Naringenin upregulates GTPCH1/eNOS to ameliorate high glucose-induced retinal endothelial cell injury

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Abstract. Diabetic retinopathy (DR) is a microvascular complication of diabetes, while retinal endothelial cell (REC) dysfunction is considered the primary pathological process of DR. Naringenin, a natural flavonoid compound, exhibits therapeutic potential against multiple types of endothelial cell injury. To the best of our knowledge, however, its effect on REC injury has not been previously investigated. Therefore, the aim of the present study was to investigate the effect of naringenin on high glucose (HG)-induced REC injury and assess the underlying mechanism. To establish a retinal injury model, human (H)RECs were treated with 30 mM glucose. Cell Counting Kit-8 assay and TUNEL staining were used to assess the effects of naringenin on cell proliferation and apoptosis, respectively. Reactive oxygen species (ROS) levels and concentration of tetrahydrobiopterin (BH4), the essential cofactor of endothelial nitric oxide synthase (eNOS), were measured using a ROS detection kit and ELISA, respectively. The transfection efficiency of HRECs with guanosine triphosphate cyclohydrolase-1 (GTPCH1) interfering plasmid was examined by reverse transcription-quantitative PCR. The protein expression levels of Ki67, proliferative cell nuclear antigen (PCNA), eNOS and GTPCH1 were determined by western blot analysis. Compared with the HG-induced group alone, co-treatment with naringenin inhibited HG-induced HREC apoptosis in a dose-dependent manner, increased expression levels of the proliferation-associated proteins Ki67 and PCNA and effectively decreased intracellular ROS levels. Furthermore, naringenin upregulated GTPCH1/eNOS signaling and promoted release of BH4. However, GTPCH1 knockdown partially reversed the ameliorative effect of naringenin on HG-induced HREC injury. In summary, the present study suggested that naringenin effectively inhibited HG-induced HREC apoptosis and intracellular oxidative stress, which may be associated with naringenin-mediated GTPCH1/eNOS upregulation.

Introduction

Diabetes mellitus is a type of metabolic disease characterized by hyperglycemia (1). Long-standing hyperglycemia may lead to multiple types of tissue injury, such as eye, kidney and heart dysfunction and chronic damage of blood vessels and nerves (2-4). Diabetic retinopathy (DR) is characterized by retinal lesions and is often accompanied by abnormal angiogenesis (5). DR involves pathological characteristics, including loss of pericytes, thickening of the basement membrane and adhesion of white blood cells (6,7). Endothelial cell (EC) dysfunction serves a key role in the structure and pathophysiology of the retina (8). Therefore, novel studies and the development of drugs for improving retinal (R)EC dysfunction may promote the effective treatment of DR.

Naringenin (4',5,7-trihydroxyflavanone) is a natural flavonoid compound that is found in grapefruit, tomato and citrus fruits of the Rutaceae family (9,10). Compared with other flavonoids, naringenin is easily absorbed by the gastrointestinal tract and is characterized by its high bioavailability and low toxicity (11). Naringenin exhibits biological effects, such as antibacterial, anti-inflammatory, antioxidant, immune regulation and anti-tumor activity (12-15). Naringenin is effective in treating obesity (16), atherosclerosis (17) and diabetes (18). Zeng et al (19) demonstrated that naringenin improves high glucose (HG)-induced injury of vascular ECs. Another study revealed that naringenin exerts a protective effect against alkali-induced corneal burn by attenuating secretion of inflammatory cytokines and resisting oxidation (20). To the best of our knowledge, however, the effect of naringenin on REC injury has not been previously investigated.

