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Role of vascular peroxidase 1 in senescence of endothelial cells in diabetes rats☆

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ABSTRACT

Background: Reactive oxygen species (ROS) is thought as a major reason of vascular injury in diabetes. Vascular peroxidase 1 (VPO1) is a newly found peroxidase playing an important role in inducing oxidative stress. In the present experiment, we tested the role of VPO1 in senescence of endothelial cells in streptozotocin (STZ)-induced diabetic rats and cultured endothelial cells.

Methods: Blood samples were collected from carotid arteries. Vasodilator responses to acetylcholine (Ach) in the isolated aortic rings were measured, serum concentration of glucose, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) and the expression of VPO1 in the aorta were determined. Endothelial cells were treated with high glucose or H_2O_2, the concentrations of MCP-1, TNF-α and hypochlorous acid (HOCl) and the expression of VPO1 were determined. shRNA of VPO1 was used for mechanism research in cultured cells.

Results: Vasodilator responses to Ach were impaired markedly and the serum concentrations of glucose, TNF-α and MCP-1 were significantly increased in diabetic rats. The expression of VPO1 in the aorta was upregulated in diabetic rats. High glucose treatment significantly decreased cell viability and elevated the levels of MCP-1, TNF-α and HOCl and upregulated the expression of VPO1. H_2O_2 treatment significantly induced cellular senescence, inhibited eNOS expression and NO production. The effects of high glucose and H_2O_2 were attenuated by shRNA interference of VPO1.

Conclusions: VPO1 plays an important role in senescence of endothelial cells and endothelial dysfunction by induction of oxidative stress and inflammatory reaction in type 2 diabetic rats.

1. Introduction

Vascular injury is a major complication of diabetes mellitus and usually leads to a poor prognosis. The impairment of vascular endothelium is thought as a hallmark of diabetic vascular injury which has been documented in animals and patients with diabetes mellitus [1]. Previous studies have also demonstrated that increased vascular endothelial cellular senescence contributes to the impaired endothelial function and serves as a key player in the pathogenesis of diabetic vascular injury [2], but the underlying mechanism of endothelial cellular senescence in diabetic vascular injury has not been fully investigated. Generally, activated oxidative stress is thought as a major reason contributing to the initiation and progression of endothelial cellular senescence [3]. The senescence of cells induced by reactive oxygen species (ROS) has also been seen in endothelial progenitor cells (EPCs) [4]. As an important member of superoxide anion produced in ROS, hypochlorous acid (HOCl) is able to induce endothelial dysfunction in atherosclerosis and vascular restenosis. Recently, studies have demonstrated that myeloperoxidase (MPO)-derived oxidant HOCl induces endothelial cell death [5–7]. The effects of these oxidants on endothelial cells senescence have strong implications for the development of vascular injury [8].

Vascular peroxidase 1 (VPO1) is a newly identified isozyme of MPO, as a member of the peroxidase–cyclooxygenase superfamily of proteins. It is mainly expressed in vascular wall, liver and lung while MPO exists only in neutrophils, monocytes and macrophages [9]. However, as same as MPO, VPO1 is capable to catalyze H_2O_2 and chloride to produce HOCl [10]. In our laboratory, we have identified that VPO1 pathogenically contributes to several cardiovascular diseases including hypertension, atherosclerosis and coronary heart disease. It has been shown that Angtension II and ox-LDL, which can induce oxidative stress, significantly upregulates VPO1 expression in vascular smooth muscle cells or endothelial cells [10–12]. According as generation of ROS induced by

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hyperglycemia, it is likely that VPO1/HOCl pathway is involved in senescence of endothelial cells in diabetes mellitus.

It is well known that there are interactions of oxidative stress with inflammatory reaction. Animal experiments and clinical studies have documented that inflammatory reaction is involved in ROS-induced endothelial dysfunction in diabetes mellitus [13,14]. In cultured endothelial cells, high glucose treatment induced cellular injury with an increase of inflammatory factors such as tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) [15,16]. Base on the regulatory effect of ROS on inflammatory responses, we speculated that VPO1-mediated inflammatory response play an important role in endothelial dysfunction of type 2 diabetic mellitus (T2DM). For these reasons, the present study was designed to explore the effect of VPO1 on endothelial cells senescence and endothelial dysfunction in type 2 diabetic rats. According as the facilitation of ROS on inflammatory reaction, we also investigated the effect of VPO1 on inflammatory reaction in diabetes mellitus.

2. Matrials and methods

2.1. Animals

Male Sprague–Dawley rats (200–220 g) were obtained from the Laboratory Animal Center, Xiang-Ya School of Medicine, Central South University, China. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH publication 85–23, revised 1996), and the experiments were approved by the Central South University Veterinary Medicine Animal Care and Use Committee. The animals were randomly allocated to two groups (n = 7 per group): the control group, rats were fed with normal diet for 1 month and then received vehicle (0.1 mol/L citrate buffer, pH 4.5) alone with normal diet for 2 months; the diabetic group, rats were fed with high fat diet for 1 month and then received STZ (35 mg/kg, dissolved in pH 4.5 citrate buffer, pH 4.5) for one time and five days after STZ injection, hyperglycemia was documented by measuring the glucose content of tail vein blood with a blood glucose monitoring system (Thera Sense, Inc., Alameda, CA). The rats in diabetic group were continually fed with high fat diet for 2 months. The fasting blood glucose levels ≥7.8 mg/dL were considered to be diabetic.

