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Luteolin decreases atherosclerosis in LDL receptor-deficient mice via a mechanism including decreasing AMPK-SIRT1 signaling in macrophages

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Abstract. Lipid metabolism dysfunction and inflammatory infiltration into arterial walls are associated with the initiation and progression of atherosclerosis. Luteolin has been reported to possess anti-inflammatory actions and protect against tumor necrosis factor-α (TNF-α)-induced vascular inflammation, monocyte adhesion to endothelial cells and the formation of lipid-laden macrophages *in vitro*. However, the role of luteolin in atherosclerosis and the associated vascular inflammatory remains to be elucidated. The aim of the present study was to investigate the effects of luteolin on plaque development, lipid accumulation and macrophage inflammation low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice with atherosclerosis, as well as the underlying mechanisms in ox-induced THP-1-derived macrophages. Firstly, 9-week-old male C57BL/6 mice were fed a standard chow diet, western diet or western diet supplemented with 100 mg/kg luteolin for 14 weeks. The results of histological staining revealed that 100 mg/kg dietary luteolin ameliorated western diet-induced atherosclerotic plaque development and lipid accumulation in the abdominal aorta. Furthermore, total cholesterol, triglyceride and LDL-cholesterol levels were decreased in the plasma of western diet + luteolin mice compared with those fed with a western diet alone. Quantitative polymerase chain reaction analysis revealed that dietary luteolin inhibited the expression of cluster of differentiation 68, macrophage chemoattractant protein 2 and inflammatory cytokines, including interleukin-6 (IL-6) and TNF-α. Mechanistically, luteolin decreased the total cholesterol level as well as macrophage chemokine and inflammatory cytokine expression in THP-1-derived macrophages via AMP-activated protein kinase (AMPK)-Sirtuin (SIRT)1 signaling following induction with oxidized low-density lipoprotein. The results of the present study suggest that luteolin prevents plaque development and lipid accumulation in the abdominal aorta by decreasing macrophage inflammation during atherosclerosis, which is mediated by mechanisms including AMPK-SIRT1 signaling.

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Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; CCL2, macrophage chemoattractant protein 2; CD, chow diet; HDL, high-density lipoprotein; IL-6, interleukin-6; LDL, low-density lipoprotein; $LDLR^{-/-}$, low-density lipoprotein receptor-deficient; SIRT1, silent information regulator 1; TNF-α, tumor necrosis factor α; WD, western diet; WD+L, western diet supplemented with luteolin

Key words: luteolin, atherosclerosis, aorta, lipid accumulation, macrophage

Introduction

Atherosclerosis is a multi-factorial process and a major cause of coronary heart disease (1,2). Atherosclerosis is accompanied by lipid metabolism dysregulation and inflammatory infiltrates in the arterial walls (3). These processes are vital in the initiation and progression of atherosclerosis, eventually leading to rupture of the atherosclerotic plaque (4). Atherosclerosis is therefore considered to be a chronic cytokine-mediated inflammatory disease that involves substantial remodeling of the arteries (3-5). During the initiation of atherosclerosis, monocytes migrate into the area in response to locally produced chemokines, differentiate into macrophages and increase the expression of several pattern recognition receptors (6). These macrophages subsequently accumulate cholesterol and lipids and become foam cells (7-9). Monocytes, macrophages and other immune cells, as well as inflammatory cytokines, growth factors and the accumulation of cholesterol and lipids serve a role in the pathogenesis of atherosclerosis (6-9).

Flavonoids have been reported to exhibit diverse health benefits; a number of epidemiological studies have reported the potential effects of flavonoids in ameliorating cardiovascular disease (CVD) (10-13). Luteolin is a natural flavone, a subtype of flavonoid, which is abundant in edible plants, including broccoli, green chilies, onion leaf, French beans, carrots, white radish, clover blossom and ragweed pollen (14). A number of previous studies have reported that luteolin possesses beneficial medicinal properties, including anti-oxidant,

anti-inflammatory and anti-aging actions (15-17). However, the effect of luteolin on atherosclerosis remains unclear. Recently, a number of studies have demonstrated that luteolin protects against tumor necrosis factor-α (TNF-α)-induced vascular inflammation and monocyte adhesion to endothelial cells (ECs) *in vitro* and *in vivo* (18-20). In addition, luteolin suppresses THP-1 cell differentiation and inhibits transendothelial migration of monocytes and the formation of lipid-laden macrophages (21,22). These observations suggest that luteolin serves a protective role in the vasculature and inflammatory process during atherosclerosis development.