Guanosine triphosphate cyclohydrolase 1 (GTPCH1), a key enzyme that catalyzes production of tetrahydrobiopterin (BH4), is involved in the synthesis of numerous hormones and neurotransmitters and serves a vital role in a series of pathophysiological processes in the body (21,22). For instance, inhibition of GTPCH1 reduces the inflammation of microglia (23). GTPCH1 participates in endothelial dysfunction in atherosclerosis (24). Nitric oxide (NO) produced by endothelial NO synthase (eNOS) serves a key role in
maintaining EC homeostasis due to its anti-inflammatory and antioxidant effects. Furthermore, BH4 is a key factor involved in maintaining eNOS activity and determining the balance of NO and eNOS-produced superoxide (25). eNOS should be fully saturated with BH4 to be fully coupled with reduced nicotinamide adenine dinucleotide phosphate to be oxidized into NO. Under BH4 deficiency, eNOS functions in an ‘uncoupled’ form, resulting in generation of superoxide and H₂O₂ and aggravating oxidative stress responses in organisms (26,27).

Previous studies showed that BH4 supplementation decreases endothelial dysfunction in patients with atherosclerosis and diabetes mellitus (28,29). Furthermore, GTPCH1 is down-regulated in ECs isolated from diabetic rats with decreased BH4 levels and uncoupled eNOS (30). Multiple studies have demonstrated that GTPCH1 upregulation improves different types of EC injury, such as brain microvascular (31), palmitic acid-induced islet (32) and HG-induced aortic EC injury (22).

Therefore, the present study aimed to investigate the effect of naringenin on HG-induced REC injury and whether the effects of naringenin are associated with regulation of the GTPCH1/eNOS axis.

Materials and methods

Cell culture and treatment. Human (H)RECs were purchased from Ningbo Mingzhou Biotechnology Co., Ltd. (cat. no. MZ-1174). Cells were cultured in Endothelial Cell Medium (cat. no. 1001; HyClone; Cytiva) supplemented with 5% FBS (cat. no. 10091141; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO₂. Cells in the logarithmic growth phase were firstly treated with naringenin (1, 10, 20, 50 and 100 µM). Naringenin at concentrations of 1 and 10 µM were selected for subsequent experiments as naringenin was cytotoxic at ≥20 µM. Subsequently, cells were treated with 30 mM HG (cat. no. 50-99-7; MilliporeSigma), HG + 1 µM naringenin (cat. no. 67604-48-2; MilliporeSigma) or HG + 10 µM naringenin at 37˚C for 24, 48 and 72 h. After GTPCH1 was silenced, cells were further divided into the following five groups: i) Control; ii) 30 mM HG; iii) HG + 10 µM naringenin; iv) HG + 10 µM naringenin + siRNA-NC; and v) HG + 10 µM naringenin + siRNA-GTPCH1. Untreated cells served as the control group.

Cell Counting Kit-8 (CCK-8) assay. HRECs were seeded into a 96-well cell culture plate at a density of 1x10⁴ cells/well and incubated overnight at 37˚C with 5% CO₂. Following treatment as aforementioned, HRECs in each well were supplemented with 10 µl CCK-8 solution (cat. no. C1005; Beyotime Institute of Biotechnology) at room temperature for 3-5 min, followed by washing with PBS 2-3 times for 3-5 min each. The cells were treated with 0.3% Triton-X-100 at room temperature for 5 min. Subsequently, cells were supplemented with 50 µl TUNEL assay solution (cat. no. C1086; Beyotime Institute of Biotechnology) and incubated at 37˚C in the dark for 1 h according to the manufacturer’s instructions. Cell nuclei were stained with DAPI (1 mg/ml) at room temperature for 10 min in the dark. Following incubation, the detection solution was discarded and cells were washed three times with PBS. Finally, cells were sealed with anti-fluorescence quenched sealing solution and observed in three randomly selected fields of view with a total of 300-500 cells under a fluorescence microscope (Zeiss GmbH; magnification, x200). The excitation wavelength range used was 450-500 nm and the emission wavelength range was 515-565 nm (green fluorescence).