2.2. Cell experiments

The human umbilical vein endothelial cells (HUVECs; ATCC, CRL-2480) were seeded at constant density ($1 \times 10^4/cm^2$) and grown to 70–80% in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were passaged into 6-well culture dishes until fully confluent and were serum starved for 24 h in DMEM containing 1% FBS before treatments. Cells were then treated with added Glucose/Sucrose (0, 10, 20, 30 m mol/L) for 48 h or exposed to 100 μ mol/L H_2O_2 (Sigma, St. Louis, MO) for 1 h and then culture for another 24 h.

2.3. Silencing of VPO1 expression

Human VPO1-shRNA (5′-gcuccgaauuguggaaauatt-3′) was cloned into the vector of PGCs-shRNA so that EGFP gene was translated from a single bicistronic mRNA, facilitating the selection for positively transfected cells. The sequence was tested by Shanghai BioAsia Biotechnology. Before transfection, 4×10^5 cells were seeded in 3 ml of 20% EGM-2. The dishes should be 40–80% confluent on the day of transfection. Transfection experiments were performed using FUGENE HD (Roche). As a control, scrambled shRNA not exhibiting homology to any coding region was used. Transfection efficiency was determined by the ratio of EGFP-expressed cells and VPO1 protein expression. Culture dishes with 60% or more transfection efficiency were used for the further experiments 48 h after the transfection.

2.4. Acetylcholine-induced vasorelaxation

Aortas were cleared of periadventitial tissue and cut transversely into rings 1.5 to 2.0 mm in diameter. Vascular rings, handled carefully to avoid damage to the inner surface, were mounted on wires in the chambers of a multivessel myograph (J.P. Trading) and bathed in Krebs' buffer. The medium was gassed with 95% O2 and 5% CO_2 and maintained at 37 °C (PH 7.4). After equilibration (30 min), the rings were set to an internal circumference equivalent to 90% of full relaxation under a transmural pressure of 100 mm Hg and allowed to stabilize for 20 to 30 min. The rings were then depolarized with potassium chloride (KCl; 60 m mol/L) to evaluate maximal contraction. After washing with a Krebs' buffer, the vascular preparations were contracted with phenylephrine (10^{-6} mol/L), and when the contractile response was stabilized (steady-state phase, 12 to 15 min), vasorelaxing responses to cumulative increments in the concentration of acetylcholine were examined.

2.5. Measurements

2.5.1. Determination of plasma lipid and glucose concentration

Plasma glucose was determined using a commercially available enzyme kit (Baoding Changcheng Clinical Agent Co. Hebei, China). Plasma lipid was determined in Xiangya Hospital. Glucose tolerance was determined by OGTT on 61st day after the injection of STZ in diabetic group and vehicle (0.1 mol/L citrate buffer, pH 4.5). Twelve-hour fasted animals were administered glucose (2 g/kg, p.o.) and blood samples were collected from the tail tip incision at 0, 30, 60, 90, and 120 min after glucose administration. Blood glucose was estimated by using a glucometer (Accu-chek, Roche).

2.5.2. Analysis of cellular senescence

Thoracic aortas were fixed in 4% paraformaldehyde for 24 h, rinsed with phosphate-buffered saline (PBS) repeatedly, and subjected to β-galactosidase (β-gal) staining overnight using a commercial kit (Beyotime Institute of Biotechnology). The stained abdominal aortas were washed with PBS and then photographed with a Sony digital camera. The percentage of the β-gal staining area in comparison with the entire thoracic aorta area was quantified using the Image J Software.

Senescence-associated β-galactosidase (SA-β-gal) staining was performed using a Senescence Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Briefly, cells were washed with PBS, fixed using a fixative solution for 10–15 min, again washed with PBS, and then incubated with Staining Solution Mix overnight at 37 °C. Cells were imaged under a bright-field microscope at a magnification of 100×. The number of positive cells with blue color was counted and normalized to the number of total cells in the same field.

2.5.3. Immunohistochemistry of VPO1 expression in aortas

Anesthetized rats were rapidly excised and the aortas were quickly frozen in liquid nitrogen. The aortas were sectioned at 5-μm thickness in a cryostat. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 for 10 min. Sections were washed with PBS and incubated with 10% horse serum for 60 min, then added VPO1 antibody (1:500) in 0.1% TBST overnight at 37 °C. The anti-rabbit antibody (1:1000) was added to the sections and incubated for 1 h. Avidin-biotin-peroxidase complex (ABC Elite Kit, Vector, USA) was added to the sections and incubated for 30 min. Tyramide signal amplification biotin system was used according to manufacturer's instructions. Antibody binding was visualized with 3, 39-diaminobenzidine (DAB).