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Beneficial effects of evodiamine on $P2X_4$-mediated inflammatory injury of human umbilical vein endothelial cells due to high glucose

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ABSTRACT

Evodiamine has been reported to exhibit anti-inflammatory and anti-nociceptive effects, but the underlying mechanisms remain to be defined. $P2X_4$ receptor ($P2X_4R$) is a subtype of ATP receptors and plays important roles in pain, inflammatory and immune responses. We aimed to investigate whether evodiamine has beneficial effects on endothelial inflammatory injury mediated by chronic high glucose condition. We found that culturing human umbilical vein endothelial cells (HUVECs) with high glucose significantly increased the expression of $P2X_4$ receptor in HUVECs, cytosolic Ca^{2+} concentrations and intracellular reactive oxygen species (ROS) while decreasing nitric oxide (NO); these effects could be reversed by evodiamine. High glucose also significantly increased the expression of the pro-inflammatory activators (NF-κB) and TNFR-α, which was accompanied by the elevation of $P2X_4R$ levels. Evodiamine was able to down-regulate the elevated NF-κB, TNFR-α, $P2X_4R$ and ROS, and up-regulate the decreased NO. Thus the evodiamine may exert the anti-inflammation activity on high-glucose challenge HUVEC via suppressing the P2X4R signaling pathway, exhibiting beneficial ability to protect HUVECs from glucotoxicity.

1. Introduction

Vascular endothelial cells play an important role in maintaining cardiovascular homeostasis. Endothelial dysfunction is the initial event and foundation of diabetes-related cardiovascular complications. Studies have found that high glucose can induce oxidative stress and produce large amounts of ROS, which may result in endothelial cell damage, provoke a series of inflammatory reactions, and eventually promote vascular endothelial cell apoptosis and atherosclerosis [1,2]. These adverse effects caused by chronic high glucose are also called glucotoxicity. Atherosclerosis can lead to plaque initiation and thrombosis, considered as the consequences of chronic low-grade inflammation [3]. Protecting vascular endothelial cells is of great importance to prevent diabetes-related vascular complications.

$P2X_4R$ is a subtype of ATP receptors, playing important roles in pain, inflammatory and immune responses. It can increase intracellular free Ca^{2+} levels, modulating expression of the early inflammatory transcriptional activator (NF-κB) which controls cytokine expression and cell apoptosis [4,5]. $P2X_4R$-mediated communication between microglia and neurons under inflammatory response has been investigated [6,7]. However, the roles of $P2X_4R$ on human umbilical vein endothelial cells (HUVECs) under high glucose condition are unknown.

Evodiamine is a kind of alkaloid extracted from the Chinese medicinal herb Wu-Zhu-Yu (Evodiae fructus; Evodia rutaecarpa Benth, Rutaceae), which has demonstrated various valuable therapeutic effects, including vasodilatory, uterotonic, thermoregulatory, anti-obesity and anti-inflammation [8,9]. Some studies also found that it can inhibit adipocyte differentiation, increasing insulin resistance [10,11]. Evodiamine can activate transient receptor potential vanilloid type 1 (TRPV1) through AMPK-dependent signaling, and thus increase endothelial nitric oxide synthase (eNOS) activity and NO production [12,13]. However, the anti-inflammation effects of evodiamine remain poorly understood. In this study, we investigated whether $P2X_4R$ is involved in high glucose-induced HUVEC dysfunction and whether evodiamine plays a potential role in protecting vascular endothelial cells from high glucose-induced inflammatory response possibly mediated by $P2X_4R$.

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2. Materials and methods

2.1. HUVEC culture and treatment

HUVECs were cultured in RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich Inc., St. Louis, MO, USA) and penicillin (100 U/mL) / streptomycin (100 μg/mL) in a humidified atmosphere containing 5% CO_2 at 37 °C. Medium was changed every two days and cell concentrations were maintained at 0.5–1.0 × 10^6 cells / ml. After seeded into six-well plates for 24 h, the cells was treated with control (5.5 mM) or high concentration of glucose (44.4 mM) for 5 days while the concentration of FBS in culture medium was reduced to 2% to keep the cells in quiescence to minimize the influence of cell growth [14]. After exposed to high glucose for 48 h, evodiamine was present during the subsequent 3 days.

2.2. Cell viability assay

The effects of evodiamine on cell viability were tested by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolim (MTS) assays (Promega, USA). Evodiamine, purchased from Nanjing Zhelang Medical Technology, Co., Nanjing, China, was dissolved in dimethyl sulfoxide (DMSO), and 0.2% DMSO was used as vehicle control for all experiments. Briefly, HUVECs were seeded in 96-well culture plates at a density of 4000 cells/well in a final volume of 200 μl. After culturing for 1 day, cells were treated with various doses of evodiamine (0–8 × 10^{-6} M) for 24, 48 and 72 h. Then HUVECs were incubated in serum-free medium mixed with 20% MTS solution for 2 h at 37 °C. The absorbance by formed formazan was determined at 490 nm using a microplate reader (Sunrise, TECAN, Mannedorf, Switzerland). Cell viability rate was calculated as percentage in compared to the control cells: (absorbance of the treated wells) / (absorbance of the control wells) × 100%.

