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Apigenin and naringenin ameliorate PKCβII-associated endothelial dysfunction *via* regulating ROS/caspase-3 and NO pathway in endothelial cells exposed to high glucose

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ABSTRACT

Endothelial dysfunction is a key event in the progression of atherosclerosis with diabetes. Increasing cell apoptosis may lead to endothelial dysfunction. Apigenin and naringenin are two kinds of widely used flavones. In the present study, we investigated whether and how apigenin and naringenin reduced endothelial dysfunction induced by high glucose in endothelial cells. We showed that apigenin and naringenin protected against endothelial dysfunction *via* inhibiting phosphorylation of protein kinase C βII (PKCβII) expression and downstream reactive oxygen species (ROS) production in endothelial cells exposed to high glucose. Furthermore, we demonstrated that apigenin and naringenin reduced high glucose-increased apoptosis, Bax expression, caspase-3 activity and phosphorylation of NF-κB in endothelial cells. Moreover, apigenin and naringenin effectively restored high glucose-reduced Bcl-2 expression and Akt phosphorylation. Importantly, apigenin and naringenin significantly increased NO production in endothelial cells subjected to high glucose challenge. Consistently, high glucose stimulation impaired acetylcholine (ACh)-mediated vasodilation in the rat aorta, apigenin and naringenin treatment restored the impaired endothelium-dependent vasodilation *via* dramatically increasing eNOS activity and nitric oxide (NO) level. Taken together, our results manifest that apigenin and naringenin can ameliorate endothelial dysfunction *via* regulating ROS/caspase-3 and NO pathway.

1. Introduction

Diabetes mellitus is characterized by inappropriate hyperglycaemia due to lack of or resistance to insulin. Endothelial dysfunction is a hallmark of diabetes mellitus and obesity, which contributes to cardiovascular diseases [1]. We now know that endothelial dysfunction is a chronic inflammatory process with multiple risk factors, such as hyperglycaemia, dyslipidemia, insulin resistance, atherosclerosis and coronary artery disease [2]. Recently it has been shown that hyperglycaemia is an independent risk factor for augmented morbidity and mortality [3,4]. Vascular endothelial cells prolonged exposure to hyperglycaemia will overproduce reactive oxygen species (ROS) *via* PKCβII activation, then, promote endothelial cell apoptosis, decrease nitric oxide (NO) bioactivity, impair acetylcholine (ACh)-mediated endothelium-dependent relaxation, finally result in endothelial dysfunction [5–8]. It has been established that hyperglycaemia-mediated oxidative stress and apoptosis play vital roles in the development of vascular complications in diabetes, whose early damage marker is endothelial dysfunction [6,9]. Intracellular hyperglycaemia causes serious metabolic dysfunction, because there is no auto-regulation system for adapting high glucose environment. Clinical and experimental studies have shown that high glucose-triggered endothelial dysfunction is a potential mechanism for atherosclerosis and coronary artery disease [10]. Besides, endothelial dysfunction in cardiovascular diseases, which is

Abbreviations: PKCβII, protein kinase C βII; ROS, reactive oxygen species; HUVECs, human umbilical vein endothelial cells; HAECs, human aortic endothelial cells; NF-κB, nuclear factor-κB; NO, nitric oxide; DMSO, dimethyl sulfoxide; ACh, acetylcholine; PE, phenylephrine; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B.

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independent of other known risk factors, is considered to be the principle cause for the initial lesion in the vessel [11]. Consequently, abrogation of endothelial dysfunction is an attractive strategy for preventing diabetic complications.

Apigenin and naringenin are two main constituents of ethyl acetate extract of *Clinopodium chinense (Benth.) O. Kuntze*, which has been used as a folk medicine for diabetes treatment [12,13]. Previous studies have indicated that apigenin and naringenin have multitude pharmacological properties, including antispasmodic [14], anti-inflammatory [15], antioxidant [16], antiatherogenic [17], hypolipidemic [18], and hypoglycemia [19] effects. Meanwhile, due to their cardiovascular protective effects *via* correction of hyperglycaemia and dyslipidemia, apigenin and naringenin have recently gained lots of attention in the cardiovascular research field [2,20]. Yet, there are few reports investigated their effects on endothelial dysfunction induced by high glucose. Hence, in this study, we investigated whether and how apigenin and naringenin protected against high glucose-induced endothelial dysfunction in endothelial cells.

In the present study, we examined the effects of apigenin and naringenin on high glucose-induced PKCβII activation and subsequent imbalance between the ROS/caspase-3 and NO/vasodilation pathway involved in endothelial dysfunction. Furthermore, we examined the effects of apigenin and naringenin on ACh-mediated vasodilation and eNOS-derived NO level in the rat aorta subjected to high glucose challenge. Our study revealed that apigenin and naringenin exerted their protective function mainly *via* inhibition of high glucose-induced oxidative stress and the pro-apoptotic molecular events. Verification and confirmation of their anti-dysfunction activity should be beneficial for the possible application of apigenin and naringenin in the treatment of high glucose-induced endothelial dysfunction in diabetic complications.

