1PR2 were significantly increased and decreased, respectively, by transfection with the specific plasmids (P<0.05). In addition, the results from western blot analysis demonstrated that the protein levels of S1PR1 and S1PR2 were upregulated and downregulated accordingly (Fig. 3B).

Role of S1PR1 and S1PR2 in hyperglycemia-induced oxidative stress and morphogenesis in HUVECs. To explore the possibility that the hyperglycemia-induced adverse effects in HUVECs are mediated by S1PR1 and S1PR2, the HUVECs were treated with HG for 48 h and the ROS and NO levels were estimated. As shown in Fig. 4A, the levels of ROS induced by HG were significantly decreased when either S1PR1 was overexpressed or S1PR2 was knocked down (P<0.05; Fig. 4A). By contrast, the NO content was markedly increased in the HUVECs by these same manipulations (P<0.05; Fig. 4B). In addition, the organized network of tubule-like structures interrupted by hyperglycemia was markedly restored by either S1PR1 overexpression or S1RP2 knockdown (Fig. 4C; P<0.05). Together, these results demonstrate that S1PR1 and S1PR2 play a crucial role in hyperglycemia-induced EC dysfunction.

Discussion

To the best of our knowledge, in the present study, for the first time, we investigated the effects of S1PRs on hyperglycemia-induced vascular EC dysfunction. The main findings of our study were the following: i) following exposure to HG, the expression levels of S1PR1 and S1PR2 in the HUVECs were significantly decreased and increased, respectively, in parallel with a marked increase in oxidative stress and hyperglycemia-induced cell dysfunction; and ii) the effects of HG on the levels of S1PR1 and S1PR2 were reversed by either the overexpression of S1PR1 by transfection with pAd-S1PR1 or by the knockdown of S1PR2 using shRNA; this not only attenuated oxidative stress, but also HUVEC dysfunction.

Diabetes is known as both a metabolic syndrome and a vascular disease due to the hyperglycemic effects on the macro- and microcirculation of the vascular beds (18,19). The link between diabetes and the increased incidence of cardiovascular disease has been well established (1). The functional impairment of the vascular endothelium observed in all forms of cardiovascular disease has also been identified in patients with insulin resistance, obesity and diabetes (20-22). One of the hallmarks of endothelial dysfunction is impaired NO bioavailability (23,24). Endothelial dysfunction is characterized by one or more features, including reduced endothelium-mediated vasorelaxation, hemodynamic deregulation, the increased expression of adhesion molecules and inflammatory mediators, the excessive generation of ROS and increased oxidative stress (25-27). It is widely accepted that ROS play critical roles in vascular EC dysfunction by elevating the calcium concentration in the cytoplasm, reducing both production and vasodilatation (28,29). Endothelial dysfunction precedes the pathogenesis of vascular complications in diabetes. There is a growing consensus that hyperglycemia is a key cause of the oxidative stress of endothelial cells which may lead to cell dysfunction. Patel *et al* (30) found that the O_2^- level increased in both HUVECs and human microvascular endothelial cells (HMVECs) with hyperglycemia. In the HUVECs, hyperglycemia increased intracellular O_2^- production, and decreased the expression of ROS-neutralizing enzymes SOD2 and CAT (30). Karbach *et al* (31) demonstrated that hyperglycemia results in elevated oxidative stress and impaired NO-cGMP signaling in HUVECs. Consistent with the results reported by Patel *et al* and Karbach *et al*, we observed increased ROS levels and decreased NO production in the HUVECs under hyperglycemic conditions. Consistently, tube formation and morphogenesis were also impaired in the ECs following exposure to high concentrations of glucose. These results demonstrate the concomitant increase in oxidative stress and cell dysfunction in HUVECs under hyperglycemic conditions.

Multiple lines of evidence have indicated that S1PRs are involved in both the generation of oxidative stress and oxidative stress-mediated injuries (32-34). S1PR1 affects EC function through its anti-inflammatory effects in diabetic ECs (35,36). S1PR2 has been shown to be upregulated in diabetic kidneys and in mesangial cells following exposure to high concentrations of glucose (16). Both S1PR1 and S1PR2 play pivotal roles in pathological angiogenesis leading to diabetic nephropathy (37). In the present study, we observed that S1PR1 and S1PR2 in HUVECs were significantly decreased and increased, respectively, when the cells were treated with high concentrations of glucose. These results were consistent with the findings of previous studies demonstrating that S1PR2 and S1PR1 were significantly upregulated and downregulated, respectively, in diabetic patients (16,35,36). In addition, in our study, increased oxidative stress and impaired function were observed in the ECs exposed to high concentrations of glucose. Furthermore, the effects of hyperglycemia on the levels of ROS and NO, as well as the morphogenesis of HUVECs were significantly reversed by either S1PR1 overexpression or S1PR2 silencing. To the best of our knowledge, this is the first report examining the effects of S1PRs on hyperglycemia-induced endothelial dysfunction.

In conclusion, we demonstrated that both S1PR1 and S1PR2 play a pivotal role in hyperglycemia-induced EC dysfunction and endothelial injury by reducing and enhancing the production of oxidative stress, respectively. This finding opens a new avenue in the study of the role of S1P1R and S1P2R in hyperglycemia-induced endothelial cell dysfunction. A better understanding of the mechanisms involved may provide both a new perspective of cardiovascular disease in diabetes and a novel target for therapeutics.

Acknowledgements

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