Western blot analysis. HRECs were washed three times with pre-cooled PBS and lysed with RIPA lysis buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) for 30 min on ice. Subsequently, the cell lysate was collected and centrifuged at 400 x g for 15-20 min at 4˚C and the protein supernatant from each group was transferred to Eppendorf tubes. The total protein concentration was measured using the compat-Able™ BCA protein assay kit (cat. no. 23229; Thermo Fisher Scientific, Inc.). The protein samples from each group (30 µg per lane) were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (cat. no. FFP24;
Following blocking with 5% skimmed milk powder at room temperature for 4 h, the membranes were washed three times with 1X TBS-0.1% Tween-20 followed by incubation with primary antibodies (all 1:1,000; all Abcam) against Bcl-2 (cat. no. ab194583), Bax (cat. no. ab32503), cleaved-caspase 3 (cat. no. ab32042), caspase 3 (cat. no. ab32351), eNOS (cat. no. ab252439), GTPCH1 (cat. no. ab236387), Ki67 (cat. no. ab15580), PCNA (cat. no. ab92552) and β-actin (cat. no. ab8226) at 4˚C over night. Subsequently, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:1,000; cat. no. ab288151; Abcam) for 4 h at room temperature and the protein bands were visualized using ECL reagent (Thermo Fisher Scientific, Inc.). The protein expression levels were semi-quantified using ImageJ (version 1.8.0; National Institutes of Health).

**RT-qPCR.** Total RNA was extracted from HRECs using RNAzol RT reagent (MilliporeSigma), according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Following digestion with DNase I, total RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription kit (Qiagen GmbH), according to the manufacturer's protocol. qPCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen GmbH), according to the manufacturer's instructions. The thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 1 min. The primer sequences used (all GenScript) were as follows: GTPCH1 forward, 5'-CGAGCGTTGCTAGT-3' and reverse, 5'-CTCAGGCGAGTCTTG-3'; TNF-α forward, 5'-GGGCTAGATGTTAC-3' and reverse, 5'-GGCCTGAGCGTGAT-3'; IL-1β forward, 5'-GGGCTAGATGTTAC-3' and reverse, 5'-GGAATTCGCTG-3'; IL-6 forward, 5'-CTCAGGCGAGTCTTG-3'; and reverse, 5'-GGCCTGAGCGTGAT-3'. The mRNA expression levels were quantified using the 2^ΔΔCq method (33); β-actin served as the internal reference gene.

**Statistical analysis.** Data are presented as the mean ± SD from ≥3 independent experiments. All statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc.). The differences between multiple groups were compared using one-way ANOVA followed by post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Naringenin ameliorates HG-induced HREC damage. The molecular structure of naringenin is presented in Fig. 1A. To evaluate the effect of different concentrations of naringenin (1, 10, 20, 50 and 100 µM) on HREC viability, a CCK-8 assay was performed. The results showed that high concentrations of naringenin (20, 50 and 100 µM) exerted an inhibitory effect on HREC viability, while low concentrations of naringenin (1 and 10 µM) had no significant effect on HREC viability. Therefore, final concentrations of 1 and 10 µM naringenin were selected to assess the protective effect of naringenin on HRECs for
the reason that naringenin was cytotoxic at ≥20 µM (Fig. 1B). Compared with that of the control, the viability of HRECs treated with HG for 24, 48 and 72 h significantly decreased, while co-treatment with naringenin increased HREC viability under HG conditions, which implied that naringenin attenuated HG-induced cell injury in a concentration-dependent manner (Fig. 1C). To evaluate the proliferative ability of HRECs, protein expression levels of intracellular proliferation markers Ki67 and PCNA were determined (Fig. 1D). HG significantly inhibited protein expression levels of Ki67 and PCNA in HRECs, while co-treatment with 1 or 10 µM naringenin improved HG-reduced intracellular proliferation-associated protein expression to varying degrees. Overall, naringenin attenuated HG-elicited HREC viability injury.

Naringenin inhibits HG-induced HREC apoptosis. HREC apoptosis was assessed using TUNEL assay. Cell apoptosis was significantly increased in the HG compared with the control group. Treatment with 1 or 10 µM naringenin partially reversed HG-induced HREC apoptosis. In summary, naringenin suppressed HG-enhanced HREC apoptosis.