The aim of the present study was to investigate the effects of luteolin on atherosclerotic plaque development and lipid accumulation in the abdominal aortas and plasma of low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice fed with a western diet. The protective effect of monocyte migration and inflammation in the aortal root was also investigated. Furthermore, the effect of luteolin on lipid deposition, inflammation and AMP-activated protein kinase (AMPK)-Sirtuin (SIRT)1 signaling in ox-LDL-induced THP-1-derived macrophages was assessed. The results indicate that luteolin as a dietary supplement has potential protective effects, preventing the development of atherosclerosis via decreasing macrophage inflammation.

Materials and methods

Animal experiments. A total of 30 9-week-old male $LDLR^{-/-}$ knockout mice with a C57BL/6 background (Body weight, 24.5±0.37 g) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and housed in ventilated cages maintained at 22±2˚C, 55±5% relative humidity with a 12 h light/dark cycle. Food and water were administered *ad libitum*. Mice were randomly divided into three groups (n=10) and fed with the following; chow diet, western-type diet (Research Diets D12108C formula containing 4.5 kcal/g, 40% of energy from fat and 1.25% cholesterol; Research Diets, Inc., New Brunswick, NJ, USA) or western-type diet supplemented with luteolin (>98% purity; 100 mg/kg diet; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) for 14 weeks. All animal experimental procedure protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Capital Medical University (Beijing, China). At the end of the treatment period, mice were sacrificed and the plasma and tissues were collected and immediately frozen.

Assessment of aortic atherosclerosis. Whole abdominal aortas were collected, fixed with 10% formalin for 24 h and stained with oil red O for 2.5 h at room temperature (25˚C) to detect the lipid deposition in lesions. Images of the entire aortic intimal surface were captured using a digital camera and digitally scanned (Nikon Corporation; Tokyo, Japan). Oil red O-positive stained lesions were identified by assessing staining intensity with Image-Pro Plus Version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). Quantification of the atherosclerotic lesion area was expressed as a positive area percentage.

Lipoprotein measurement. After 14 weeks, mice were euthanized with CO_2 asphyxiation in a 7.07-l chamber with 1.5 l/min flow rate and a final concentration of 100% CO_2. To confirm sacrifice was successful, mice remained in the chamber filled with 100% CO_2 for an additional time of ~3 min. Subsequently, their blood were harvested. To obtain plasma, blood was left overnight at 4˚C and centrifuged at 5,000 g (4˚C) for 15 min. Plasma total cholesterol, triglyceride, high density lipoprotein-cholesterol, LDL-cholesterol (LDL-c) were measured using a colorimetric enzymatic kit including triglyceride, cholesterol reagents or HDL cholesterol precipitating reagent kits (ALPCO, Salem, NH, USA) according to the manufacturer's protocol.

