Luteolin Attenuates Foam Cell Formation and Apoptosis in Ox-LDL-Stimulated Macrophages by Enhancing Autophagy

Bu-Chun Zhang¹ Cong-Wei Zhang² Cheng Wang¹ De-Feng Pan¹ Tong-Da Xu²
Dong-Ye Li¹²

¹Department of Cardiology, The Affiliated Hospital of Xuzhou Medical University, ²Institute of Cardiovascular Disease Research, Xuzhou Medical University, Xuzhou, PR China

Key words
Autophagy • Foam cells • Apoptosis • Atherosclerosis • Luteolin

Abstract
Background: Our previous studies demonstrated that luteolin, which is rich in flavones, has various biological properties and can exert anti-oxidant, anti-inflammatory and anti-apoptotic activities. However, its effect on ox-LDL-induced macrophage lipid accumulation and apoptosis has not been revealed. Aims: This study aimed to explore the role of luteolin in ox-LDL-induced macrophage-derived foam cell formation and apoptosis and to delineate the underlying mechanism. Methods: Murine RAW264.7 cells were stimulated with oxidized low-density lipoprotein (ox-LDL) (50 μg/ml) for 24 h and then pretreated with 25 μM luteolin for another 24 h. The effects of luteolin on lipid accumulation in RAW264.7 cells induced by ox-LDL were assayed using Oil red O staining and high performance liquid chromatography (HPLC). Apoptosis was confirmed by acridine orange/ethidium bromide (AO/EB) staining, flow cytometric analysis and the TUNEL assay. Immunofluorescence, Western blot and monodansylcadaverine (MDC) staining analyses were then used to further investigate the molecular mechanisms by which luteolin protects macrophages from ox-LDL-induced foam cell formation and apoptosis. 3-Methyladenine (3-MA), an autophagy inhibitor, was used as a positive control. Results: Treatment with 25 μM luteolin not only significantly attenuated ox-LDL-induced macrophage lipid accumulation but also decreased the apoptotic rate of RAW264.7 cells, the number of TUNEL-positive macrophages and the expression of Bax, Bak, cleaved caspase-9 and cleaved caspase-3. In addition, luteolin pretreatment significantly increased autophagosome formation and Beclin-1 activity, thus increasing the ratio of LC3-II/LC3-I. Moreover, these effects were abolished by 3-MA. Conclusions: Taken together, these results highlight that luteolin treatment attenuates foam cell formation and macrophage apoptosis by promoting autophagy and provide new insights into the molecular mechanism of luteolin and its therapeutic potential in the treatment of atherosclerosis.

B.-C. Zhang and C.-W. Zhang contributed equally to this paper.

Institute of Cardiovascular Disease Research, Xuzhou Medical University
84 West Huaihai Road, 221002, Xuzhou, Jiangsu, (PR China)
Tel. +86-516-85582763, Fax +86-516-85582753, E-Mail ldy@xzmc.edu.cn
Introduction

Macrophage foam cell formation and apoptosis have been implicated as key mediators during the development of atherosclerosis [1, 2]. Many experimental studies show that lesional macrophage apoptosis participates in the formation and expansion of the lipid core and gives rise to inflammation and necrosis, both of which lead to plaque instability [3, 4]. Therefore, protecting macrophages against apoptosis is believed to be an effective approach to attenuate plaque instability and combat acute vascular events.

Luteolin (3,4,5,7-tetrahydroxy-flavone), a naturally occurring polyphenolic compound found in many fruits, vegetables and medicinal herbs [5], has anti-apoptotic and anti-atherogenic properties [6]. Our previous studies have provided preliminary evidence that luteolin protects macrophages from oxidative stress, inflammation and apoptosis [7, 8]. However, the precise mechanisms concerning the anti-atherosclerotic effects of luteolin remain unclear.

Autophagy is a self-protecting catabolic cellular pathway. Since its discovery, autophagy has been found to be associated with a variety of diseases, including cardiovascular disease [9, 10]. Autophagy dysfunction previously has been shown to increase lipid accumulation and apoptosis in mouse atherosclerosis models [11]. These findings suggest the induction of autophagy may be exploited as a potential strategy for the treatment of atherosclerosis.

Currently, the role of luteolin in macrophage autophagy, foam cell formation and apoptosis in atherosclerosis has not been investigated. Therefore, we hypothesize that luteolin may protect macrophages from ox-LDL-induced foam cell formation and apoptosis by enhancing autophagy. In this study, we developed a cell model to evaluate the role of luteolin in protecting against foam cell formation and apoptosis as well as to identify the underlying mechanisms.

