Role of apigenin in high glucose-induced retinal microvascular endothelial cell dysfunction via regulating NOX4/p38 MAPK pathway in vitro

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Abstract

- AIM: To investigate the retinoprotective role of Apigenin (Api) against high glucose (HG)-induced human retinal microvascular endothelial cells (HRMECs), and to explore its regulatory mechanism.

- METHODS: HRMECs were stimulated by HG for 48h to establish the in vitro cell model. Different concentrations of Api (2.5, 5, and 10 μmol/L) were applied for treatment. Cell counting kit-8 (CCK-8), Transwell, and tube formation assays were performed to examine the effects of Api on the viability, migration, and angiogenesis in HG-induced HRMECs. Vascular permeability was evaluated by Evans blue dye. The inflammatory cytokines and oxidative stress-related factors were measured using their commercial kits. Protein expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) and p38 mitogen-activated protein kinase (MAPK) was measured by Western blot.

- RESULTS: Api prevented HG-induced HRMECs viability, migration, angiogenesis, and vascular permeability in a concentration-dependent manner. Meanwhile, Api also concentration-dependently inhibited inflammation and oxidative stress in HRMECs exposed to HG. In addition, HG caused an elevated expression of NOX4, which was retarded by Api treatment. HG stimulation facilitated the activation of p38 MAPK signaling in HRMECs, and Api could weaken this activation partly via downregulating NOX4 expression. Furthermore, overexpression of NOX4 or activation of p38 MAPK signaling greatly weakened the protective role of Api against HG-stimulated HRMECs.

- CONCLUSION: Api might exert a beneficial role in HG-stimulated HRMECs through regulating NOX4/p38 MAPK pathway.

- KEYWORDS: apigenin; retinal microvascular endothelial cell; glucose; NOX4; p38 MAPK

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INTRODUCTION

Diabetic retinopathy (DR) is a common microvascular complication of diabetes mellitus that triggers irreversible retinal damage. It has been widely recognized that DR is the leading of vision loss and preventable blindness in working-age adults and elderly people worldwide[1]. With the increasing prevalence of diabetes, the population of people with DR is estimated to rise to 628 million in 2045, which will cause great public health burdens[2]. The pathogenesis of DR is complicated, and multi-faceted. Oxidative stress, inflammatory response and advanced glycation end products induced by long-term hyperglycaemia can lead to retinal microvascular endothelial cell dysfunction and blood-retinal barrier breakdown, eventually contributing to the progression of DR[3-6]. Up to date, the main therapeutic approaches for DR, such as anti-vascular endothelial growth factor (VEGF) agents and laser photocoagulation, significantly reduce the occurrence of severe vision loss; however, the troublesome side effects and serious complications after these treatments strictly limit their clinical application[7]. Hence, a better understanding of DR pathogenesis and effective therapeutic approaches to repair the damaged retina and restore visual function are urgently required.

Flavonoids, the largest group of naturally-occurring polyphenols existing in almost all plants tissues, possess broad biological activities in numerous mammalian systems. Apigenin [Api; 5,7-dihydroxy2-(4-hydroxyphenyl)-4H-1-
benzopyran-4-one] is an edible flavonoid widely distributed in most vegetables and fruits, especially celery and parsley\[8-9\]. In the past decades, considerable efforts have been made towards unveiling the antioxidant, anti-inflammatory, anti-hyperglycemic, anti-atherogenic and antimicrobial properties of Api, which has been confirmed to play a protective role against multiple human diseases, including cancers, Alzheimer’s disease, chronic inflammatory diseases, and metabolic diseases\[10-14\]. Of note, accumulating evidence demonstrated that Api not only exerted a protective effect in diabetes, but also ameliorated a series of diabetic complications, such as diabetic nephropathy, diabetic cardiomyopathy, and diabetes-associated cognitive decline\[15-18\]. Nevertheless, there is little information about the role of Api during the development of DR, and whether Api can play a protective role in DR raises our interest. The previous studies identified that Api had a potential therapeutic efficacy in retinopathy via inhibiting retinal oxidative damage and enhancing retinal vascular barrier function\[19-20\]. Hence, we speculated that Api possessed the potential to prevent from DR. On this basis, the main objectives of the current study were to examine whether Api might exert a beneficial role in repressing the progression of DR and to elucidate the potential regulatory mechanism.