Cell culture. Human THP-1 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium and maintained at 37˚C in humidified atmosphere containing 5% CO_2 in air and differentiation into adherent macrophages was induced by incubating at 37˚C with 100 nM phorbol myristate acetate for 48 h. The medium was replaced with new medium containing 0, 5, 10 and 20 μM of luteolin for 24 h. Next, the cells were treated with 100 μg/ml ox-LDL (Alfa Aesar, Tewksbury, MA, USA) for 5 h at 37˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the mice proximal aorta or cultured cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. cDNA was synthesized by RT using reverse transcriptase (Takara Biotechnology Co., Ltd., Dalian, China). For reverse transcription, 2.5 μl of 20 μM primer stock was combined with RNA sample and heated with 70˚C for 3 min. A total of 4 μl 5X First-Strand Buffer, 2 μl dNTP mix, 2 μl 100 mM DTT and 0.5 μl SMART MMLV reverse transcriptase were mixed and incubated at 42˚C for 60 min. The reaction was terminated by heating at 70˚C for 15 min. qPCR was performed on an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a SYBR PCR kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The thermocycling conditions used were as follows: qPCR was performed at 42˚C for 5 min, 95˚C 10 sec for one cycle, followed by 40 cycles at 95˚C for 5 sec, 60˚C for 20 sec; and melting curve analysis was performed at 65˚C for 15 sec, and increased to 95˚C by 0.1˚C/sec. Results were quantified using the $2^{-\Delta\Delta Cq}$ method (23). β-actin served as the housekeeping gene for the comparisons of the gene expression data. The primer sequences for qPCR are listed in Table I.

Western blotting. Following treatment, cells were lysed in radioimmunoprecipitation assay buffer containing 10 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China). Total cellular protein (30 μg) determined by the BCA method was separated by 10% SDS-PAGE, transferred onto nitrocellulose membranes and blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 2 h at room temperature. Subsequently, the membrane was incubated with primary antibodies directed against SIRT1 (1:1,000; cat. no. 2028; Cell Signaling Technology, Inc., Danvers, MA, USA), AMPK (1:1,000; cat. no. 5832; Cell Signaling Technology, Inc.) and Thr172 phosphorylated-AMPK (pAMPK; 1:1,000;

Table I. Primers for the quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primers (5'-3')
CD68	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
CCL2	CCCCAAGAAGGAATGGGTCC	GGTTGTGGAAAAGGTAGTGG
IL-6	CCTTCCTACCCCAATTTCCAA	AGATGAATTGGATGGTCTTGGTC
TNF-α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

CD68, cluster of differentiation 68; CCL2, macrophage chemoattractant protein 2; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

cat. no. 2535; Cell Signaling Technology, Inc.). Following incubation with the rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000, cat. no. 7074; Cell Signaling Technology, Inc.). Following incubation with the secondary antibodies, the immunoreactive bands were detected with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). Immunoblots were quantified using ImageQuant TL 7.0 software (GE Healthcare, Chicago, IL, USA) and expressed at a ratio of pAMPK to AMPK or SIRT1 to β-actin (1:1,000, cat. no. 4970; Cell Signaling Technology, Inc.).

Statistical analysis. Data are presented as the mean ± standard error of the mean. One-way analysis of variance with Duncan's post hoc tests were used for the mouse assays and *in vitro* assays P<0.05 was considered to indicate a statistically significant difference. SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used as statistical software.

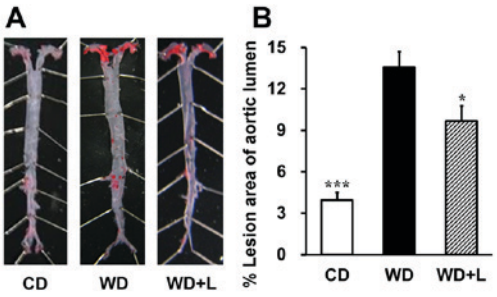

Figure 1. Luteolin reduces the area of aortic lipid accumulation in LDLR$^{-/-}$ mice fed with a WD. (A) Representative images and (B) quantitative analysis of atherosclerotic lesions stained with oil red O in the abdominal aortas of LDLR$^{-/-}$ mice fed with CD, WD and WD+L. n=10 per group. *P<0.05 and ***P<0.001 vs. WD mice. LDLR$^{-/-}$, low-density lipoprotein receptor-deficient; WD, western diet; CD, chow diet; WD+L, WD supplemented with 100 mg/kg luteolin.

