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Dihydromyricetin ameliorates atherosclerosis in LDL receptor deficient mice

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ABSTRACT

Background and aims: Dihydromyricetin, the most abundant flavonoid in *Ampelopsis grossedentata*, exerts numerous pharmacological activities, including anti-inflammatory, antioxidant, hepatoprotective, and lipid regulatory activities; however, its protective effect against atherosclerosis remains poorly understood. The aim of the present study was to evaluate the effects of dihydromyricetin on high fat diet (HFD)-induced atherosclerosis using LDL receptor deficient ($LDLr^{-/-}$) mice.

Methods: Blood samples were collected for determination of serum lipid profiles, oxidized LDL (ox-LDL) and pro-inflammatory cytokines. Histology, hepatic lipid content, quantification of atherosclerosis, assessment of oxidative stress and inflammation were performed on liver and aorta samples by molecular biology methods. The effects of dihydromyricetin on ox-LDL-induced human umbilical vein endothelial cells (HUVECs) dysfunction and foam cell formation were further studied.

Results: (1) Dihydromyricetin ameliorated hyperlipidemia, reduced serum ox-LDL, IL-6 and TNF-α levels in HFD-fed $LDLr^{-/-}$ mice. Moreover, (2) dihydromyricetin suppressed hepatic lipid accumulation and increased protein expressions of PPARα, LXRα and ABCA1. (3) It inhibited atherosclerotic lesion formation and favoured features of plaque stability. (4) Dihydromyricetin prevented hepatic and aortic inflammation as evidenced by the reduced *IL-6* and *TNF-α* mRNA expression; (5) it prevented hepatic and aortic oxidative stress by normalizing activities of antioxidant enzymes in the liver and suppressing reactive oxygen species generation and NOX2 protein expression in both liver and aorta; (6) it inhibited oxLDL-induced injury, monocytes adhesion and oxidative stress in HUVECs and (7) inhibited macrophage foam cell formation and enhanced cholesterol efflux.

Conclusions: These findings suggest that dihydromyricetin could reduce atherosclerosis via its pleiotropic effects, including improvement of endothelial dysfunction, inhibition of macrophage foam cell formation, amelioration of lipid profiles, anti-inflammatory action and anti-oxidative effect.

1. Introduction

Atherosclerosis is a vascular disease characterized by accumulation of lipids in the arterial wall. The development of atherosclerosis is a complex cascade, in which an altered lipid metabolism, oxidative stress and persistent inflammation play predominant roles [1]. One critical event in the initiation of atherosclerosis is endothelial cell activation with the expression of adhesion molecules within the vessel wall by various stimuli, including high levels of reactive oxygen species (ROS), oxidized low density lipoprotein (ox-LDL) and pro-inflammatory cytokines. Subsequently, it promotes adherence of circulating monocytes to the vascular endothelium and their transmigration into the intima, which further recruit more inflammatory cells to the site of injury, and finally, form atherosclerotic plaque with its lipid-rich core [2,3]. The uptake of modified LDL by macrophages in the subendothelial arterial space leads to the formation of foam cells, which also are a key determinant of atherosclerotic lesion occurrence [4]. In addition, atherosclerosis is usually associated with other metabolic disorders such as fatty liver. The liver plays a central role in regulating the homeostasis of lipid metabolism and redox status. Hepatic dysfunction could promote the development of atherosclerosis via deterioration of dyslipidemia and systemic inflammation [5,6].

Natural products are important sources of therapeutic agents

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and are also good lead compounds suitable to further modification during drug development. Epidemiological studies suggest that increased consumption of dietary flavonoids can contribute to beneficial effects on the cardiovascular system [7]. Dihydromyricetin (DMY), also known as ampelopsin, is the most abundant flavonoid in vine tea, which has been used as herbal medicine or health tea (Tengcha) for the prevention and treatment of hypertension, common colds, sore throat, and jaundice hepatitis for hundreds of years in Asia [8]. DMY has been reported to exert numerous pharmacological activities, including anti-inflammatory, antibacterial, antioxidant, hepatoprotective, lipid and blood glucose regulatory and anti-carcinogenic effects [8–12]. Li et al. reported constant drinking of vine tea can regulate hyperlipidemia rat serum lipid and lipoprotein and clean oxygen free radical [13]. A recent study has demonstrated that DMY decreases the serum total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-c) content, increases the high-density lipoprotein cholesterol (HDL-c) level in high fat diet (HFD)-fed rats [8]. Williams et al. reported that DMY prevents formation of plaque in aortas of ApoE knockout mice fed a western diet [14]. A randomized controlled trial reported that dihydromyricetin supplementation improves glucose and lipid metabolism and exerts anti-inflammatory effects in patients with nonalcoholic fatty liver disease [15]. Together, these data led us to the hypothesis that DMY may be able to improve atherosclerosis and its associated metabolic disorders. To test this hypothesis, we aimed at evaluating the effects of DMY on HFD-induced atherosclerosis using LDL receptor deficient ($LDLr^{-/-}$) mice, a well-established animal model used to study atherosclerosis and explore the mechanisms involved.