MATERIALS AND METHODS

Cell Culture and Treatment Human retinal microvascular endothelial cells (HRMECs, cat no. CP-H130) were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in a complete culture medium (cat no. CM-H130; Procell Life Science & Technology Co., Ltd.) supplemented with 10% fetal bovine serum (FBS), 1% growth supplement and 1% penicillin/streptomycin mixture in a humidified atmosphere of 5% CO₂ at 37°C. For treatment, HRMECs were treated with 5 mmol/L glucose as a normal glucose (NG) group, 30 mmol/L glucose as a high glucose (HG) group, and 5 mmol/L glucose plus 25 mmol/L mannitol as an osmotic control group (MA). Meanwhile, HRMECs were treated with different concentrations of Api (0, 2.5, 5, and 10 μmol/L; Sigma-Aldrich, Saint Louis, MO, USA) for 48h. In addition, cells were pre-treated with 50 μmol/L of P79350 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), a selective agonist of p38 mitogen-activated protein kinase (MAPK) for the investigation of regulatory mechanism.

Cell Transfection The pcDNA3.1-based nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) overexpression vector (pc-NOX4) and the scramble vector (pcDNA3.1) were obtained from GenePharma (Shanghai, China). Cells were transfected with pcDNA3.1 or pc-NOX4 using Lipofectamine 3000 reagent (Invitrogen) in accordance with the manufacturer’s instructions. Post transfection 48h, cells were collected for analysis.

Cell viability The cell viability of HRMECs was assessed using cell counting kit-8 (CCK-8) assay. Briefly, cells were cultured in 96-well plates (5×10^4 cells/well) at 37°C overnight and then treated as the above grouping method. Subsequently, 10 μL of CCK-8 solution (Beyotime, Shanghai, China) was added to each well and the cells were cultured at 37°C for another 2h. Finally, the absorbance at 450 nm of each well was measured using a microplate reader (Multiskan MK3, Thermo Fisher Scientific).

Measurement of Reactive Oxygen Species, Glutathione S-Transferase, and Malondialdehyde The intracellular reactive oxygen species (ROS) level was measured by the commercial kits (Molecular Probes, Eugene, OR, USA) based on the turn out of the 2’,7’-dichlorofluorescin diacetate (DCF-DA) into highly fluorescent 2’,7’-dichlorofluorescein. After incubation with DCF-DA for 30min, ROS staining was observed under a fluorescence microscope (IX53 Olympus, Tokyo, Japan). For glutathione S-transferase (GSH-ST) and malondialdehyde (MDA) assays, after indicated treatment, the supernatants from HRMECs of each group were harvested, and the GSH-ST and MDA levels were detected adopting GSH-ST and MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. The absorbance was detected with a microplate reader (Multiskan MK3, Thermo Fisher Scientific) at 412 nm for GSH-ST and 532 nm for MDA.

Measurement of Inflammatory Cytokines After indicated treatment, the supernatants from HRMECs of each group were harvested. The concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-18 was measured using their corresponding enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN, USA) in line with the guidelines.

Cell Migration Assay Cell migration assay was performed using a 24-well Transwell chamber (BD Biosciences, USA). In brief, 200 μL of HRMECs (2×10^5 cells/mL) were seeded in upper chamber of the Transwell, and 500 μL of the complete medium containing 10% FBS was added to the lower chamber. After incubation at 37°C for 48h, cells on the bottom of the transwell membrane were fixed with 4% formaldehyde for 30min and then stained with 0.5% crystal violet for 20min at room temperature. Finally, the images of the migrated cells were obtained under a light microscope (IX51 Olympus, Tokyo, Japan).

Tube Formation Assay HRMECs were seeded into 24-well plates (2×10^5 cells/well) which was pre-coated with 200 μL matrigel (BD Bioscience) per well and the plates were cultured at 37°C for 20h. Finally, the tube formation was captured under a light microscope (IX51 Olympus, Tokyo, Japan) and analyzed using Image J software (version 1.49; NIH, Bethesda, MD, USA).
Apigenin ameliorates retinal endothelial cell damage

Measurement of Vascular Permeability  After indicated treatment, HRMECs were stained with Evans blue dye (Sigma-Aldrich) for 2h. After washing, the dye was extracted by formamide at 70°C for 18h, followed by centrifugation at low temperature for 30min. The optical density (OD) value of the supernatant was then detected at 620 nm.

Western Blot  Total protein was extracted from HRMECs using radio-immunoprecipitation assay buffer (Thermo Fisher Scientific) containing 1% protease and phosphatase inhibitors (Thermo Fisher Scientific). After quantification of protein concentration using the bicinchoninic acid assay (Pierce, Rockford, IL, USA), the same amount of proteins (30 μg/lane) was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation, followed by transferring onto polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% skimmed milk for 2h at room temperature, and then probed with primary antibodies at 4°C overnight and the corresponding secondary antibody for 2h at room temperature. The blots were visualized using enhanced chemiluminescence reagents (4A Biotech Co., Beijing, China).

