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Puerarin inhibits expression of tissue factor induced by oxidative low-density lipoprotein through activating the PI3K/Akt/eNOS pathway and inhibiting activation of ERK1/2 and NF-κB

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ABSTRACT

Aims: The present study aimed to investigate whether puerarin regulated tissue factor (TF) expression induced by oxidative low-density lipoprotein (ox-LDL), an independent risk factor for atherosclerosis, and its mechanisms.

Main methods: TF expression at the mRNA level was determined by reverse transcription-quantitative polymerase chain reaction, and its expression at the protein level, as well as other target proteins, was assessed by western blotting. Nitric oxide (NO) production was measured by a nitrate reduction method.

Key findings: Results demonstrated that treatment with ox-LDL (50 mg/l) for 24 h significantly increased ($P < 0.01$) TF expression at the mRNA and protein levels in human umbilical vein endothelial cells (HUVECs). Such an ox-LDL exposure also triggered the dephosphorylation of Akt, resulting in a reduction of NO production and activated the extracellular signal-regulated kinase (ERK)1/2 and nuclear factor (NF)-κB signaling pathways. Pre-treatment with puerarin (50–200 μM) for 1 h significantly attenuated the ox-LDL-induced TF expression, augmented the phosphorylation of Akt, with a resultant increase of the NO production, and inhibited the activation of ERK1/2 and NF-κB ($P < 0.01$). However, this beneficial effect of puerarin (100 μM) was abolished by LY294002 (10 μM), an inhibitor of phosphoinositide 3-kinase (PI3K), or NG-nitro-L-arginine methyl ester (100 μM), an inhibitor of NO synthase.

Significance: These results suggested that puerarin suppressed TF expression in HUVECs through activating the PI3K/Akt/endothelial nitric oxide synthase signaling pathway and inhibiting the activation of ERK1/2 and NF-κB. These findings suggested that puerarin possessed certain anticoagulation and may be a potential novel therapeutic drug for thrombosis in coronary artery disease.

1. Introduction

The coagulation feedback triggered by tissue factor (TF) serves a key role in thrombus formation, which is the main cause of acute coronary events [1]. Although TF is normally absent in intact endothelial cells, numerous experiments have indicated that its expression may be induced by certain cardiovascular risk factors [2–5]. Oxidative low-density lipoprotein (ox-LDL), an oxidative product of native LDL in vivo, is believed to participate in atherogenesis, and it exhibits a wide variety of biological properties, including vascular endothelial injury and coagulation disorders [6,7]. Ox-LDL has been identified in atherosclerotic plaques, and TF has also been observed in cracked plaques, leading to fibrin deposition and thrombosis [8]. In fact, ox-LDL has been demonstrated to elicit TF expression, followed by thrombosis formation on atherosclerotic lesions [4,5]. It remains unclear how ox-LDL induces TF expression in endothelial cells. The mitogen-activated protein kinase (MAPK) family [extracellular regulated protein kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK] is the most common signaling pathway regulating the expression of cytokines [9]. C-reactive protein (CRP) [3], ox-LDL [5] and lipopolysaccharide (LPS) [10] have been indicated to elicit endothelial TF expression accompanied by activation of the MAPK pathway and the classic nuclear transcription factor-κB (NF-κB) signaling pathway. However, unlike the MAPK and NF-κB pathways, phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB; also known as Akt) has a negative regulatory role in TF expression of endothelial cells [10,11]. Endothelial nitric oxide synthase (eNOS) may be phosphorylated and activated by Akt, the downstream effector of PI3K, and such activation promotes the production of nitric oxide (NO),

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which is not only a powerful and potent inhibitor of platelet aggregation, but also a depressor of TF expression in human vascular endothelial cells [12].

Puerarin is a primary isoflavone extracted from Chinese medicinal herb Gegen, which is also called “Kudzu” in the West [13]. A number of studies have demonstrated that puerarin has a physiological and pharmacological role in regulating cardiovascular functions, including protection of endothelial cell function [14,15], regulation of vascular tone [16] and inhibition of smooth muscle cell proliferation [17]. The vascular protection and anti-atherosclerotic effects of puerarin are predominantly related to activated PI3K/Akt/eNOS and subsequently increased NO production [15,16,18,19], inhibited NF-κB pathway [14,20] and suppressed ERK1/2 phosphorylation [17]. Researchers have demonstrated that puerarin possesses anticoagulant effects and is able to prolong the prothrombin time of rats with hyperlipidemia [21]. However, the anticoagulant mechanism of puerarin remains unclear. Further studies have displayed that puerarin may reduce TF expression in peripheral blood [22] and macrophages derived from patients with acute coronary syndrome (ACS) [23]. A study by Ran et al. [24] indicated that puerarin may attenuate TF expression induced by angiotensin II in a dose- and time-dependent manner, and its underlying mechanism may be related to reduced nuclear transport of NF-κB. Therefore, in the present study, it was hypothesized that puerarin may inhibit ox-LDL-induced TF expression. To verify this hypothesis, the effect of puerarin on ox-LDL-induced TF expression at the mRNA and protein levels in human umbilical vein endothelial cells (HUVECs) was assessed. Additionally, whether the intracellular signaling pathways, including PI3K/Akt/eNOS, MAPK or NF-κB, participated in the inhibitory effects of puerarin on ox-LDL-induced TF expression were also investigated.