Naringenin upregulates eNOS and GTPCH1 in HG-induced HRECs. Subsequently, the effect of naringenin on ROS overproduction in HG-induced HRECs was investigated. Treatment with naringenin reversed HG-induced ROS overproduction in a dose-dependent manner (Fig. 3A). The effect of naringenin on BH4 (Fig. 3B), GTPCH1 and eNOS levels (Fig. 3C) in HRECs was assessed. Compared with the control, HG significantly decreased BH4 contents and protein expression levels of GTPCH1 and eNOS in HRECs; this was partially reversed by naringenin. In addition, RT-qPCR showed that naringenin also reversed the HG-induced increase in inflammatory factors (TNF-α, IL-1β and IL-6) in a concentration-dependent manner (Fig. 3D-F). These results indicated that naringenin ameliorated HG-induced HREC oxidative stress and inflammatory response by enhancing GTPCH1/eNOS signaling.

GTPCH1 knockdown reverses the inhibitory effect of naringenin on HG-induced HREC injury. To uncover the mechanism underlying the effect of naringenin on
improving HG-induced HREC injury via upregulation of GTPCH1, its role in GTPCH1-knockdown HRECs was investigated. Western blotting and RT-qPCR showed that expression of GTPCH1 was significantly decreased in the siRNA-GTPCH1 compared with the siRNA-NC group (Fig. 4A and B). HREC viability, proliferative ability and apoptosis were then assessed. HREC viability and expression levels of Ki67 and PCNA (Fig. 4C and D) were significantly decreased in the HG + 10 µM naringenin + siRNA-GTPCH1 compared with the HG + 10 µM naringenin + siRNA-NC group. Additionally, the inhibitory effect of naringenin on HG-induced HREC apoptosis was reversed in the HG + 10 µM naringenin + siRNA-GTPCH1 group compared with the HG + 10 µM naringenin + siRNA-NC group (Fig. 4E and F).
Figure 4. siRNA-GTPCH1 reverses the inhibitory effect of naringenin on HG-induced HREC injury. (A) Western blotting and (B) reverse transcription-quantitative PCR were used to detect GTPCH1 protein and mRNA expression levels, respectively. Effect of siRNA-GTPCH1 on naringenin-enhanced (C) viability and (D) proliferation of HG-induced HRECs. (E) Apoptosis of HRECs was detected by TUNEL staining. (F) Western blotting was used to assess expression of apoptosis-associated proteins (Bcl-2, Bax and cleaved-caspase 3). *P<0.05, **P<0.01 and ***P<0.001. si, small interfering; GTPCH1, guanosine triphosphate cyclohydrolase-1; HG, high glucose; HREC, human retinal endothelial cell; NC, negative control.
Naringenin-reduced ROS generation in HG-insulted HRECs was improved again after silencing of GTPCH1 (Fig. 5A), and GTPCH1 depletion reversed the elevated BH4 content, and GTPCH1 and eNOS expression imposed by naringenin administration in HG-treated HRECs (Fig. 5B and C).

Discussion

It has been reported that exposure to HG promotes overproduction of intracellular ROS, leading to oxidative stress, apoptosis and dysfunction of ECs (34). Levels of BH4, a key co-factor of eNOS, are regulated by GTPCH1 (35). In the absence of BH4, eNOS produces ROS instead of NO, which is also referred to as eNOS uncoupling (27). ROS are partially derived from eNOS uncoupling. Studies have shown that naringenin exerts different cytotoxic effects on different types of cells, including polymorphonuclear leukocytes and Wilms tumor cells (36,37). In the present study, naringenin was cytotoxic at 20 µM. Therefore, concentrations of 1 and 10 µM were selected for subsequent experiments. The present results demonstrated that HG promoted HREC apoptosis, increased ROS production, downregulated protein expression levels of GTPCH1 and eNOS and attenuated BH4 secretion, suggesting that HG induced oxidative stress and dysfunction of BH4 secretion, thus promoting cell apoptosis. Naringenin inhibited HG-induced HREC apoptosis, upregulated Ki67 and PCNA expression and effectively decreased intracellular ROS levels in a dose-dependent manner. Furthermore, naringenin upregulated GTPCH1/eNOS signaling, promoted release of BH4 and notably alleviated HREC injury. GTPCH1 knockdown confirmed that the GTPCH1/eNOS signaling pathway was involved in the protective role of naringenin in HG-induced HRECs.