Materials and Methods

Reagents and antibodies

Luteolin (purity >98%) was purchased from Sigma-Aldrich (Fluka, Germany, Cat#: 491-70-3), dissolved in dehydrated alcohol as 50 mM stock solutions, and serially diluted in PBS immediately prior to experiments. The autophagy inhibitor 3-methyladenine (3-MA) was obtained from Sigma-Aldrich Co (St. Louis, MO, USA, Cat#: 5142-23-4). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan, Cat#: 3-41-07761). Ox-LDL was purchased from AbD Serotec (Kidlington, Oxford, UK). Oil red O (ORO), AO EB staining (Cat#: KG2A13), MDC staining (Cat#: KGATG002) and the AnnexinV-APC Apoptosis Detection Kit (Cat#: KG2A14) were purchased from the KeyGEN Bioengineering Institute (Nanjing, China). A terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay kit was obtained from Roche (Mannheim, Germany, Cat#: 11767291910). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco BRL (NY, USA). Antibodies against Bax (Cat#: ab10813), Bak (Cat#: ab32371) and ABCA1 (Cat#: ab18180) were purchased from Abcam (Cambridge, UK). Beclin-1 (Cat#: 3738), LC3 (Cat#: 12741), caspase-9 (Cat#: 9502), cleaved caspase-9 (Cat#: 9501), caspase-3 (Cat#: 9665), and cleaved caspase-3 (Cat#: 9661) antibodies were purchased from Cell Signaling Technology (MA, USA).

Cell culture and treatment

RAW264.7 murine macrophage cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

For experiments, the aforementioned cells (seeded at 3 × 10⁵ cells/ml) were treated with 50 μg/ml ox-LDL for 24 hours as described previously [12, 13]. Cells treated with PBS were used as controls. In some experiments, cells were exposed to luteolin for an additional 24 hours in the presence of 50 μg/ml ox-LDL. Cells were pretreated for 2 hours with the autophagy inducer 3-MA to explore the possible mechanisms of action. After the treatments, the cells were collected and analyzed as described below.
Cell viability assays

Cell viability was determined using the CCK-8 assay according to the manufacturer’s instructions. Cells were grown and treated in 96-well plates followed by incubation with CCK-8 reagent for 3 h at 37 °C. Absorbance was measured at 450 nm and expressed as an arbitrary unit proportional to cell toxicity. For each of these experiments, at least three parallel measurements were performed.

Oil red O staining

ORO staining of lipid droplets was performed as previously described [14]. Cells were fixed with 10% formalin in PBS and then stained for 20 min with 0.05% ORO in isopropanol and water (3:2 by volume). Cells were then washed with PBS for 2 min and immediately counterstained with Harris’s hematoxylin before cell drying. The cells were observed and photographed using a fluorescence microscope (Olympus, Japan). Lipid-laden macrophages were counted using ORO staining, and the absolute number of ORO-positive macrophages was collected and compared with the total cell counts for each group. The ratio of ORO-positive macrophages to total cells was quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Lipid analysis by high performance liquid chromatography (HPLC) assays

Cellular cholesterol levels were analyzed by high performance liquid chromatography (HPLC) as described previously [15, 16]. Cells were detached in PBS supplemented with 1% EDTA (T4174; Sigma). Sterols were analyzed using an HPLC system (model 2790; Waters Corp.). A photodiode array detector equipped with a 4 μL cell was applied to detect the sterols (model 996; Waters Corp.). Analysis of cholesterol and cholesteryl esters was performed with an acetonitrile-isopropanol solution (30:70 v/v) after elution. The absorbance at 210 nm was detected. Total Chrom software from PerkinElmer was used to analyze all data.

Western blot analysis

Protein samples were obtained from the lysates of cultured cells, and the protein concentration was determined. The samples were separated by SDS-PAGE, transferred to a PVDF membrane and blocked with TBS containing 0.05% Tween-20 (TBST) and 5% nonfat milk powder for 2 h. The membranes were then incubated with appropriate primary antibodies overnight at 4 °C. After 3 washes with TBST, membranes were incubated with secondary antibodies for 2 h at room temperature. The protein samples were visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; Amersham Biosciences, Foster City, CA, USA).

Morphological analysis by acridine orange/ethidium bromide (AO/EB) double staining

At the end of the treatment, cells were stained with acridine orange (AO, 200 μg/mL) and ethidium bromide (EB, 200 μg/mL) for 10 min and then washed with PBS to remove background staining. The cells were then observed under a fluorescence microscope (Olympus, Japan).

Monodansylcadaverine (MDC) staining

The autofluorescent agent monodansylcadaverine (MDC) was introduced as a specific autophagolysosome marker to analyze the autophagic process [17]. After treatment, the cells were stained with MDC (50 μmol/L) at 37 °C for 40 min. After incubation, the cells were washed three times with PBS, fixed with 5% paraformaldehyde, and immediately observed under a fluorescence microscope (Olympus, Japan).

