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Exogenous SERP1 attenuates restenosis by restoring GLP-1 receptor activity in diabetic rats following vascular injury

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ABSTRACT

The activity of glucagon-like peptide 1 (GLP-1R) is essential for preventing restenosis following vascular injury; however, the mechanism of dysfunctional GLP-1R glycosylation and ways to enhance the activity of GLP-1R on vascular surfaces in diabetic patients are poorly understood. In the present study, we investigated the N-glycosylation level and role of stress-associated endoplasmic reticulum protein 1 (SERP1) in preventing restenosis following carotid injury in diabetic rats. Our results showed that N-glycosylation levels in both rat aortic endothelial cells (RAOECs) and rat vascular smooth muscle cells (VSMCs) decreased gradually following glucose treatment in a concentration dependant manner. Furthermore, co-immunoprecipitation (Co-IP) analyses indicated that SERP1 could interact with GLP-1R in RAOECs and VSMCs. Moreover, SERP1 enhanced GLP-1R N-glycosylation and increased the production of phosphorylated endothelial nitric oxide synthase (eNOS) as well as proliferation of RAOECs. SERP1 also increased phosphorylated adenosine monophosphate activated protein kinase (AMPK) and decreased the migration of VSMCs. Importantly, intima media thickness (IMT) and neointimal hyperplasia were alleviated in the carotid artery of diabetic rats injected with SERP1 following balloon injury. We also found an increase in re-endothelialization and a decrease in VSMC proliferation in the carotid artery of diabetic rats injected with SERP1. In summary, the remarkable effects of SERP1 on reducing restenosis following vascular injury may contribute to future advancements in the treatment of diabetic vascular complications.

1. Introduction

Cadiovascular accidents are the main cause of death in patients with type 2 diabetes mellitus (T2DM) [1]. Almost 80% of T2DM patients suffer from vascular diseases including neuropathy, retinopathy, and peripheral artery disease (PAD) [1]. Due to its benefits and advantages such as, minimally invasive nature and no requirement of general anaesthesia, intravascular therapy has become an indispensable option for such patients [2]. However, it is also plagued by many limitations, especially the high rate of vascular restenosis in diabetic patients [3]. Vascular restenosis, induced by neointimal hyperplasia, is the major cause of the failure of endovascular therapy [4,5]. Triggered by intravascular therapy, arterial injury leads to the exposure of vascular smooth muscle cells (VSMCs) under inflammatory factors, and excessive proliferation and migration of VSMCs could lead to neointimal hyperplasia [6]. Hyperglycaemia delays reparation of endothelial cells (ECs), and weakens the protective effects of ECs in preventing the stimulation of VSMCs by inflammatory factors [7]. Furthermore, due to hyperglycaemia, VSMCs are more inclined to migrate to the intima [8]. Based on these factors, elucidating the underlying mechanism and planning treatment protocols, particularly for preventing restenosis in diabetic patients following intravascular therapy are crucial to their prognosis.

Glucagon-like peptide 1 (GLP-1) is a gut-derived hormone secreted in response to oral nutrient intake, and is beneficial to the cardiovascular system, maintaining body weight, as well as bone and central nervous system beyond glycaemic control in T2DM patients [9]. Recent studies have demonstrated that activation of GLP-1R not only accelerates re-endothelialization by promoting the phosphorylation of endothelial nitric oxide synthase (eNOS) at Ser 1177 [10], but also inhibits the proliferation of VSMCs by inducing the phosphorylation of adenosine monophosphate activated protein kinase (AMPK) at Thr 172 [11]. These evidences imply GLP-1R activation as an effective way to prevent vascular restenosis after injury [12]. Due to rapid degradation of GLP-1 by the enzyme dipeptidyl peptidase 4 (DPP-4), GLP-1R agonists tolerant to this degradation and DPP-4 inhibitors have been introduced within the past decade to prevent the degradation of GLP-1 and extend its short half-life [13,14]. However, recent studies have

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shown that longer durations of hyperglycaemia inhibit the protective effect of GLP-1 by disrupting the activity of GLP-1R [15,16]. For instance, N-glycosylation is a key post-translational modification process which regulates the expression of many G-protein coupled receptors (GPCRs) from the endoplasmic reticulum (ER) and delivery to the plasma membrane [17]. As GLP-1R is a member of GPCRs, scientists have found that protein folding, stability, sorting, secretion and expression of GLP-1R are also associated with N-glycosylation [18]. N-glycosylation of GLP-1R occurs on residues Asn 63, Asn 82, and Asn 115 with Asn-X-Ser/Thr (X not Pro) motifs in extracellular domains, and such post-translational modifications are necessary for high affinity ligand activity, which can be inhibited by the N-glycosylation inhibitor-tunicamycin [19]. A few studies have found that excessive production of reactive oxygen species (ROS) due to hyperglycaemia, upregulates hypoxia-inducible factor 1 (HIF-1α) thus, contributing to increased glycolysis or glutaminolysis, which in-turn restrains the N-glycosylation of proteins in cells [20].

To date, many accessory proteins have been shown to interact directly with GLP-1R to form a functional protein complex [21,22]. Previous findings have reported that GPCRs can achieve N-glycosylation by specifically interacting with a small, tail-anchored, 66 amino acid long, stress-associated endoplasmic reticulum protein 1 (SERP1) [23,24]. SERP1 was found to be identical to ribosome-associated membrane protein 4 (RAMP4), with respect to stabilizing membrane proteins and facilitating subsequent glycosylation [25]. However, whether and how SERP1 plays a role in preventing vascular complications by inducing GLP-1R under diabetic conditions is currently unknown. Since GLP-1R contains a large extracellular N-terminus consensus site for N-glycosylation, it is possible that an exogenous strategy facilitates the N-glycosylation of GLP-1R. Based on this, we hypothesized that therapeutic administration of exogenous SERP1 can prevent restenosis by restoring GLP-1 receptor activity in diabetic rats following vascular injury.