Results

Luteolin prevents atherosclerotic plaque development in LDLR$^{-/-}$ mice fed with *a western diet*. To investigate the role of luteolin in atherosclerosis, the effects of luteolin on lesion formation in LDLR$^{-/-}$ mice fed with a western diet were examined. Oil red O staining revealed that LDLR$^{-/-}$ mice had significant atherosclerotic lesions compared with chow diet-fed mice (P<0.001; Fig. 1). Notably, supplementation with 100 mg/kg luteolin for 14 weeks significantly reduced the lesion area by 28.8% in mice fed with a western diet (P<0.05; Fig. 1). The results demonstrate that dietary luteolin prevents aortic lipid accumulation and atherosclerotic plaque development in LDLR$^{-/-}$ mice fed with a western diet.

Luteolin normalizes metabolic parameters in LDLR$^{-/-}$ mice fed with a western diet. Plasma total cholesterol (P<0.001; Fig. 2A) and triglyceride (P<0.01; Fig. 2B) levels were significantly higher in mice fed with a western diet compared to the control group; however, total cholesterol (P<0.05; Fig. 2A) and triglyceride ~30% following luteolin treatment. Consistently, plasma LDL-c levels exhibited a 32.8% reduction in the luteolin treatment group compared with the western diet group (P<0.05; Fig. 2C). However, dietary luteolin had no significant effect on high-density lipoprotein cholesterol levels in mice fed with a western diet (Fig. 2D). These data indicate that luteolin ameliorates lipid accumulation in the plasma and prevents dyslipidemia in LDLR$^{-/-}$ mice fed with a western diet.

Luteolin attenuates the macrophage number and vascular inflammation in the aortic root. The activation of monocytes and macrophages is an important initial step in atherosclerosis, sustaining inflammation within atheromasia and possibly influencing plaque stability (7-9). The macrophage recruitment and inflammation in the aortic root was therefore examined in the present study. RT-qPCR revealed that the macrophage marker cluster of differentiation 68 (CD68; P<0.01; Fig. 2E) and macrophage chemoattractant protein 2 (CCL2; P<0.05; Fig. 2F) were significantly reduced in the aortic root of mice fed a western diet supplemented with luteolin compared with mice fed with a western diet alone. Additionally, the levels of inflammatory cytokine interleukin (IL)-6 and TNF-α were significantly decreased in the aortic root following luteolin treatment (P<0.01; Fig. 2G and H). These results demonstrate that luteolin ameliorates atherosclerosis by decreasing macrophage infiltration into the plaque and inflammation in the aorta of LDLR$^{-/-}$ mice.

Luteolin decreases foam cell formation and inflammatory factors in ox-LDL induced THP-1-derived macrophages. In order to verify the effect of luteolin on macrophage inflammation in atherosclerosis, differentiated THP-1-derived macrophages were treated with increasing concentrations of luteolin. Incubation of THP-1 cells with 100 μg/ml ox-LDL significantly increased the uptake of lipids, as demonstrated by the higher total cholesterol level (P<0.05; Fig. 3A). Treatment with 10 or 20 μM of luteolin for 24 h significantly reduced the