Statistical Analysis  Statistical analysis was carried out using GraphPad Prism software (version 8.0; GraphPad Software Inc., La Jolla, CA, USA). All experimental data were presented as mean±standard deviation (SD). The difference comparison was conducted using one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. In all comparisons, \( P<0.05 \) was considered statistically significant.

RESULTS

Api Inhibits Cell Viability of HRMECs Under the HG Environment  First, to examine the cytotoxicity of Api, HRMECs were treated with increasing concentrations of Api (0, 2.5, 5, and 10 μmol/L) for 48h. The results obtained from CCK-8 assay revealed that there was no significant difference among these groups, suggesting that Api (no more than 10 μmol/L) was non-toxic to HRMECs (Figure 1A). Then, HRMECs were stimulated by HG for 48h to establish the in vitro DR cell model, with or without treatment of Api. A statistically significant difference existed in cell viability between NG and HG groups, confirming that the cell viability of HRMECs was elevated in response to HG stimulation. Meanwhile, Api at 5 and 10 μmol/L caused an inhibition of the elevated cell viability after HG stimulation (Figure 1B).

Api Prevents HG-Induced HRMECs Migration, Angiogenesis and Vascular Permeability  Then, the effects of Api on HRMECs migration and angiogenesis were examined. The results from Transwell assay revealed that the migrated cells were sharply boosted following HG stimulation. Api treatment remarkably lessened the migrated cells in a concentration-dependent manner (Figure 2A, 2B). Meanwhile, the tube formation assay was carried out to evaluate the effect of Api on angiogenesis of HRMECs in HG environment. As exhibited in Figure 2C, 2D, the junctions of HRMECs were increased following HG exposure, which were partly hindered by Api treatment concentration-dependently, indicating that Api slowed angiogenesis of HRMECs in HG environment. In addition, consistent with previous study that increased vascular permeability is a characteristic alteration in early diabetic microangiopathy, the vascular permeability was also elevated after HG stimulation, while Api could effectively retard this elevation (Figure 2E).

Api Reduces HG-Induced Oxidative Stress and Inflammation in HRMECs  Subsequently, we also evaluate the effects of Api on inflammation and oxidative stress in HG-induced HRMECs. As expected, HG triggered oxidative stress in HRMECs, evidenced by the elevated ROS generation and MDA activity, as well as the reduced GSH-ST activity, whereas Api treatment greatly restricted these alternations in a concentration-dependent manner (Figure 3A–3D). In addition, the excessive production of TNF-α, IL-6, and IL-18 stimulated by HG in HRMECs was also repressed by Api treatment (Figure 3E–3G). Therefore, Api treatment could alleviate oxidative stress and inflammation in HRMECs exposed to HG.

Api Inactivates HG-Stimulated p38 MAPK Signaling via Targeting NOX4 in HRMECs  To explore the potential molecular mechanism of the protection of Api against DR, it was predicted from SwissTargetPrediction website (http://www.swisstargetprediction.ch) that NOX4 was a potential downstream target of Api, which was verified by concentration-dependent reduction of NOX4 expression following Api treatment in HG-induced HRMECs, despite of the elevated NOX4 expression in response to HG stimulation (Figure 4A). In addition, NOX4 overexpression as achieved by transfection with pc-NOX4 (Figure 4B). As NOX4 is a crucial upstream modulator of p38 MAPK signaling\(^{[21]}\), the p38 MAPK signaling was examined. As presented in Figure 4C, the protein expression of p-p38 was remarkably elevated following HG exposure, which was inhibited by Api (10 μmol/L) treatment; however, this inhibition was partially abolished when NOX4 was over-expressed. These findings suggested that the HG stimulation facilitated the activation of p38 MAPK signaling in HRMECs, and Api could weaken this activation partly via downregulating NOX4 expression.