2. Materials and methods

2.1. Reagents

Apigenin and naringenin (purity ≥98%) were extracted from the herbs of *C. chinense (Benth.) O. Kuntze* (Putian, China) in our laboratory and dissolved in dimethyl sulfoxide (DMSO, 0.1% v/v). Glucose was purchased from Nanjing Chemical Reagent (Nanjing, China) and dissolved in medium. Monoclonal antibodies were procured from the cited commercial sources: rabbit anti-Akt, anti-p-Akt (Ser473), anti-NF-kB p65, anti-p-NF-kB p65 (Ser536), anti-Bcl-2, anti-Bax, anti-eNOS (4963), anti-p-eNOS (Ser1177) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology (Beverly, MA, USA); anti-PKCβII and anti-p-PKCβII (Ser660) were purchased from Abcam (Cambridge, MA, USA); Goat anti-rabbit IgG (H + L) HRP from Bioworld Technology (St. Paul, MN, USA). The caspase-3 activity assay kit, Bicinchoninic Acid (BCA) Protein Assay kit, DCFH-DA (a fluorescence probe for detection of intracellular ROS production) and DAF-FM DA (a fluorescence probe for detection of intracellular NO production) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). The Annexin V-FITC/propidium iodine (PI) apoptosis detection kit was purchased from KeyGEN Biotech Co., Ltd. (Nanjing, China). 3-(4,5-dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) obtained from Sigma (St Louis, MO, USA) and was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml as stock solution. The kit for determination of NO level was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs, KeyGEN Biotech Co., Ltd., Nanjing, China) and Human aortic endothelial cells (HAECs, Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China) were respectively cultured in low glucose Dulbecco's Modified Eagle Medium (L-DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2 and 95% air. When cells grew to confluence, they were starved in medium containing 2% serum and 33 mM glucose [21,22] to follow our experiment.

2.3. Cell viability assay

HUVECs or HAECs (5×10^3 cells/well) were seeded into a 96-well plate, and cell viability was evaluated by MTT assay. To observe different glucose-mediated HUVECs damage, HUVECs were seeded into a 96-well plate, and incubated with glucose (5.5, 11, 22, 33 and 44 mM) or mannitol (33, 44 mM) for 72 h, respectively. Secondly, HUVECs were cultured with glucose (5.5, 33 mM) for 24, 48 and 72 h, respectively. To examine the protective effects of apigenin and naringenin against HUVECs or HAECs damage induced by high glucose, HUVECs or HAECs were seeded in a 96-well plate, and treated with apigenin (3 or 30 μM) and naringenin (3 or 30 μM) in the presence or absence of high glucose (33 mM) or mannitol (33 mM) for 72 h [13,21]. Then, cells were incubated with MTT solution (0.5 mg/ml) at 37 °C for 4 h and the formazan crystals was dissolved in DMSO. The absorbance measured at 490 nm was used to calculate the relative cell viability ratio and cell viability was normalized to the normal control.

2.4. Measurement of intracellular ROS

DCFH-DA fluorescent probe was used to measure the intracellular ROS generation. HUVECs (5×10^4 cells/well) were seeded in 6-well plates and maintained at 37 °C. When cells grew to confluence, they were co-cultured with apigenin (3 or 30 μM) and naringenin (3 or 30 μM) in the presence or absence of high glucose (33 mM) for 48 h. Then cells were incubated with DCFH-DA (10 μM) for 30 min at 37 °C. After rinsing with cold PBS three times, cells were collected and analyzed immediately by flow cytometer (FACS Calibur, BD Bioscience) [21].

2.5. Analysis of apoptosis by Annexin V-PI staining

HUVECs or HAECs (5×10^4 cells/well) were seeded in 6-well plates and maintained at 37 °C. When cells grew to confluence, they were co-cultured with apigenin (3 or 30 μM) and naringenin (3 or 30 μM) in the presence or absence of high glucose (33 mM) for 48 h. The number of apoptotic cells was analyzed using the Annexin V-FITC apoptosis detection kit, according to manufacturer's instruction. The detection of Annexin V-FITC was performed by flow cytometry analysis (FACS Calibur).

2.6. Cell morphology assay by Hoechst staining

HUVECs (5×10^4 cells/well) were seeded in 6-well plates and maintained at 37 °C. When cells grew to confluence, they were co-cultured with apigenin (3 or 30 μM) and naringenin (3 or 30 μM) subjected to high glucose (33 mM) challenge for 48 h. Morphology of apoptotic cells was detected by nuclear staining with Hoechst 33258. The cells were stained with Hoechst 33258 (10 mg/l) for 20 min at 37 °C. Stained cells were imaged under a fluorescent microscope (Olympus IX81, Tokyo, Japan).

2.7. Assay of caspase-3 activity

Caspase-3 (cell apoptosis execution protease) activity was measured using an assay kit following the manufacturer's instruction. After various treatments with test materials for 48 h, HUVECs or HAECs were lysed in ice-cold lysis buffer. The supernatants were collected and incubated with the substrate acetyl-Asp-Glu-Val-Asp Þ-nitroanilide (Ac-DEVD-ÞNA, 200 mM) for 2 h at 37 °C. Samples were measured at an absorbance of 405 nm.

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