2. Materials and methods

An expanded Materials and methods section is available in the Supplemental Materials. Experimental protocols were approved by the Animal Care and Use Committee of Nantong University. Male $LDLr^{-/-}$ mice were housed under standard conditions with a 12-h light/dark cycle and free access to food and water. Starting from 8 weeks, the mice were randomly separated into five groups: a control group, a HFD group, two DMY-treated groups (250, 500 mg/kg/day) and a positive-control group (simvastatin, 30 mg/kg/day). 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 three drug treatment groups were given the same high fat diet, dosed daily via intragastric gavage with 250, 500 mg/kg/day DMY or 30 mg/kg/day simvastatin by weight for 8 weeks. After 8 weeks of treatment, the animals were sacrificed. Blood samples were collected for serum lipid profiles, ox-LDL and pro-inflammatory cytokines determination. Mouse peritoneal macrophages were collected. Histology, hepatic lipid content, quantification of atherosclerosis, assessment of oxidative stress, real-time PCR and Western blot were performed on liver and aorta samples.

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords as described previously by our laboratory [16]. Cell viability was determined by MTT assay. Cell injury was further confirmed by measuring the activity of LDH. The endothelial-monocyte adhesion assay was carried out as described in our previous report [16]. ROS generation in HUVECs was monitored using the fluorescence of the dihydroethidium (DHE) probe. Oil Red O staining and intracellular cholesterol quantitative assay were used to observe lipid accumulation in foam cells derived from macrophages. Cholesterol efflux was tested by fluorescent assay in RAW264.7 cells.

2.1. Statistical analysis

Data were reported as mean ± SD. All values were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test using Graphpad Prism 5 software. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. DMY ameliorated hyperlipidemia and reduced serum ox-LDL level and pro-inflammatory cytokines in HFD-fed $LDLr^{-/-}$ mice

As expected, after 8 weeks of feeding on a HFD, $LDLr^{-/-}$ mice had significantly higher body weight, serum levels of TC, TG and LDL-c and lower HDL-c serum level compared with those fed a standard chow (Supplemental Fig. 1 and Fig. 1A–D). Notably, compared with the HFD alone group, DMY treatment (250 and 500 mg/kg/day) markedly ameliorated the above serum lipid profiles (Fig. 1A–D). In addition, $LDLr^{-/-}$ mice fed a HFD developed a high ox-LDL level and serum levels of pro-inflammatory cytokines IL-6 and TNF-α compared with those of the control group, and these effects were significantly reduced by treatment with DMY (Fig. 1E and F). Simvastatin used as positive control had similar effects as DMY.

3.2. DMY suppressed hepatic lipid accumulation and steatosis in HFD-fed $LDLr^{-/-}$ mice

As the liver is the main source of triglyceride and cholesterol synthesis, the hepatic tissue was examined. HFD-fed $LDLr^{-/-}$ mice exhibited a uniformly pale yellow liver associated with a slight enlargement, implying lipid accumulation (Fig. 2A). Next, the cross-sections of frozen liver tissue were stained for neutral lipid content using Oil Red O. The results showed an accumulation of many lipid droplets (part of red color) in the livers of HFD-fed $LDLr^{-/-}$ mice compared to the control (Fig. 2B). This finding was confirmed by H&E staining, indicating an increased number and size of intracytoplasmic micro and macro-vacuoles within the liver of HFD-fed $LDLr^{-/-}$ mice associated with slight inflammatory cell infiltration (Fig. 2C). However, livers from HFD-fed $LDLr^{-/-}$ mice treated with DMY or simvastatin displayed alleviation of these pathological changes compared with the HFD-fed alone group (Fig. 2A–C). Consistent with these hepatic histological observations, the inhibitory effects of DMY on cholesterol and triglyceride deposition in the liver were also revealed by the biochemical analysis (Fig. 2D and E). Furthermore, protein expression of genes involved in lipid metabolism was measured in the liver. The results showed that compared to the HFD alone group, the protein levels of PPARα, LXRα and ABCA1 were markedly up-regulated in the HFD + DMY or simvastatin group (Fig. 2F–H), but the expression levels of FAS and HMGCR were not significantly affected by DMY (Supplemental Fig. 2).

3.3. DMY prevented hepatic inflammation and oxidative stress in HFD-fed $LDLr^{-/-}$ mice

Next, the effect of DMY treatment on hepatic inflammation was examined. Liver macrophages are key drivers of liver inflammation. Detailed immunohistochemical analysis on liver sections stained with the macrophage marker CD68 and gene analysis revealed that after 8 weeks of HFD, the area of the macrophages and *CD68* mRNA expression were increased in the liver of $LDLr^{-/-}$ mice (Fig. 3A–C). Hepatic mRNA expression of pro-inflammatory cytokines TNF-α and IL-6 confirmed this finding (Fig. 3D and E). Treatment with DMY or simvastatin significantly reduced hepatic CD68-positive macrophages content together with *IL-6* and *TNF-α* mRNA