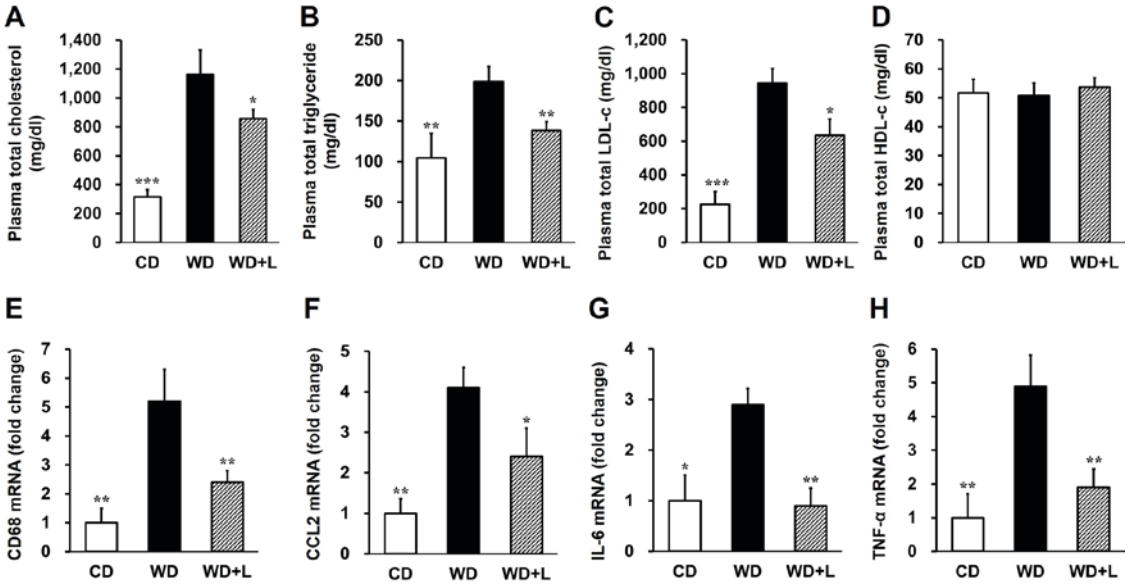

Figure 2. Luteolin improves the metabolic parameters of plasma in LDLR$^{-/-}$ mice fed with a WD. (A) Total plasma cholesterol, (B) total plasma triglycerides, (C) total plasma LDL-c and (D) total plasma HDL-c were quantified in LDLR$^{-/-}$ mice fed with CD, WD and WD+L. mRNA expression of (E) CD68, (F) CCL2, (G) IL-6 and (H) TNF-α in the aortic root of LDLR$^{-/-}$ mice fed with CD, WD and WD+L. β-actin was used as a reference gene. n=10 per group. *P<0.05, **P<0.01 and ***P<0.001 vs. WD mice. LDLR$^{-/-}$, low-density lipoprotein receptor-deficient; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; CD, chow diet; WD, western diet; WD+L, WD supplemented with 100 mg/kg luteolin; CD68, cluster of differentiation 68; CCL2, macrophage chemoattractant protein 2; IL, interleukin; TNF-α, tumor necrosis factor-α.

