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Full paper

Mangiferin inhibits high-fat diet induced vascular injury via regulation of PTEN/AKT/eNOS pathway

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ABSTRACT

Mangiferin (MAN), a naturally occurring polyphenol commonly found in mango and papaya. However, little is known its anti-vascular injury effects and the underlying mechanisms. This paper investigated the anti-vascular injury effect of MAN and the mechanisms in high-fat diet (HFD)-induced C57BL/6J mice and oxidized low-density lipoprotein (ox-LDL) induced human umbilical vein endothelial cells (HUVECs). The levels of plasma lipid, inflammatory factors and nitric oxide (NO) in mice were evaluated. The expression levels of PI3K, AKT, eNOS, PTEN and their phosphorylated proteins were measured by western blots. In addition, the PTEN-siRNA HUVECs were also used. The result showed that MAN markedly decreased the plasma lipid, inflammatory level in HFD-induced vascular injury mice respectively. Furthermore, MAN alleviate ox-LDL-stimulated dysfunction of HUVECs, restored the diminished NO release, decreased the ROS generation, significantly increased the expression of p-Akt, p-eNOS, and decreased the expression of PTEN, but have no effect on PI3K. However, the protective effects of MAN were significantly reduced by co-treatment with PI3K inhibitor or abolished by eNOS inhibitor. In addition, MAN has no protective effect on ox-LDL induced PTEN-siRNA HUVECs injury. Collectively, MAN appeared to alleviate ox-LDL-stimulated dysfunction of HUVECs via the PTEN/Akt/eNOS signaling pathway, thus decrease vascular injury in HFD-administrated mice.

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1. Introduction

Obesity often contributes to cardiovascular disease such as hypertension, heart disease, and vascular diseases, resulting in unpreventable death.[1,2] Obesity can lead to disorder of lipid metabolism and cause vascular endothelial cells dysfunction.[3] Vascular endothelial cell damage can lead to abnormal proliferation of vascular smooth muscle cells, cause vascular wall thickening, and eventually cause hypertension and atherosclerotic disease.[4] Endothelial dysfunction which is characterized by reduced activity of endothelial nitric oxide synthase (eNOS) and bioavailability of nitric oxide (NO), plays a key role in the pathogenesis of obesity induced cardiovascular complications.[5,6]

Mangiferin (MAN), 1, 3, 6, 7-tetrahydroxyxanthone-C2-b-Dglucoside, a naturally occurring polyphenol commonly found in both mango and papaya is a natural immunomodulator.[7] Mangiferin exerts anti-diabetic action with the ability to improve lipid profiles in experimental animals, demonstrating well its beneficial effects on metabolic homeostasis.[8,9] However, the effects of mangiferin on diet-induced vascular injury were still unclear. Furthermore, the detailed molecular mechanisms underlying the cardiovascular protection effects of mangiferin are not completely understood. So purpose of this study is thus to define the functional and the protection effects of mangiferin in diet-induced vascular injury model, the mechanism was also discussed.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine plasma (FBS) were purchased from Gibco-BRL (NY, USA). The assay

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kits of LDH (Catalog No: A02-2) and NO (Catalog No: A013-2) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, CHN). Reactive oxygen species (ROS) (Catalog No: E004), TNF-α(Catalog No: PT518), IL-6 (Catalog No: PI330), IL-10 (Catalog No: KLC009) assay kit were purchased from Beyotime Biotechnology(Shanghai, CHN). Mangiferin, LY294002, N-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. Anti-eNOS phosphor-S1177 (ab184154); Anti-eNOS(ab5589); Anti-pan-AKT (ab8805); Anti-PTEN (ab32199); Anti-PI3K (ab191606), host animal: rabbit, these antibody were produced by Abcam,UK. Anti-Phospho-PI3K PY-458 (#4228); Anti-Phospho-Akt (Ser 473) (#4060), host animal: rabbit, these antibody were produced by Cell Signaling Technology, USA; GAPDH (AT0002,CMCTAG, USA).

2.2. Animals and diets

Male C57 BL/6 J, weighing 18–22 g, were obtained from Hebei Medical University. The Principles of Laboratory Animal Care (NIH Publication 85e23, revised 1996) were followed. Animal study protocols were approved by the Ethics Committee of Hebei Medical University. housed under controlled environment (22 ± 2 °C, 12 h light/dark cycle, free access to food and water). Mice were fed with high-fat diet (HFD, 5062 kcal; carbohydrate/protein/fat/fiber = 25.3:23:35:6.6; Oriental Yeast Co., Ltd.) for 12 weeks. To study the protected effect of MAN on the HFD induced vascular injury, Mice were randomly divided into five groups (n = 12 for each group), There were groups of control, model, and mice were fed with high-fat diet (HFD) mixed with MAN (5 mg/kg, 20 mg/kg) or atorvastatin (2.5 mg/kg). Dosages of MAN applied in this study were calculated based on the Food and Drug Administration (FDA) Draft Guidelines. The mice were fasted for 12 h before the experimental procedures.