2.3. Determination of total NO

HUVECs were cultured for 5 days with different treatments in 12-well culture plates. Then, the culture solution was collected for measuring the concentrations of NO by a kit named Nitrate reductase method supplied by Nanjing Jiancheng Bioengineering Institute. Endothelial dysfunction can lead to the lower bioavailability of NO. Nitrate reductase can turn NO-derived nitrate into nitrite. The absorbance of nitrite was analyzed by a spectrophotometer set at 540 nm. The concentration of NO was calculated as following: concentration of NO (μM) = [(absorbance of treated wells − absorbance of blank wells) / (absorbance of standard wells − absorbance of blank wells) × standard concentration (100 μM)].

2.4. Measurement of intracellular ROS

DCFH-DA method was used to detect intracellular ROS [15]. HUVECs were seeded on 24-well microplates for 5 days with different treatments. Then, the medium was replaced by serum-free medium containing 10 μM DCFH-DA (Sigma-Aldrich) for 25 min at 37 °C to load the fluorescent dye. The microplates were shaked every 8 min to mix cells with fluorescence probe fully. Then, cells were washed three times with phosphate-buffered saline (PBS) to completely remove fluorescence probe that did not enter cells. Fluorescence was measured using a fluorescence plate reader at excitation wavelengths of 485 and emission wavelengths of 535 nm. Each sample was run in triplicate and repeated three times.

2.5. Detection of intracellular free Ca^{2+}

Intracellular free Ca^{2+} levels can be detected by fluro-3/AM (Beyotime Biotechnology) [16]. After various treatments, the cells were washed with PBS and incubated in serum-free medium containing 5 μM flura-3/AM for 60 min at 37 °C. Then cells were washed with PBS for three times and cultured for another 25 min to ensure fluro-3/AM converted into fluro-3 in cells. The excitation and emission wavelengths were set at 488 and 530 nm respectively for measuring fluro-3 fluorescence.

2.6. Quantitative real-time RT-PCR

Total RNA was extracted by using Trizol reagent (TIANGEN, China) following the manufacturer's instructions. The reverse transcription reaction was performed through Revert Aid First Strand cDNA Synthesis Kit (Promega Corp., Madison, WI, USA). Real-time PCR was performed by using the SYBR Green reagent (Applied Biosystems, Fostercity, CA, USA). Briefly, SYBR green master mix, forward and reverse primers, and cDNA template were mixed thoroughly, and then results were generated by ABI 7500 Fast System using SDS software. Sense and anti-sense primers were 5′GGAGAACGCAGGACACAGTT3′ and 5′GAGT ACCTGGGCAAGCAGAG3′ for $P2X_4$; sense 5′TGACGTGGACATCCGCAA AG3′ and anti-sense 5′CTGGAAGGTGGACAGCGAGG3′ for GADPH. The relative expression levels of $P2X_4$ were analyzed after normalized to the expression levels of GADPH in the same group.

2.7. Western-blotting

Following different treatments for 5 days, the cells were rinsed with PBS and incubated in lysis buffer (Solarbio, China) containing 1% PMSF on ice for 15 min. After scraped and pelleted, the cell lysate was centrifuged at 15,000 × g for 10 min at 4 °C to collect the supernatant. Samples containing the same amount of protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) and transferred onto nitrocellulose membrane (Millipore, Billerica, MA, USA). The membranes were blocked for 2 h at room temperature with 5% skimmed milk and incubated overnight at 4 °C with the following respective primary antibodies: rabbit polyclonal anti-$P2X_4$ (1:200, ab99154, Abcam), anti-TNFR-α (1:500, ab19139, Abcam), anti-NF-κB (1:1000, Cell Signalling) and monoclonal anti-β-actin (1:700; Beijing Zhongshan Biotech Co.). The membranes were washed before incubated with horseradish peroxidase conjugated secondary antibodies at room temperature for 2 h. Then the protein bands were visualized by chemiluminescence development kit (ECL, Thermo technology, Rockford, USA) using an imaging system (ChemiDoc XRS from BioRad Lab., Hercules, CA, USA). The intensity of bands was analyzed by a digital imaging computer software (Image-Pro Plus Version 6.0, Media Cybernetics, MD, USA). After normalized to the corresponding β-actin, the changes of expression of target proteins ware determined.

2.8. Statistical analysis

All values were presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Duncan's multiple range t-test was conducted by using SPSS 17.0 to determine the significance of differences among different treatments. A P-value of <0.05 was deemed to be statistically significant. All data were repeated thrice.

3. Results

3.1. Effects of evodiamine on proliferation of HUVECs

To avoid the cytotoxicity of evodiamine on HUVECs and determine proper dosage and treatment time, MTS was performed after cells were treated with different concentrations of evodiamine for various periods. The results showed that evodiamine could affect HUVEC growth in time- and dose-dependent manner (Fig. 1A-C). Co-cultured with 4 μM evodiamine for 24 h could markedly inhibit the proliferation