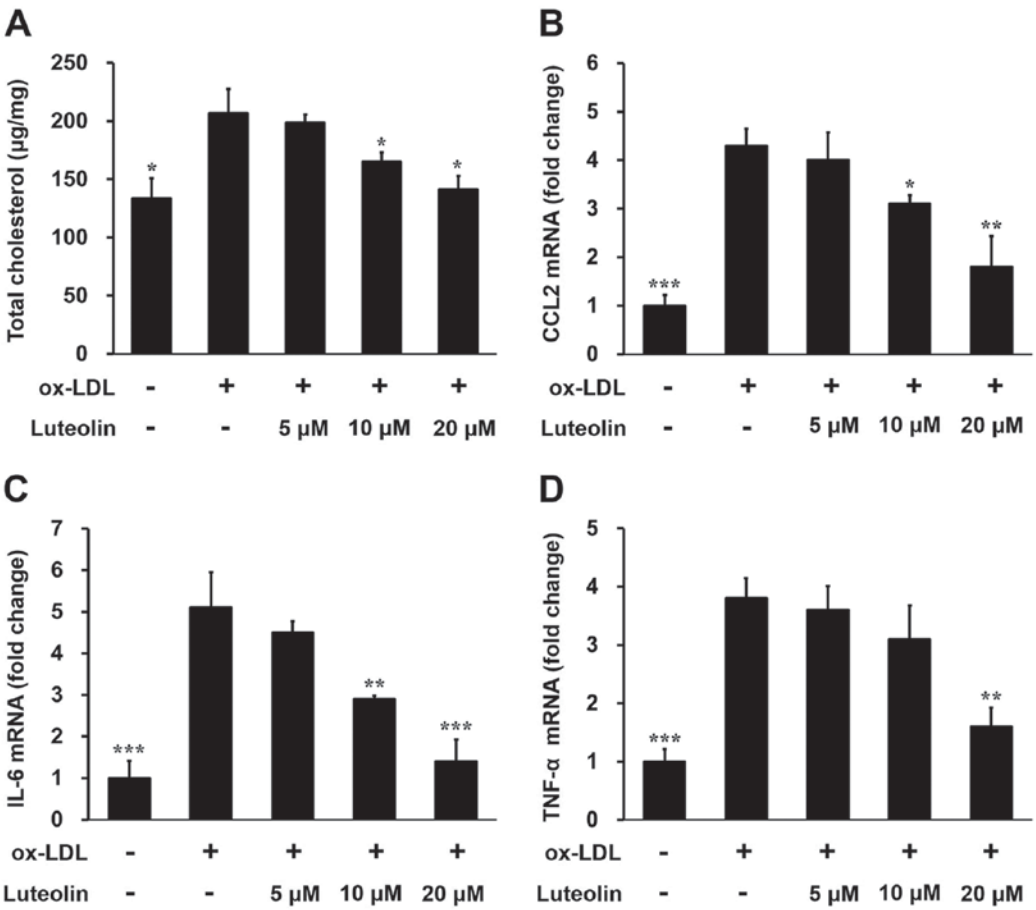

Figure 3. Luteolin decreases lipid uptake and inflammation in ox-LDL induced THP-1-derived macrophages. Differentiated THP-1-derived macrophage cells were induced with 5, 10 and 20 μM of luteolin for 24 h. Next, the cells were treated with 100 μg/ml ox-LDL for 5 h. (A) Total cholesterol level in THP-1 cells was quantified by enzymatic assays. Quantitative polymerase chain reaction mRNA expression of (B) CCL2, (C) IL-6 and (D) TNF-α in THP-1 cells. β-actin was used as a reference gene. n=4 per group. *P<0.05, **P<0.01 and ***P<0.001 vs. ox-LDL only. ox-LDL, oxidized low-density lipoprotein; CCL2, macrophage chemoattractant protein 2; IL, interleukin; TNF-α, tumor necrosis factor-α.

