European Journal of Pharmacology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Anti-atherosclerotic effect of hesperidin in LDLr$^{-/-}$ mice and its possible mechanism

Ye-Zi Sun[a,*], Jian-Fei Chen[b], Li-Min Shen[a], Ji Zhou[a], Cui-Fang Wang[c]

[a] Department of Endocrinology, Zhang Jia Gang First People's Hospital, Zhang Jia Gang, People's Republic of China
[b] Department of Traditional Chinese Medicine, Zhang Jia Gang First People's Hospital, Zhang Jia Gang, People's Republic of China
[c] Department of Endocrinology, Affiliated Hospital of Nantong University, Nantong, People's Republic of China

ARTICLE INFO

Keywords:
Hesperidin
Atherosclerosis
Hepatic steatosis

ABSTRACT

Hesperidin, a citrus bioflavonoid, exerts numerous pharmacological activities. However, its protective effect against atherosclerosis in vivo remains poorly understood. In the present study, we aimed to observe the effects of hesperidin on high fat diet (HFD)-induced atherosclerosis using LDL receptor deficient (LDLr$^{-/-}$) mice. After 12 weeks of treatment, the animals were sacrificed. The blood samples were collected for further analysis. Mouse peritoneal macrophages were collected. Hepatic lipid content, quantification of atherosclerosis, assessment of oxidative stress and inflammation, gene expressions were performed on liver and aorta samples. The data showed that hesperidin ameliorated HFD-induced weight gain, improved insulin resistance and ameliorated hyperlipidemia. Hesperidin suppressed HFD-induced hepatic steatosis, atherosclerotic plaque area and macrophage foam cell formation. Further study showed that hesperidin down-regulated expressions of acetyl coenzyme A carboxylase alpha (ACCα) and fatty acid synthase (FAS) which are two key enzymes in fatty acid and triglyceride synthesis in liver; and upregulated expression of hepatic ATP-binding cassette transporters G8 (ABCG8), macrophage ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1) which are transporters involved in the process of reverse cholesterol transport. Hesperidin also reduced oxidative stress by normalizing activities of antioxidant enzymes and inflammation in HFD-fed LDLr$^{-/-}$ mice. These findings suggest that hesperidin reduced atherosclerosis via its pleiotropic effects, including improvement of insulin resistance, amelioration of lipid profiles, inhibition of macrophage foam cell formation, anti-oxidative effect and anti-inflammatory action.

1. Introduction

Atherosclerosis is a chronic vascular disease process that is characterized by focal narrowing and thickening of the lumen of blood vessels due to the formation of lipid-laden plaques in the luminal surface of arterial wall (Weber et al., 2017). Dyslipidemia, oxidative stress and persistent inflammation are well-known risk factors in the development of atherosclerosis (Hurtubise et al., 2016). In addition, atherosclerosis is usually associated with other metabolic disorders such as insulin resistance and fatty liver (Xu et al., 2015; Zeadin et al., 2013). Insulin has been reported to induce forceful effects on lipid accumulation as well as expressions of genes involved in lipogenesis (Zeadin et al., 2013). The liver plays a critical metabolic role in regulating lipid homeostasis through many processes including uptake of cholesterol from the peripheral tissues, synthesis of cholesterol and triglycerides, and excretion of cholesterol into the bile. Hepatic dysfunction can accelerate atherosclerosis via deterioration of dyslipidemia, imbalanced redox status and increased levels of circulating proinflammatory factors (Xu et al., 2015; Targher et al., 2010). Thus, manipulation of hepatic lipid metabolism, oxidative stress and inflammation is a commonly pursued strategy to inhibit the progression of atherosclerosis.

Reverse cholesterol transport (RCT) is a cholesterol metabolism pathway ensuring the effux of cholesterol from lipid-laden macrophages in the artery wall and its transport back to the liver for eventual excretion (Favari et al., 2015). RCT is believed to be an important defense mechanism in atherogenesis. Cholesterol eflux from the macrophage is the first step in RCT, which is mediated by various membrane transporters, such as ATP-binding cassette transporters G1 and A1 (ABCG1 and ABCA1) on the membrane of the macrophage. ABCG1 mediates the effux of cholesterol and phospholipids to HDL particles, while ABCA1 induces cholesterol effux to lipid-poor apolipoprotein A-I (apoA-I). After uptaking of HDL/apoA-I-accociated cholesterol or cholesterol ester into hepatocytes by hepatic scavenger receptor class B type I (SR-BI), secretion of cholesterol and bile acids into the bile is

* Corresponding author.
E-mail address: sunyezi1976@163.com (Y.-Z. Sun).

http://dx.doi.org/10.1016/j.ejphar.2017.09.010
Received 20 June 2017; Received in revised form 30 August 2017; Accepted 8 September 2017

regulated by the respective hepatic transporters ABCG5 and ABCG8 (Zhang et al., 2016).

Hesperidin, a specific flavonoid glycoside, can be isolated in large amounts from the rinds of some citrus species. Preclinical studies and clinical trials have demonstrated hesperidin has anti-inflammatory, anti-oxidant, lipid-lowering and insulin-sensitizing properties, contributing to its therapeutical effects in various diseases (Li and Schluesener, 2017; Mulvihill et al., 2016). But, until now, no study has reported the direct effect of hesperidin on atherosclerosis using LDL receptor deficient ($LDLr^{-/-}$) mice, which is a well established animal model with many characteristics of the metabolic syndrome. Thus, the aim of the present study was to determine the potential effects of hesperidin on atherosclerosis and its associated metabolic disorders in $LDLr^{-/-}$ mice, and also explore the possible mechanisms involved.

