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Rutin protects endothelial dysfunction by disturbing Nox4 and ROS-sensitive NLRP3 inflammasome

Wei Wang[1], Qiu-hong Wu[1], Yong Sui, Yan Wang, Xin Qiu*

Department of Pharmacy, Maternal and Child Health Care Hospital of Zaozhuang, Zaozhuang, 277100, China

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ABSTRACT

High glucose induced endothelial dysfunction is blamed for initiation of vascular complication in Type 2 diabetes mellitus. Rutin has been described in possessing comprehensive pharmacological activities, but the mechanism involved in endothelial protection through regulating oxidative stress and damage is still ambiguous. In the present study, rutin was evaluated in high glucose stimulated human umbilical vein endothelial cells (HUVECs) and high glucose diet-treated SD rats were applied to explored the molecular mechanism in rutin counteracting oxidative stress and damage. Firstly, 30 and 100 μM rutin effectively increased HUVECs viability in high glucose challenge. Then we found that rutin could dose-dependently reduced high glucose mediated mRNA and protein expressions of Nox4. With Nox4 and Nox2 inhibitors, we further confirm that Nox4, but not Nox2, was responsible for reactive oxygen species (ROS) production in high glucose environment. Moreover, rutin and Nox4 inhibitor significantly ameliorated ROS generation and TXNIP, NLRP3, caspase-1 and IL-1β proteins expression in vivo. Furthermore, rutin substantially recovered nitric oxide production in HUVECs under high glucose condition. However, rutin could not inhibited inflammasome protein expressions and improved nitric oxide production in Nox4-overexpressed HUVECs under high glucose challenges. Finally, we found that rutin restored phenylephrine-mediated contractions and acetylcholine induced relaxations in aortic tissue of high glucose diet treated rats. In vitro, expressions of TXNIP, NLRP3, and caspase-1 in aortic tissue of high glucose diet treated rats were decreased under rutin administration. In summary, rutin may protect endothelial dysfunction through inhibiting Nox4 responsive oxidative stress and ROS-sensitive NLRP3 signaling pathway under high glucose stress both in vivo and vitro.

1. Introduction

Type 2 diabetes mellitus (T2DM) induced vascular complication is still a life- threatening event that characterized by heterogeneous disorders [1–3]. Under sustained glucose stress, normal vascular endothelium changes its homeostasis role into the initiator of vascular diseases, including retinopathy, nephropathy, etc [4]. Thus, endothelial dysfunction is considered to play a key role in occurrence and development of vascular complication.

Imbalance between oxidative and anti-oxidative systems leads to cellular oxidative stress in diabetes. As to endothelial cells, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, rather than cytochrome p450, xanthine oxidase, etc., has been implicated as the major source of reactive oxygen species (ROS) production in high glucose stress [5]. Although several NADPH isoforms have been identified, Nox4 was believed to mainly implicate in producing ROS in endothelial cells [6,7]. Recently, increasing studies proved that ROS is not just the by-product of metabolism, but also an agonist to activate nucleotide-binding domain-like receptor 3 (NLRP3) inflammasome to further disturb endothelial function [8]. Therefore, inhibiting NADPH oxidase to restrict ROS production and NLRP3 inflammasome activation is of great significance to control glucose risk [9].

3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside, also known as rutin, is one kind of flavonoids that can be obtain in different dietary sources [10]. Studies showed that rutin did well in protecting rats from stress damage [11,12]. Also, accumulating studies indicated rutin tablets as the therapeutic agent for many diseases including diabetes [13–15]. Although rutin has been proved to regulate inflammation, oxidative stress, and nitric oxide (NO) production, exact mechanism is still ambiguous. Thus, in this study, we try to determine whether Nox4-ROS-NLRP3 was

* Corresponding author at: Department of Pharmacy, Maternal and Child Health Care Hospital of Zaozhuang, No. 25 East Cultural Road, Zaozhuang, 277100, China.
E-mail address: pluxud@sina.com (X. Qiu).
[1] Both authors have contributed equally to the paper.

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implicated in rutin protecting human umbilical vein endothelial cells (HUVECs) dysfunction under high glucose environment, which help replenishing new mechanism to rutin's endothelial protective function.

2. Materials and methods

2.1. Materials

2-(2-chlorophenyl)-4-(3-(dimethylamino)phenyl)-5-methyl-1,2-dihydro-5H-pyrazolo[4,3-*c*]pyridine-3,6-dione (GKT137831, Nox4 inhibitor) was purchased from MedChem Express (New Jersey, USA). Nox2ds-tat (also known as gp91ds-tat, Nox2 inhibitor) was purchased from Absin Bioscience Inc. (Shanghai, China). Rutin (98% purity) was purchased from Chinese Institute for Drug and Biological Product Control (Beijing, China). Anti-Nox4 (67 kD) (UOTR1B493), anti-thioredoxin-interacting protein (TXNIP) (46 kD) (EPR14774), anti-NLRP3 (114 kD) (EPR4777), anti-caspase-1 (45 kD) (EPR4321), and glyceraldehyde-3-phosphate de-hydrogenase (GAPDH) (37 kD) monoclonal antibodies were obtained from Abcam (Cambridge, MA, USA). IL-1β Enzyme-linked Immunosorbent Assay (ELISA) Kit was purchased from Shanghai Gaochuang Chemical Co., Itd (Shanghai, China). Phosphate buffer saline (PBS), 3-(4,5-dime-thyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), 2,7-dichlorofluoresce in diacetate (DCFH-DA), 3-Amino, 4-aminomethyl-2′,7′-difluorescein (DAF-FM DA), Membrane and Cytosol Protein Extraction Kit, and BCA Protein Assay Kits were purchased from Beyotime Biotechnology Corporation (Shanghai, Chin-a). PCR related Kits (including Transzol Up Kit, TransScript® II First-Strand cDNA Synthesis SuperMix Kit and TransStart® Top Green qPCR Super Mix Kit) were obtained from TransGen Bioteach (Beijing, China).