2.3. Determination of plasma total-cholesterol, triglyceride, HDL cholesterol, LDL cholesterol

Plasma lipids level of triglycerides (TG), total-cholesterol (TC), high density lipoprotein-cholesterol (HDLc) and low density lipoprotein cholesterol (LDLc) were measured using the corresponding commercial kits (Biolabo, France). The blood sample was transferred into a 1.5 mL tube and kept in room temperature for 2 h, and then centrifuged for 5 min at 2000 g, then loaded onto an automatic biochemical analyzer (Hitachi, Tokyo, Japan) to complete the assay.

2.4. Analysis of abdominal aorta

Abdominal aorta samples were obtained after mice were sacrificed at the end of experiment. The abdominal aortas were fixed in a 4% formalin solution, followed by sequential dehydration (70% ethanol, 100% ethanol, and acetone), xylene clearance, and paraffin embedding. The sliced aortas were stained with Harris hematoxylin and eosinY (H&E).

2.5. Cell culture and cell transfection

The human umbilical vein endothelial cells (HUVECs) were purchased from China infrastructure of cell line resources. The cells were cultivated in DMEM medium with 10% heat-inactivated fetal bovine plasma (Gibco-BRL NY, USA), penicillin (100 U/ml), and streptomycin (100 μg/mL) and cultured at 37 °C in a humidified atmosphere with 5% CO_2.

PTEN-siRNA HUVECs: The HUVECs cells were transfected with PTEN siRNA, (PTEN-3′-UTR-WT, F: 5′-TTGTGGCAACAGATAAGTT TGCAGTTGGCTAAGA GAGGTT-3′R: 5′-CATTCCCCTAACCCGAATA CATGCATTAGAATGTAGCAAA-3′; PTEN-3′-UTR-MT, F: 5′-TTGTG GCAACAGCTGAATCTGCAGTTGGCTAAGAGAGGTT-3′R: 5′-ATGTA GCAAAACCCTTCGGAAACCTCTCTTAGCCAACTGC-3)

Cells were seeded in 25 cm^2 culture flask and grew in complete medium to the density of 30–50%. Plasmid at a final concentration of 30 nM was accomplished with lipofectamine 2000 according to the manufacturer's protocol. After 48 h of siRNA transfection, the cells were harvested for further studies.

2.6. Cellular viability assay

HUVECs were plated at a density of 1.0×10^4 cells/well in a 96-well plate. The cells were pretreated with MAN (5, 10, and 20 μM) for 12 h, and then treated with 100 μg/mL of ox-LDL for 24 h. These cells were then stained with 20 μl of 5 mg/mL MTT (Sigma–Aldrich) per well and incubated for 4 h at 37 °C. Continuously, the intracellular MTT purple formazan was solubilized with 150 μl of DMSO (Life, USA). The absorbance was detected at optical density(OD) 490 nm with a universal microplate reader (Mpro200, Tecan, USA). Each experiment was performed three times.

LDL (Yiyuan Biotechnology Co., Ltd., Guangzhou, China) at 1 mg/mL^{-1} was incubated 24 h at 37 °C with oxidants: $CuSO_4$ at 10 μM. The oxidation was stopped by the addition of 1% EDTA. The extent of lipid peroxidation was determined by the TBARS method, and the values are expressed as nM of MDA.

2.7. Cytokine/chemokine assay

Levels of TNF-α, IL-6, IL-10, LDH in plasma and the culture medium were determined using the corresponding ELISA kit according to the manufacturer's instruction.

2.8. Measurement of NO release

Nitrite and nitrate levels in the plasma and cell supernatants are commonly considered as an indicator of NO production. The levels of nitrite and nitrate were determined by an assay kit according to the manufacturer's instruction. The optical density values (OD) were determined at 550 nm with a microplate reader (Mpro200, Tecan, USA).

2.9. Assay of ROS levels

The intracellular ROS levels were determined using 2–,7-dichlorofluorescin diacetate (DCFH-DA), a peroxide-sensitive fluorescent probe, according to the manufacturer's instructions. Briefly, the treated cells were harvested, washed with PBS and loaded with 10 mM DCFH-DA in plasma-free DMEM in the dark for 30 min at 37 °C. Fluorescence was observed under an inverted fluorescence microscope (Olympus, Japan) and detected on a microplate reader (Mpro200, Tecan, USA).

2.10. Western blotting

Cells were harvested after treatment and lysed with lysis buffer on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The protein samples were separated on SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membrane. Membranes were blocked with TBST buffer containing 5% free fat milk for 1 h, and then incubated with specific primary antibodies overnight at 4 °C PI3K (1:1000), pY458-

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