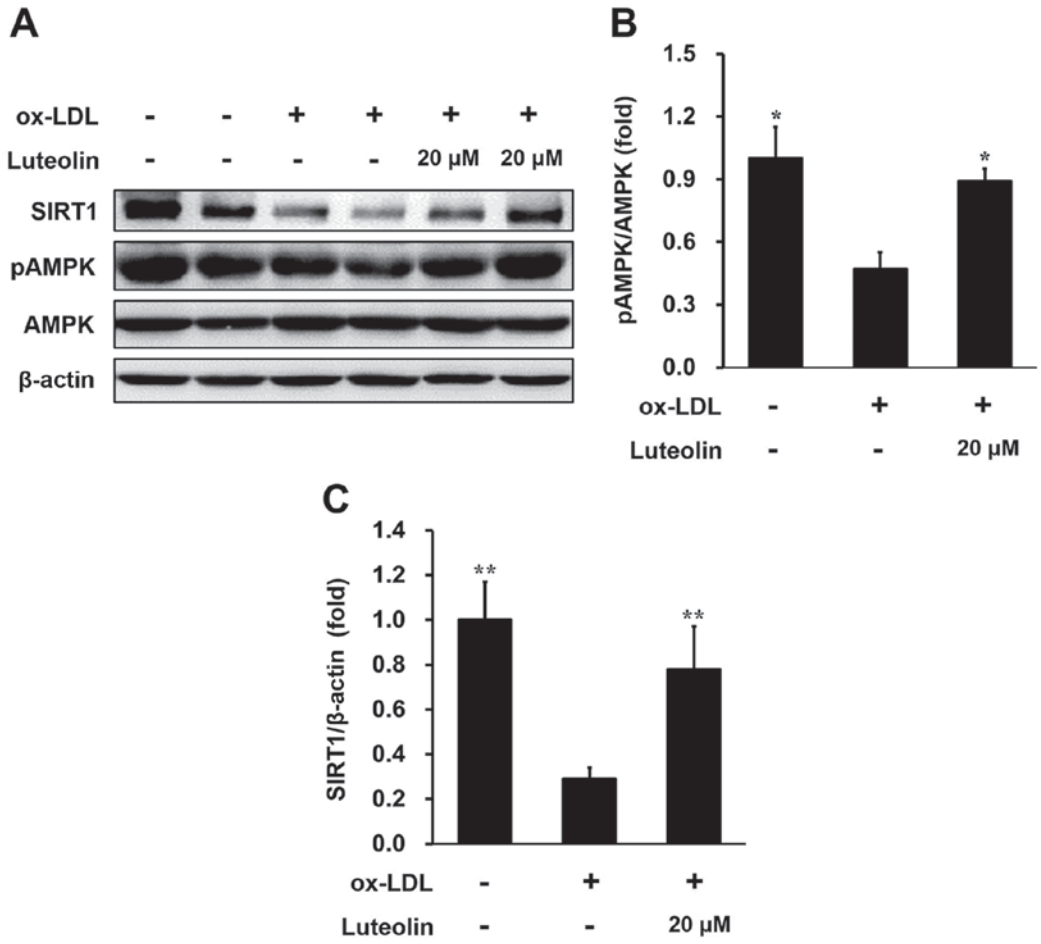

Figure 4. Luteolin inhibits macrophage inflammation via a mechanism that includes activating AMPK-SIRT1 signaling in ox-LDL-induced THP-1-derived macrophages. Differentiated THP-1-derived macrophages cells were induced with 20 μM of luteolin for 24 h. Cells were treated with 100 μg/ml oxLDL for 5 h. (A) Immunoblot of AMPK, pAMPK and SIRT1. (B) Ratio of pAMPK to total AMPK and (C) of SIRT1 to β-actin in THP-1 cells. n=4 per group. *P<0.05 and **P<0.01 vs. ox-LDL only. AMPK, adenosine monophosphate-activated protein kinase; SIRT1, Sirtuin1; ox-LDL, oxidized low-density lipoprotein; p, phosphorylated.

cholesterol content in THP-1 cells in a dose-dependent manner (P<0.05; Fig. 3A). Consistent with these results, luteolin also inhibited CCL2 (P<0.05; Fig. 3B), IL-6 (P<0.01; Fig. 3C) and TNF-α (P<0.01; Fig. 3D) expression in ox-LDL induced THP-1 cells in a dose-dependent manner. These results provide further evidence that luteolin decreases foam cell formation and inflammatory factors in macrophages, alleviating atherosclerosis.

Luteolin inhibits lipid deposition and inflammation and activates AMPK-SIRT1 signaling in ox-LDL-induced THP-1-derived macrophages. AMPK-SIRT1 signaling has been reported to be associated with the attenuation of atherosclerosis and vascular dysfunction (21-25); luteolin may be able to activate this signaling in macrophages (26). In order to explore the effect of luteolin on lipid deposition and inflammation in macrophages, AMPK-SIRT1 signaling in luteolin-treated THP-1 cells was assessed. Western blot analysis revealed that 20 μM luteolin, an effective concentration for the inhibition of inflammation in THP-1 cells, significantly increased the levels of phosphorylated AMPK compared with cells without luteolin (P<0.05; Fig. 4A and B). Furthermore, luteolin significantly ameliorated the ox-LDL-induced decrease in SIRT1 expression in THP-1-derived macrophages (P<0.01; Fig. 4C). In aggregates, these results demonstrated that luteolin inhibits macrophage inflammation via a mechanism that is associated with activation of AMPK-SIRT1 signaling.