NOX4 and p38 MAPK Signaling Involved in the Protective Role of Api Against HG-Stimulated HRMECs  Eventually, to confirm the involvement of NOX4 and p38 MAPK signaling behind the protective role of Api, HRMECs were transfected with pc-NOX4 to overexpress NOX4 or pre-treated with the p38 MAPK signaling agonist P79350, followed by Api treatment and HG induction. It was observed from Figure 5A–5D...
that the inhibitory effects of Api on the HRMECs migration and tube formation in HG environment were weakened by NOX4 overexpression or P79350 treatment. Meanwhile, the decreased vascular permeability caused by Api was rebounded by NOX4 overexpression or P79350 (Figure 5E). In addition, NOX4 overexpression or P79350 also restrained the protection of Api against oxidative stress and inflammation in HRMECs in the HG environment, as reflected by the elevated ROS generation and MDA activity and the reduced GSH-ST activity, as well as the upregulated production of TNF-α, IL-6, and IL-18 (Figure 6).

**DISCUSSION**

Diabetes is a serious and common chronic metabolic disease, which will lead to multiple diabetes-related vascular complications. It is reported that approximately one-third of people with diabetes will develop DR, mainly accounting for the blindness among working-age individuals. In the present study, we investigated whether Api could play
Figure 3 Apigenin ameliorates retinal endothelial cell damage

Figure 4 Apigenin ameliorates retinal endothelial cell damage via targeting NOX4 in HRMECs

A: HRMECs were stimulated by HG for 48h, with or without treatment of Ap. The protein expression of NOX4 was detected using Western blot. *P<0.001 vs NG; †P<0.001 vs HG. B: HRMECs were transfected with pcDNA3.1 or pc-NOX4, and the expression level of NOX4 was detected by Western blot. *P<0.001 vs pcDNA3.1. C: HRMECs were transfected with pc-NOX4 to overexpress NOX4, followed by Ap treatment and HG induction. The expression level of p-p38 and p38 was examined using Western blot. *P<0.001 vs NG; †P<0.001 vs HG; ‡P<0.01 vs HG+ Api+pcDNA3.1. MAPK: Mitogen-activated protein kinase; Api: Apigenin; HRMECs: Human retinal microvascular endothelial cells; HG: High glucose; NG: Normal glucose; MA: Mannitol; NOX4: NADPH oxidase 4; pc-NOX4: pcDNA3.1-based NOX4 overexpression vector.
a protective role in DR based on HG-induced HRMECs as the in vitro DR cell model, as well as the potential molecular mechanism. The findings from a series of cellular experiments confirmed that Api could ameliorate HG-induced retinal endothelial cell dysfunction by not only preventing HRMECs proliferation, migration, tube formation and vascular permeability, but also reducing oxidative stress and inflammation under HG environment. In addition, the molecular mechanism that underlie these beneficial effects of Api may involve the targeted downregulation of NOX4 and the inactivation of p38 MAPK signaling. These novel findings suggest that Api may be a potential drug for curtailing the progression of DR.

It has been well recognized that increased vascular permeability and neovascularization are main characteristics of DR, which will lead to the loss of blood-retinal barrier and, consequently, irreversible vision loss or even blindness\[^{24-26}\]. Meanwhile, hyperglycemia boosted ROS production in retinal micro-vessels, consequently triggering oxidative stress\[^{27}\]. Oxidative stress has been evidenced as the main target in DR pathophysiology\[^{28}\]. In addition, the excessive accumulation of ROS is a pivotal factor contributing to retinal endothelial cell dysfunction and inflammation processes under hyperglycemia\[^{29}\]. Therefore, vascular permeability, neovascularization, oxidative stress and inflammation are the main research directions for discovering therapeutic strategies for the treatment of DR. For example, Yu et al\[^{30}\] disclosed circ-UBAP2 as a promising therapeutic target for DR as knockdown of circ-UBAP2 relieved HG-induced oxidative stress and vascular dysfunction of HRMECs. Fang and Chang\[^{31}\] proved the protective role of celastrol in HG-induced in vitro DR cell model by inhibiting the proliferation, invasion and angiogenesis of retinal endothelial cells. It was demonstrated by Mei et al\[^{32}\] that growth differentiation factor 11 (GDF11) protected against DR by alleviating retinal cell death, inflammatory reaction and blood-retinal barrier breakdown. Consistently, it was uncovered in this study that Api greatly relieved HG-induced HRMECs migration and angiogenesis, elevated vascular permeability and pro-inflammatory cytokines production, and high ROS generation and oxidative stress, supporting that protective role of Api against DR pathophysiology.