2.2. Cell culture and plasmid transfection

HUVECs were obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (DMEM, 5.5 mM glucose) with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY), 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a condition of 5% CO_2 and 95% humidified air at 37 °C. Culture medium was changed properly till the cells grew to 80% confluence. pcDNA3.0-V5-NOX4, human NOX4 expression plasmid, was applied to build up Nox4-overexpressed HUVECs [16]. Through comparing with normal HUVECs in the indicated experiments, Nox4-overexpressed HUVECs that applied in western blot, nitric oxide detection, and IL-1β experiments were used to help determining rutin's effect.

2.3. Cell viability assay

MTT assay was applied to detect cell viability. In brief, HUVECs were seeded on a 96-well plate (1×10^4 cells/well). Then cells were cultured at 37 °C under the following experimental groups: (1) normal glucose (5 mM), (2) high-glucose (25 mM), (3) 25 mM mannitol (osmotic control), (4) high-glucose (25 mM) and rutin (10 μM), (5) high-glucose (25 mM) and rutin (30 μM), (6) high-glucose (25 mM) and rutin (100 μM), (7) high-glucose (25 mM) and GKT137831 (Nox4 inhibitor) (20 μM), and (8) normal glucose (5 mM) and GKT137831 (20 μM) [17]. HUVECs were pretreated with agents (PBS, rutin or GKT137831) for 0.5 h followed by 48 h high glucose or mannitol treatment in indicated group. After 48 h treatment, 20 μl MTT solution (5 mg/ml) was added into each well to incubate for 4 h. Finally, 150 μl/well DMSO was added to dissolve associated crystals. The plate was read by a scanning multi-well spectrophotometer with wave length of 570 nm.

2.4. RNA analysis by real-time quantitative PCR

HUVECs were seeded on a 6-well plate (5×10^4 cells/well). HUVECs were pretreated with indicated agents (PBS, rutin or GKT137831) for 0.5 h followed by 48 h high glucose treatment in indicated group. Then total RNA was extracted using Transzol Up Kit according to the manufacturer's protocol. With TransScript® II First-Strand cDNA Synthesis SuperMix Kit, 5 μg RNA in each group was transcribed to cDNA. Expressions of Nox4 were determined using TransStart® Top Green qPCR Super Mix Kit by improved Real-Time Quantitative PCR (BioRad, California, USA). Samples were denatured at 95 °C for 3 min, followed by 40cycles (95 °C for 13 s and 64 °C for 35 s in each cycle). The primer sequences were used as follows:

Nox4 forward: 5′-AGTCAGCTCTCTCCTTTCAGG-3′, Nox4 reverse: 5′-CTTG-CCCCCTTTGAATAAAT-3′. GAPDH forward: 5′-AGT-CAGCTCTCTCCTTTCAGG-3′, GAPD-H reverse: 5′-TCCAC-CACCCTGTTGCTGTA-3′. GADPH was u-sed as the internal control of mRNA expression.

2.5. Western blotting

HUVECs or Nox4-overexpressed HUVECs were seeded in 6-well-plates and pretreated with indicated agents (PBS, rutin, or GKT137831) for 0.5 h followed by 48 h high glucose treatment in indicated group. Protein lysates were obtained according to manufacturer's suggested protocol of Membrane and Cytosol Protein Extraction Kit, and concentration of proteins were detected by BCA Protein Assay Kit. 25 μg proteins in each group were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently electro-transferred onto polyvinylidenedifluoride membranes (Millipore, Billerica, MA). After blocked by Tris-buffered saline containing 5% skimmed milk and 0.05% Tween 20 at 37 °C for 2.5 h, the membranes were incubated with the primary antibodies (1:500 dilution) and secondary antibodies (1:1000 dilution) properly. Protein bands were analyzed by the ChemiDoc XRS system (BioRad, California, USA) and Image J software (National Institutes of Health, U.S.A.).

2.6. ROS measurement by flow cytometry

HUVECs were seeded on a 6-well plate (5×10^4 cells/well). HUVECs were pretreated with indicated agents (PBS, rutin, GKT137831, or Nox2ds-tat) for 0.5 h followed by 48 h high glucose treatment in indicated group [18]. Cells were harvested and incubated with 10 μM DCFH-DA at 37 °C for 30 min under dark condition. Then cells were washed twice with cold PBS. Finally, cells were analyzed with FACSCalibur flow cytometry (Becton, Dickinson and Company, New Jersey, USA) at an excitation wave length of 488 nm and an emission wavelength of 525 nm. DCF fluorescence distributions were calculated by Cell Quest software (Moleular Devices Corporation).

2.7. IL-1β measurement by ELISA assay

HUVECs or Nox4-overexpressed HUVECs were seeded 6-well-plates and pretreated with indicated agents (PBS, rutin, or GKT137831) for 0.5 h followed by 48 h high glucose treatment in indicated group. IL-1β protein concentrations in cell supernatants were quantified by ELISA Kits according to the manufacturer's instructions.

2.8. Intracellular NO measurement by fluorescence microscope

HUVECs or Nox4-overexpressed HUVECs were seeded and pretreated with indicated agents (PBS, rutin, or GKT137831) for 0.5 h followed by 48 h high glucose treatment in indicated group.