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Original article

Irisin protects macrophages from oxidized low density lipoprotein-induced apoptosis by inhibiting the endoplasmic reticulum stress pathway

Guanlin Zheng [a,b], Haizhen Li [a,c], Tie Zhang [d], Libo Yang [d], Shutong Yao [e], Shihong Chen [a,*], Maochuan Zheng [f], Qin Zhao [g], Hua Tian [e]

[a] Department of Endocrinology and Metabolism, Second Hospital of Shandong University, Ji'nan, Shandong Province, China
[b] Taishan Vocational College of Nursing, Taian, Shandong Province, China
[c] Department of Endocrinology, Dongying City District People Hospital, Dongying, Shandong Province, China
[d] Department of Endocrinology, Taian City Central Hospital, Taian, Shandong Province, China
[e] Key Laboratory of Atherosclerosis in Universities of Shandong and Institute of Atherosclerosis, Taishan Medical University, Taian, Shandong Province, China
[f] Graduate School of Taishan Medical University, Taian, Shandong Province, China
[g] Department 2 of Gastroenterology, Taian City Central Hospital, Taian, Shandong Province, China

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ABSTRACT

Irisin is a newly discovered myokine which can relieve metabolic disorders and resist atherosclerosis. The effects of irisin on ox-LDL-induced macrophage apoptosis and endoplasmic reticulum stress-related pathways were observed *in vitro*. RAW264.7 macrophages were cultured *in vitro* and pretreated with irisin at 20, 40 and 80 ng/ml for 30 min, followed by culture with 100 mg/L ox-LDL and 5 mg/L tunicamycin (TM) for 12 h. The cell viability and apoptosis were detected by MTT assay and annexin V-FITC double staining. The nuclear translocation of activating transcription factor 6 (ATF6) was detected by immunofluorescence assay. Western blot was used to detect the expressions of p-PERK, p-eIF2α, C/EBP homologous protein (CHOP) and Bcl-2. Irisin reduced lipid accumulation in macrophages in a concentration-dependent pattern and significantly inhibited apoptosis induced by ox-LDL and TM. Compared with ox-LDL and TM groups, the expressions of CHOP, p-PERK and p-eIF2α in the irisin group significantly decreased, the translocation of ATF6 from cytoplasm to nucleus was significantly weakened, and Bcl-2 expression significantly increased. Irisin can alleviate the apoptosis of macrophages induced by ox-LDL, which may be achieved by inhibiting the PERK/eIF2α/CHOP and ATF6/CHOP endoplasmic reticulum stress signaling pathways.

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1. Introduction

Atherosclerosis is characterized by the formation of lipid-rich atherosclerotic plaques in the vessel wall. The deposition of lipoprotein rich in apolipoprotein E to the vessel wall leads to endothelial cell dysfunction which causes the adhesion and migration of mononuclear cells to subcutaneous tissues. Mononuclear cells differentiate into macrophages which can phagocytose oxidized low density lipoprotein (ox-LDL) to form foam cells. The accumulation of ox-LDL in macrophages and oxidative stress can promote the apoptosis of macrophages and increase the fragility of plaques. The thrombosis caused by fragile plaque rupture is mainly responsible for acute cardiovascular events (Finn et al., 2010).

Endoplasmic reticulum stress (ERS) is an important pathway for macrophage apoptosis induced by ox-LDL (Scull and Tabas, 2011). Excessive or prolonged ERS can activate unfold protein response (UPR). Protein kinase RNA-like ER kinase (PERK), inositol requiring protein 1α (IRE1 α) and activating transcription factor 6 (ATF6) are considered to be three branched channels involved in UPR initiation. The activation of PERK and ATF6 can induce the expression of CHOP (C/EBP homologous protein), and IRE1 α can activate c-Jun amino-terminal kinase (JNK). Both JNK and CHOP can inhibit the expression of anti-apoptotic factor Bcl-2 and activate the

* Corresponding author.
E-mail address: chenshshsu@foxmail.com (S. Chen).

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calcium signaling pathway, causing macrophage apoptosis (Tabas and Ron, 2011).

Irisin is a hormone that is mainly secreted by muscle cells, which was reported for the first time in 2012 (Bostrom et al., 2012). It can increase energy consumption by promoting white fat browning, and relieve a variety of metabolic disorders, including obesity, insulin resistance, hyperglycemia and hyperlipidemia (Bostrom et al., 2012; Zhang et al., 2014; Xiong et al., 2015). Further *in vitro* and animal studies have demonstrated that irisin may have direct therapeutic effects on atherosclerosis. It can also inhibit high glucose-induced endothelial cell apoptosis, promote endothelial cell proliferation, and alleviate endothelial cell dysfunction (Song et al., 2014; Fu et al., 2016a; Zhang et al., 2016; Zhu et al., 2016). Lu et al. (2015) found using a model of diabetic mice with apolipoprotein E knockout that irisin treatment reduced the atherosclerotic plaque area, inflammatory cell infiltration on the vascular wall and inflammatory factor expression. In humans, irisin has been found in the paraventricular hypothalamic nucleus and cerebrospinal fluid, but its role in the central nervous system remains unclear, probably participating in the differentiation of neurons and motor coordination (Dun et al., 2013). Injecting the third ventricle of rats with recombinant irisin can activate neurons in the paraventricular hypothalamic nucleus, thereby elevating blood pressure and enhancing myocardial contraction (Wrann et al., 2013). Injecting irisin into the central nervous system also increases the activity and oxygen consumption of rats (Zhang et al., 2015).