Next, we explored the molecular mechanism that explains the protective role of Api in DR. First, it was predicted that NOX4 and p38 MAPK signaling are involved in the protective role of Api against HG-stimulated HRMECs A, B: HRMECs were transfected with pc-NOX4 to overexpress NOX4 or pre-treated with the p38 MAPK signaling agonist P79350, followed by Api treatment and HG induction. HRMECs migration was examined using Transwell assay. C, D: The tube formation assay was carried out to evaluate the effect of Api on angiogenesis of HRMECs in HG environment. E: The vascular permeability was elevated by Evens blue dye. $^a$P<0.001 vs HG; $^b$P<0.001 vs HG+Api+pcDNA3.1; $^c$P<0.001 vs HG+Api; $^d$P<0.01 vs HG+Api+pcDNA3.1. MAPK: Mitogen-activated protein kinase; Api: Apigenin; HRMECs: Human retinal microvascular endothelial cells; HG: High glucose; NG: Normal glucose; NOX4: NADPH oxidase 4; pc-NOX4: pcDNA3.1-based NOX4 overexpression vector.
was a potential downstream target of Api, which was then verified as Api remarkably suppressed NOX4 expression in HG-induced HRMECs in a concentration-dependent manner. NOX4, one of the most well-studied isoforms of the cytosolic NADPH oxidase, is upregulated during hyperglycemia condition, and is primarily responsible for ROS generation, thereby contributing to the early pathogenesis of DR \cite{33-34}. Inactivating the NOX4 signaling pathway or knockdown of NOX4 has been demonstrated to inhibit vascular permeability and neovascularization in retinopathy and alleviate pyroptosis and inflammation in DR \cite{35-36}. NOX4 interference was also verified to protect visual function in an experimental model of retinal detachment \textit{via} alleviating blood-retinal barrier damage\cite{37}, indicating that inhibition of NOX4 is a strategy for preventing from visual damage. Here, NOX4 expression was expected to be upregulated following HG stimulation. Api exerted an inhibitory effect on NOX4 expression, and the protection of Api against HG-induced endothelial cell damages was partially reversed by NOX4 overexpression, indicating that Api might exert its functions partly through downregulating NOX4 expression.

Furthermore, it is well documented that NOX4 is a crucial upstream modulator of p38 MAPK signaling\cite{21}. p38 MAPK signaling can be activated by numerous cellular stresses, such as hypoxic and oxidative stresses, and regulate multiple extracellularly stimulated processes. It is worthy to note that p38 facilitated retinal micro-angiogenesis and inflammation in DR, and inhibition of p38 MAPK offers a novel therapeutic approach to inhibiting the development of early stages of DR\cite{38-39}. The results of this study support the notion that p38 MAPK signaling was activated under HG environment, in agreement with the previous findings\cite{39-40}. However, Api

**Figure 6 NOX4 and p38 MAPK signaling are involved in the protective role of Api against HG-stimulated HRMECs**

A, B: HRMECs were transfected with pc-NOX4 to overexpress NOX4 or pre-treated with the p38 MAPK signaling agonist P79350, followed by Api treatment and HG induction. ROS generation of each group was determined by DCF-DA method. C, D: The activity of GSH-ST and MDA was measured using their corresponding commercial kits, respectively. E–G: The production of inflammatory cytokines, including TNF-α, IL-6 and IL-18, was examined by ELISA kits. *P*<0.001 vs HG; **P**<0.05, ***P***<0.01, ****P***<0.001 vs HG+Api+pcDNA3.1; ****P***<0.001 vs HG+Api. MAPK: Mitogen-activated protein kinase; Api: Apigenin; HRMECs: Human retinal microvascular endothelial cells; HG: High glucose; NOX4: NADPH oxidase 4; pc-NOX4: pcDNA3.1-based NOX4 overexpression vector; ROS: Reactive oxygen species; GSH-ST: Glutathione S-transferase; MDA: Malondialdehyde; DCF-DA: 2',7'-Dichlorofluorescin diacetate; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.
caused a reduction of the activated p38 MAPK response to HG stimulation. P79350, the agonist of p38 MAPK, partially abrogated Api-mediated protection against HG-induced microvascular dysfunction. Thus, the inactivation of p38 MAPK signaling caused by Api took a part of the responsibility for the protection of Api against DR. Given that NOX4 overexpression facilitated the activation of p38 MAPK signaling, the protective role of Api in DR might be achieved by regulating NOX4/p38 MAPK pathway.

Taken together, our study indicated that Api could effectively alleviate HG-induced microvascular dysfunction through reducing vascular permeability, angiogenesis, oxidative stress and inflammation. In terms of mechanism, NOX4 was a target protein of Api, and Api might exert its protective role against DR through regulating NOX4/p38 MAPK pathway. Our study implies a potential alternative medicine for the treatment of DR.

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