2. Materials and methods

2.1. Animal experiment

Male $LDLr^{-/-}$ mice on C57BL/6JNju were purchased from Nanjing Biomedical Research Institute of Nanjing University. All procedures were approved by the animal care and use committee of Nantong University. The mice were housed under standard laboratory conditions with a 12-h light/dark cycle and free access to water and food. Starting from 8 weeks, the mice were randomly separated into four groups: a control group, a high fat diet (HFD) model group, 100 mg/kg/day hesperidin-treated group and 200 mg/kg/day hesperidin-treated group. The dose of hesperidin used in this study was based on previous reports of its beneficial pharmacological effects in animals with different disease (Jung et al., 2004; Mahmoud et al., 2012; Çetin et al., 2016). Hesperidin (purity > 98%) was provided by XI'AN natural field biotechnioue Co., LTD, China. The control group was fed a standard chow diet. The HFD group was given a high fat diet containing 21% fat and 0.21% cholesterol (D12079B, Open Source Diets, Research Diets, Inc). The two hesperidin treatment groups were given the same high fat diet and dosed daily via intragastric gavage with 100 and 200 mg/kg/day hesperidin by weight for 12 weeks. Hesperidin was suspended in 0.5% carboxymethyl cellulose (CMC). Mice in control and HFD model group received the same volume of 0.5% CMC vehicle gastrically. At the end of the study, animals were fasted overnight and were sacrificed. The blood samples and tissues were collected for further analysis.

2.2. Biochemical measurements

Fasting insulin was determined using a commercial mouse insulin elisa kit (Millipore, MA). Fasting glucose were measured with a multifunctional biochemistry analyzer Olympus AU2700 (Olympus, Tokyo, Japan). Insulin resistance was evaluated by homeostasis model assessment (HOMA) index as described previously for mice (Mulvihill et al., 2009). Serum high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), triglyceride (TG) and total cholesterol (TC) were detected using the biochemical kits (Zhongsheng Bio-tech Co., Ltd, Beijing, China). Activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured with enzymatic colorimetric assays using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum levels of ox-LDL TNF-α and IL-6 were measured using elisa kits (Shanghai Westang Biotechnology Co. Ltd, Shanghai, China).

2.3. Determination of hepatic lipid content

For Oil Red O staining, frozen liver tissues were embedded in Tissue-Tek OCT compound, sectioned at 8 μm, and stained with Oil Red O (Sigma, St. Louis, USA) to detect lipid deposition. For hepatic lipid content measurement, total lipids were extracted from frozen hepatic tissue (approximately 50 mg) according to the modified Folch method (Folch et al., 1957). Triglyceride and total cholesterol concentrations were determined using the commercial enzyme kits (Zhongsheng Biotech Co., Ltd, Beijing, China) and were normalized to liver protein content.

2.4. Quantification of atherosclerosis

Atherosclerotic lesion severity was assessed by *en face* analysis of the aorta as previously described (Liu et al., 2017). In brief, the ascending aorta and thoracic aorta were opened longitudinally, and fixed with 4% paraformaldehyde and then stained with Oil Red O (Sigma, St. Louis, MO). Lesion and total areas were determined using Image-Pro Plus software 6.0.

2.5. Isolation of mouse peritoneal macrophages and studies of macrophage foam cell formation

In a separate study, after 12 weeks, male $LDLr-/-$ mice fed with standard chow, HFD or HFD with hesperidin as described above were injected intraperitoneally with 1 ml of 10% thioglycolate. Three days later, Isolation of mouse peritoneal macrophages was performed as described previously (Mo et al., 2014). The cells were resuspended in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin for use in subsequent experiments. Upon fixation with paraformaldehyde (4%), the peritoneal macrophages from mice were stained with 0.5% oil red O and hematoxylin was used as counterstaining. After alcohol extraction, density of the total cellular neutral lipid content was determined by a microplate reader (absorbance at 540 nm). The intracellular cholesterol was quantified using the Amplex Red cholesterol assay kit (Invitrogen, CA, USA) and were normalized to cell total protein which was determined by the BCA protein assay (Beyotime Biotech, China).

2.6. Cholesterol efflux assay

Peritoneal macrophages from untreated $LDLr^{-/-}$ mice at 8 weeks old were equilibrated with 22-NBD-cholesterol (Life Technologies, Carlsbad, CA, USA) (1 μg/ml) for 24 h. Then cells were washed with PBS and incubated with 0.1, 1 and 10 μM of hesperidin for an additional 18 h. 50 μg/ml HDL (Luwen Biotechnologies, China) or ApoA1 (Sigma, USA) was added as the receptor protein to start the efflux experiment for 6 h. The fluorescence-labeled cholesterol released from cells into the medium was measured using a microplate reader (synergy H1, BioTek, USA). Cholesterol efflux was expressed as a percentage of fluorescence in the medium relative to the total amounts of fluorescence detected in cells and the medium.

2.7. RNA analysis

mRNA expressions of targeted genes were quantified by real-time PCR with forward and reverse primers (Supplementary Table 1). Total RNA was extracted using Trizol reagent and was reverse-transcribed into cDNA using the PrimeScript RT Master Mix Kit (Takara, TaKaRa Biotechnology, Dalian, China). The real-time PCR were carried out on StepOne Plus Real-Time PCR System (Applied Biosystems) using SYBR Green PCR master mix (Applied Biosystems) with the resulting cDNAs. Housekeeping gene 18 s was used as a reference. Data were analyzed using the cycle threshold (Ct) method.

2.8. Western blot analysis

Equal amounts of protein were separated and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, USA). After blocking, the membranes were incubated with the first antibodies (all obtained from Abcam, USA) overnight. And then the membranes were incubated with the appropriate IRDye 680RD secondary antibodies (1:10000) in