2. Materials and methods

2.1. Cell culture

Rat aortic endothelial cells (RAOECs) and rat vascular smooth muscle cells (VSMCs) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2 and 95% air. A total of 5.6, 10, 15, 25 or 35 mM glucose was added to the media. In some experiments, RAOECs and VSMCs were serum deprived and then pre-incubated for 48 h with recombinant rat SERP1 (1.0, 2.0, 3.0 μg/ml) (Creative BioMart, NY, USA) and exendin-4 (10 nM) (Sigma–Aldrich, St. Louis, MO), a frequently-used GLP-1 analogue. In the N-glycosylation inhibition groups, cells were exposed to 5 μg/ml tunicamycin (Sigma-Aldrich) for 4 h before harvesting.

2.2. Cell proliferation assay

Once both RAOECs and VSMCs reached at least 70% confluence, cells were cultured in DMEM plus 0.2% FBS for 0, 6, 12, 24 or 48 h for serum deprivation, and incubated with 10 ml of Cell Counting Kit-8 (CCK8) (Yeasen, Shanghai, China) for 1 h to detect cell viability.

2.3. Flow cytometry

Following 24 h of serum deprivation, RAOECs were seeded into 6-well plates (500,000 cells/well). 12 h later, the cells were harvested by trypsinization, centrifuged (1000 *g*, 5 min), resuspended, washed twice with 1 ml pre-cooled PBS, and then fixed overnight with 70% ethanol at 4 °C. The fixed cells were washed and resuspended in PBS, consecutively incubated with 20 ml RNase A (37 °C, 30 min) and 400 ml PI staining solutions (4 °C, 60 min; Yeasen), and then analysed by flow cytometry [26].

2.4. Wound-healing assay

Following serum deprivation for 24 h, VSMCs were seeded into 6-well plates (500,000 cells/well) and treated with different concentrations of SERP1 plus 10 nM Exendin-4 for 48 h. Cells were grown for 24 h to achieve at least 70% confluence; subsequently, three parallel wounds of similar widths (< 3 mm) were created in each well using a sterile 200 μl pipette tip. Cells were incubated for another 24 h to enable migration, and then, images were obtained of each well using microscopy.

2.5. Transwell migration assay

After infection, the cells (5×10^5 cells/mL) were resuspended in 200 μl of serum-free DMEM before being added to the upper chamber of a transwell apparatus (Corning, Tewksbury, NY, USA). The lower chamber of the apparatus was filled with 500 μl serum-free DMEM with different concentrations of SERP1 plus 10 nM Exendin-4. After 16 h, the migrated cells on the bottom surface of the chamber membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

2.6. Animal protocols

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Forty-eight 8-weeks old male SD rats were randomly categorized into four groups (n = 12 per group): (i) Non-diabetic, (ii) Non-diabetic + SERP1, (iii) Diabetic, (iv) Diabetic + SERP1 based on the treatments they would receive. Rats in groups (i) and (ii) were fed a regular diet (60% carbohydrates, 22% protein, 10% fat), while rats in groups (iii) and (iv) were fed a high-fat diet (50% carbohydrates, 13% protein, 30% fat). After six weeks, rats in groups (iii) and (iv) were injected intraperitoneally with 30 mg/kg of freshly prepared streptozotocin (STZ) solution (in 0.05 M citrate buffer, pH 4.4) to induce hyperglycaemic symptoms, while the rats in groups (i) and (ii) received only the vehicle. Three days after the injection, the rats were fasted for 8 h, and fasting blood glucose (FBG) levels were measured in blood samples obtained from the tail vein. Rats with FBG higher than 11.1 mmol/L were considered diabetic. A total of 0.16 mg/kg recombinant rat SERP1 or the same dose of placebo was then injected into the tail vein of rats in respective groups. Two weeks post-injection, six rats in each group were examined by noninvasive ultrasonography of the carotid arteries and then euthanized for histomorphologic analysis. The remaining rats were anaesthetized using isoflurane. A 2-French balloon catheter (Edwards Life sciences, Irvine, CA, USA) was inserted through the left external carotid artery into the common carotid artery and insufflated three times with 2 atm pressure. Following injury, the external carotid artery was quickly ligated, and blood flow was resumed. Carotid arteries of all rats were examined by ultrasonography, and the rats were euthanized 14-days post injury. Carotid artery tissues were collected for further biochemical and pathological analysis.

2.7. Measurement of ROS generation

Production of intracellular ROS was measured using 5-(and-6)-carboxy-20, 70-dichlorodihydrofluorescein diacetate (H_2-DCFDA) (Thermo Fisher Scientific, MA, USA). After the medium was removed, cells were incubated with 100 ml of 50 mM H_2-DCFDA for 30 min at 37 °C. Next, the medium was replaced with pre-cooled phosphate-buffered saline (PBS) and after 20 min, the fluorescence was directly visualised by a fluorescence microscope using a FITC filter (excitation wavelength of 485 nm and emission wavelength of 530 nm). Cells were incubated for 3 min and stained with 0.25 mg/ml of 40, 6-diamidino-2-phenylindole (DAPI). Cells with green