DR, a common microvascular complication in patients with diabetes, is primarily characterized by retinal structure and functional abnormality, which causes blindness in severe cases (38). Retinal endothelial dysfunction is the primary pathological process of DR (39). Previous studies have suggested that long-term hyperglycemia causes multiple types of EC injury, including brain microvascular (40), aortic (41), human umbilical vein (42) and HREC injury (43). Here, a retinal injury model was established by treating HRECs with 30 mM glucose as an inducer. Previous studies suggested that this dose of glucose significantly enhances levels of intracellular inflammatory factors in HRECs and promotes EC dysfunction (43,44). Another study demonstrated that naringenin effectively decreases diabetes-induced oxidative stress response caused by impaired NO synthesis in rat ECs (45).
In addition, naringenin protects the eye by inhibiting corneal angiogenesis (20) and improving macular degeneration (46). To the best of our knowledge, the present study is the first to demonstrate that naringenin attenuates generation of ROS and oxidative injury in HG-induced HRECs.

Steady-state imbalance of NO and ROS may lead to endothelial-dependent impaired vasodilation and enhanced inflammatory responses, oxidative stress and EC injury (47). eNOS is the key rate-limiting enzyme for NO synthesis (48) and catalyzes conversion of L-arginine into NO. However, under pathological conditions, eNOS promotes conversion to superoxide instead of NO (eNOS uncoupling) (49). eNOS uncoupling is partially promoted by GTPCH1 downregulation, which leads to dysfunction of BH4 secretion and impaired NO synthesis (50). An et al (51) showed that enhanced GTPCH1-mediated eNOS recirculation alleviates HG-induced endothelial dysfunction. In addition, exogenous zinc supplementation restores diabetic endothelial dysfunction via upregulating GTPCH1 (22). In the present study, HG-mediated induction of HRECs decreased protein expression levels of GTPCH1 and eNOS, thus supporting the abnormal increase in ROS levels in HRECs. Naringenin significantly increased protein expression levels of GTPCH1 and eNOS and BH4 secretion in HRECs, thus attenuating ROS generation and cell apoptosis. However, GTPCH1 knockdown partially restored the protective effects of naringenin on HG-treated HRECs, suggesting that naringenin improved oxidative stress, cell apoptosis and impaired BH4 secretion via the GTPCH1/eNOS signaling pathway.

To the best of our knowledge, the present study is the first to report the positive effects and underlying mechanism of naringenin on diabetic REC injury; however, these findings were only supported by in vitro experiments. Therefore, in vivo studies are needed to verify the aforementioned results. The role of the GTPCH1/eNOS signaling pathway in naringenin-mediated protection of RECs was verified only in GTPCH1-knockdown HRECs. Therefore, experiments using GTPCH1 antagonists or inhibitors should be performed to confirm the results of the present study. Bai et al (53) showed that naringenin metabolism in different species is a complex multi-pathway process, therefore the specific metabolic pathway of naringenin and its toxicity in vivo need to be studied. Additionally, the involvement of pathways other than the GTPCH1/eNOS pathway in the protective effect of naringenin cannot be ruled out. To verify the accuracy of the present study, it is necessary to evaluate the potential role of GTPCH1 siRNA in control cells for detection of apoptosis in future studies.

In summary, the present study suggested that naringenin improved oxidative stress, cell apoptosis and dysfunction of BH4 secretion in HG-treated HRECs by upregulating the GTPCH1/eNOS signaling pathway.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
BX conceptualized and designed the study. YW acquired, analyzed and interpreted data. BX and YW drafted the manuscript and revised it critically for important intellectual content. All authors agreed to be held accountable for the current study in ensuring questions related to the integrity of any part of the work are appropriately investigated and resolved. All authors have read and approved the final manuscript. BX and YW confirm the authenticity of all the raw data.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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