2. Materials and methods

2.1. Reagents

Puerarin was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Ox-LDL was obtained from Shanghai Yisheng Biotechnology Co., Ltd., (Shanghai, China). Rabbit antibodies against human TF, anti-phosphorylated-p38 (p-p38), anti-p38 (T-p38), anti-JNK (T-JNK), anti-p-IκB α, anti-p-p65 and anti-histone H3 were supplied from Abcam (Cambridge, UK). Rabbit anti-p-Akt, anti-Akt (T-Akt) and anti-ERK1/2 (T-ERK1/2) were provided by Cell Signaling Technology, Inc., (Danvers, MA, USA). Rabbit anti-p-ERK1/2 was purchased from Signalway Antibody LLC (College Park, MD, USA). Rabbit anti-p-JNK (p-JNK) and mouse anti-β-actin, as well as all secondary antibodies, were obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). PI3K inhibitor, LY294002, and eNOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), were supplied from Selleck Chemicals (Houston, TX, USA). TRIzol and SYBR Green PCR Master Mix were provided by Invitrogen (San Diego, CA, USA) and Applied Biosystems (Foster City, CA, USA), respectively. TF and β-actin primers were synthesized by Sangon Biotech Co., Ltd., (Shanghai, China).

2.2. Cell culture

A HUVEC line, HUVE-12, was purchased from the Experimental Center, Xiangya School of Medicine (Changsha, China) and cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, GrandIsland, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, GrandIsland, NY, USA) at 37 °C with a 95% humidified atmosphere and 5% CO_2.

2.3. Cell morphology and viability detection

In order to detect the effect of puerarin on the morphological appearance and viability of cells, the cells were plated in 96-well plates (2000 cells/well) and treated with various concentrations of puerarin (25–200 μM) at 37 °C for 26 h. The images were obtained by an inverted light microscope (Nikon Corporation, Tokyo, Japan)(magnification, 20 × 10). Cell viability was determined using a colorimetric MTT assay kit(Sigma-Aldrich, Merck KGaA, Darmstadt Germany). For MTT reduction assays, cells were treated with MTT solution at 37 °C for 4 h. The purple formazan was dissolved with dimethyl sulfoxide, and the optical density(OD) was read at 490 nm(A490).

2.4. Experimental protocols

HUVE-12 cells were starved in medium supplemented with 1% FBS at 37 °C for 24 h and then used in the different sets of experiments. The cells were split into the following three groups: Control, ox-LDL and puerarin. The cells of the control group were cultured only in DMEM medium. The cells of the ox-LDL group were exposed to ox-LDL (50 mg/l) at 37 °C for 24 h. In the puerarin group, the cells were pre-treated with puerarin at various concentrations (25–200 μM) at 37 °C for 1 h before ox-LDL exposure. Following these treatments, TF expression at the mRNA and protein levels was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively.

To investigate whether the PI3K/Akt/eNOS pathway was involved in the suppressive effect of puerarin on ox-LDL-induced expression of TF, the cells were treated with LY294002 (10 μM), an antagonist of PI3K, or L-NAME (100 μM), an antagonist of eNOS, in the presence of puerarin (100 μM) at 37 °C for 1 h and then exposed to ox-LDL (50 mg/l) at 37 °C for 24 h. Subsequently, TF expression at the mRNA and protein levels, the phosphorylation of Akt and NO production were determined. Additionally, the phosphorylation of ERK1/2, JNK, p38 MAPK, IκB and p65 was determined to assess whether the MAPK and NF-κB pathways contributed to the suppressive effect of puerarin on ox-LDL-induced expression of TF.

2.5. RT-qPCR

Total cellular RNA was extracted from HUVE-12 cells with TRIzol reagent, and then reverse transcribed into first strand cDNA(cDNA synthesis kit; Toyobo, Japan). Subsequently, cDNA was used as a template in qPCR. Briefly, amplification was carried out with 40 cycles at a denaturation temperature of 94 °C for 20 s, an annealing temperature of 60 °C for 20 s and an extension temperature of 72 °C for 20 s. Experiments were conducted in triplicate, and the relative gene expression levels were analyzed by the $2^{-\Delta\Delta Cq}$ method [25]. β-Actin was used as an internal control. The sequences of the primers used are listed in Table 1.

Table 1
Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Target gene	Direction	Sequence (5′–3′)	Annealing temperature, °C	Product size, bp
Tissue factor	Forward	GCCAAGATGTACCTGGGCTA	60	220
	Reverse	CATTCATGATCTTGGCGATG		
β-Actin	Forward	CATTAAGGAGAAGCTGTGCT	60	208
	Reverse	GTTGAAGGTAGTTTCGTGGA		