However, the effects of irisin on macrophage apoptosis or ERS have never been evaluated hitherto. Therefore, this study aimed to explore the therapeutic effects of irisin on atherosclerosis by investigating the influence of irisin on ox-LDL-induced macrophage apoptosis, ERS and ERS-related signaling pathways.

2. Materials and methods

2.1. Materials

Mouse RAW264.7 macrophages were purchased from Shanghai Institute of Biochemistry and Cell Biology (China). Irisin was bought from Phoenix Pharmaceuticals (USA). MTT, oil red O, tunicamycin (TM) and rabbit anti-β-actin monoclonal antibody were obtained from Sigma (USA). Rabbit antibodies against CHOP, Bcl-2, p-PERK and p-eIF2α were provided by Santa Cruz (USA). Rabbit anti-ATF6 polyclonal antibody was purchased from Abcam (USA). Goat anti-rabbit IgG and ready-to-use SABC-Cy3 immunohistochemical kit were bought from Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd. (China) and Wuhan Boster Biological Technology Co., Ltd. (China) respectively. DMEM high-glucose culture medium and fetal bovine serum were provided by Gibco (USA). RIPA lysis solution and BCA protein quantification kit were purchased from Solarbio (USA). Annexin V-FITC apoptosis detection kit was bought from Nanjing Keygen Biotech. Co., Ltd. (China). Trizol reagent was obtained from Invitrogen (USA). cDNA synthesis kit and Real Master Mix (SYBR Green) kit were provided by Tiangen Biotech (Beijing) Co., Ltd. (China). ECL kit and PVDF membrane were purchased from Pierce (USA) and Millpore (USA) respectively. Other reagents were all analytically pure.

2.2. LDL separation and oxidation

Ox-LDL was prepared according to the method described previously (Yao et al., 2013). First, fresh human plasma LDL was isolated by serial ultracentrifugation, dialyzed with PBS without EDTA for 48 h, then incubated in PBS containing 10 μmol/L $CuSO_4$ (pH 7.2) at 37 °C for subsequent 18 h of dialysis, and finally dialyzed in PBS containing 100 μmol/L EDTA at 4 °C for 24 h. After sterile filtration, the protein was quantified using BCA reagent, and the protein concentration was adjusted to 1 g/L with PBS and stored at 4 °C. The concentration of the thiobarbituric acid reactive substance of MDA was measured (>30 μmol/g). Agarose gel electrophoresis showed that the electrophoretic mobility of ox-LDL was increased 2- to 2.5-fold that of LDL.

2.3. Cell culture and experimental grouping

RAW264.7 macrophages with normal growth were cultured in DMEM high glucose medium (containing 10% fetal bovine serum, penicillin 100 U/mL, streptomycin 100 μg/mL) to a cell density of 1×10^8 cells/L, and placed in a 5% CO_2 incubator at 37 °C. The cells were transferred to DMEM containing 0.1% fetal bovine serum 24 h before treatment.

Experimental grouping (1): They were randomly divided into a control group: routinely cultured in the culture medium; an ox-LDL group: 100 mg/L ox-LDL was added to the culture medium; and irisin intervention groups: irisin (20, 40 and 80 ng/ml) was added to the culture medium for pretreatment for 30 min, followed by 100 mg/L ox-LDL. Except the irisin pretreatment group, other groups were added with dimethyl sulfoxide (DMSO) with volume fraction of 0.1%, and cultured for 24 h.

Experimental grouping (2): They were divided into a control group: DMSO with volume fraction of 0.1% for conventional culture; a TM group: the culture medium was added with 5 mg/L TM; and an irisin intervention group: 80 ng/ml irisin was added to the culture medium for pretreatment for 30 min, followed by 5 mg/L TM. The cells were collected 12 h after treatment.

2.4. Observation of intracellular lipid droplet changes by oil red O staining

The cells were seeded in 6-well culture plates (built-in sterile cover slips). After treatment, the cells were rinsed with PBS, fixed with 4% calcium formaldehyde solution, stained with oil red O for 15 min, counterstained with hematoxylin for 3 min, and then observed under Olympus BX51 microscope. Intracellular lipid was red and the nucleus was blue. Five fields were randomly selected for each cover slip, and the results were analyzed by Image-Pro Plus 6.0 (Media Cybernetics) software. The cell mean integrated absorbance (IA) was used to express the content of intracellular lipid droplet. The experiment was repeated three times, two repeated wells for each group each time (Yao et al., 2013).

2.5. Detection of cell viability by MTT assay

The cells with normal growth were cultured in 96-well culture plates at a density of 1×10^4/L. After treatment, the cells were added MTT (final concentration of 0.5 g/L) and continued to be incubated for 4 h in the incubator. The supernatant was discarded, and 200 μL of DMSO was added to each well. The optical density (OD) of each well was measured at 490 nm using the Infinite F200 Multifunctional Microplate Reader (Tecan, Switzerland). With the cell viability of the control group as 100%, the viability of the other groups was expressed as a percentage of OD to that of the control group.

2.6. Detection of cell apoptosis by flow cytometry

The cells of each group were collected, with the density adjusted to 5×10^5/mL at 4 °C, centrifuged at 1000 r/min for 10 min, and then rinsed twice with ice-cold PBS. The supernatant was discarded and the cells were resuspended with 100 μL of binding buffer. Subsequently, 5 μL of annexin V-FITC and 5 μL of

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