Discussion

CVD is multifactorial and the risk of developing CVD is increased by predisposing factors, including obesity, hyperlipidemia, arterial hypertension, diabetes inadequate exercise and smoking (27,28). Imbalanced lipid metabolism and vascular inflammation serve a significant role in the pathogenesis of atherosclerosis. Early development of atherosclerosis begins with the adhesion of monocytes to the vascular endothelium, followed by accumulation of lipid and eventually maturation in the intima (6-9,29). In the present study, dietary luteolin supplements were demonstrated to have beneficial effects, preventing plaque formation by regulating macrophage recruitment and inflammation in $LDLR^{-/-}$ mice fed with a western diet. Luteolin was also revealed to inhibit macrophage inflammation via a mechanism including AMPK-SIRT1 signaling in ox-LDL induced THP-1-derived macrophages.

Luteolin is a natural flavonoid that exhibits anti-inflammatory and anti-oxidative properties (14,15). A series of studies have reported that luteolin protects against vascular inflammation, monocyte adhesion to endothelial cells (ECs) and macrophage differentiation, oxidized LDL uptake and foam cell formation of macrophages (18-20). However, the exact effect of luteolin in atherosclerosis and the underlying cellular mechanisms have not been elucidated. In order to assess the effect of luteolin on atherosclerosis, $LDLR^{-/-}$ mice were fed with a western diet for 14 weeks to develop atherosclerotic plaques. Supplementing the western diet with 100 mg/kg luteolin significantly ameliorated atherosclerotic plaque development and decreased lipid accumulation in abdominal aortas. These results demonstrate that luteolin may be an effective preventative treatment for atherosclerosis, which was further confirmed by decreased lipid concentrations in the plasma. These results promoted us to investigate the process luteolin acted on during atherosclerosis, including monocytes adhesion to the vascular endothelium, lipid accumulation or plaque development.

Luteolin has been broadly reported to inhibit macrophage inflammation *in vivo* and *in vitro* (30-32). As macrophages serve a key role in the initiation and progression of atherosclerosis (33), the present study focused on macrophages and inflammation in the atherosclerotic plaque. The results revealed that levels of the macrophage marker CD68, macrophage chemokine CCL2 and inflammatory cytokines IL-6 and TNF-α expression in the aortic root were decreased with luteolin supplementation. Luteolin was also demonstrated to inhibit macrophage chemokine and inflammatory cytokine expression in THP-1-derived macrophages in a dose dependent manner. These results are in agreement with earlier observations that reveal luteolin suppresses macrophage differentiation and inhibits lipid accumulation in macrophages (21,22). However, luteolin has also been reported have inhibitory effects on monocyte adhesion to ECs *in vitro* (18). Further studies are required to explore whether luteolin affects the adhesive properties of ECs of the arteries. The activated ECs in arteries also release chemokines and cytokines, which recruits circulating immune cells, including T cells, B cells and macrophages in atherosclerotic plaques. In addition, ECs express adhesion proteins, including intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, which are associated with the recruitment of immune cells (34). Consequently, the importance of luteolin in the regulation of vascular adhesion requires further investigation.

AMPK is important in regulating cellular glucose and lipid metabolism and has been reported to serve a role in the attenuation of atherosclerosis and vascular dysfunction (24-26). The present study demonstrated that luteolin promotes AMPK phosphorylation in ox-LDL induced THP-1 macrophages. It has also been reported that luteolin is able to increase AMPK phosphorylation in macrophages (30). SIRT1 is also an important regulatory sensor of nutrient availability and a target in catabolic metabolism, mitochondrial activation and angiogenesis (35,36). AMPK negatively regulates lipid-induced inflammation via SIRT1 in macrophages (37). Consequently, luteolin may increase SIRT1 expression in THP-1 cells. These data suggest that luteolin regulates inflammation and lipid metabolism in macrophages via a mechanism that includes the AMPK-SIRT1 signaling pathway.

In conclusion, the results of the present study indicate that dietary luteolin ameliorates atherosclerotic plaque development and lipid accumulation in the abdomen and plasma of $LDLR^{-/-}$ mice fed with a western diet. Dietary luteolin also protects the aorta from monocyte migration and inflammation. Finally, using ox-LDL-induced THP-1-derived macrophages, the present study demonstrated that luteolin prevents inflammation through a mechanism that includes AMPK-SIRT1 signaling.

Acknowledgements

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