Flow cytometry analysis of apoptotic cells

The annexin V-FITC/propidium iodide (PI) double-staining assay was used to quantify apoptosis. Cells from each group were collected, washed twice with ice-cold PBS and centrifuged at 4 °C. Approximately 500 μL of binding buffer was added to resuspend the cells followed by 5 μL of annexin V-FITC and 5 μL of PI. The cells were incubated for 15 min in the dark at room temperature, and the apoptosis rates were analyzed on a FACScan flow cytometer using Cell Quest software (Becton Dickinson, San Jose, CA, USA).
TUNEL staining for apoptosis

Apoptotic cells were detected by using the TUNEL staining kit according to the manufacturer’s instructions. Cells were fixed with 4 % paraformaldehyde for 30 min at room temperature, rinsed with PBS, and then incubated in a 0.1 % Triton X-100 permeabilization solution for 2 min on ice. The TUNEL reaction mixture was added to the cells and incubated for 1 h in a dark humidified chamber at 37 °C, after which the cells were washed twice for 5 min in PBS and stained with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI). Cells were detected using a fluorescence microscope (Olympus, Japan), and the ratio of TUNEL-positive cells to total cells was calculated.

Immunofluorescence assay for Beclin-1 expression

The treated cells were fixed with 4 % (w/v) paraformaldehyde, blocked with BSA, and then incubated with a Beclin-1 antibody (1:150) overnight at 4 °C. After incubation with a secondary FITC-conjugated antibody and DAPI, the cells were washed with PBS, mounted in antifade reagent and then observed using an Olympus fluorescence microscope.

Statistical analysis

All data are expressed as the mean ±standard error of mean (SEM), and all experiments were independently repeated three times. Student’s t-test was used to analyze differences between groups. A P-value < 0.05 was considered to be statistically significant.

Statistical calculations were performed using GraphPad Prism 5.0 statistical software (San Diego, California, USA).

Results

Effects of luteolin on the viability of RAW264.7 cells

The cytotoxicity of luteolin in RAW264.7 cells was assessed by the CCK-8 assay. RAW264.7 cells were incubated with different concentrations (0, 6.25, 12.5, 25, 50, 100 and 200 μM) of luteolin for 24 h. As shown in Fig. 1, luteolin significantly decreased cell viability at concentrations of 50, 100 or 200 μM while the viability of RAW264.7 cells was not affected by luteolin at concentrations below 25 μM. Therefore, all subsequent experiments used 25 μM luteolin as the treatment concentration.

Effects of luteolin on autophagy-related cellular lipid accumulation and ABCA1 expression in ox-LDL-stimulated RAW264.7 cells

Lipid droplets stained by ORO were significantly observed in RAW264.7 cells treated with 50 μg/ml of ox-LDL for 24 h. However, the ox-LDL-induced accumulation of lipid droplets was reduced by luteolin. In contrast, the beneficial effects of luteolin could be blocked by 3-MA (Fig. 2A and B). Lipid accumulation was further certified by high-performance liquid

![Image of Fig. 1](image-url)
chromatography assays (HPLC). As shown in Table 1, the level of total cholesterol (TC) and esterified cholesterol (CE) increased significantly in the ox-LDL group compared with the control group. As anticipated, luteolin reduced the intracellular levels of TC and CE; conversely, the inhibition of autophagy by 3-MA exerted the opposite effect.
Reverse cholesterol transport (RCT) moves cholesterol from peripheral tissues such as the vessel wall to the liver for final excretion, thus preventing atherosclerosis. RCT is initiated by ATP-binding cassette transporter A1 (ABCA1) [18, 19]. We next examined whether ABCA1 is involved in luteolin-induced macrophage foam cell formation. The results demonstrated that luteolin rescued ABCA1 downregulation in ox-LDL-induced macrophages. By comparison, ABCA1 expression was notably inhibited in RAW264.7 cells treated with 3-MA (Fig. 2C and D).

Taken together, these data indicate that luteolin treatment reduced macrophage lipid accumulation and upregulated ABCA1 expression through autophagy activation.

Effects of luteolin on apoptosis of ox-LDL-stimulated RAW264.7 cells via autophagy

To test whether luteolin treatment for 24 h affected apoptosis in macrophages induced by ox-LDL, the apoptotic rate of macrophages was assessed by using AO/EB staining and flow cytometry to detect annexin V and PI staining. The quantitative analysis of AO/EB-positive macrophages indicated that the apoptotic rate increased in the ox-LDL group compared with
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the control group, while 25 μM luteolin treatment significantly reduced the rate of apoptosis. The addition of 3-MA abolished the protective effect of luteolin on macrophage apoptosis (Fig. 3A and B). The flow cytometry results indicated that the percentage of apoptotic cells increased from 9.3% in the control group to 31.7% in the ox-LDL group, and pretreatment with 25 μM luteolin significantly inhibited macrophage apoptosis (21.2%). However, ox-LDL-stimulated cells pretreated with 3-MA and luteolin had a higher percentage of apoptotic cells (48.8%) (Fig. 3C). Meanwhile, TUNEL staining revealed reduced apoptosis in ox-LDL-stimulated RAW264.7 cells treated with luteolin compared to the ox-LDL-stimulated cells alone. In contrast, cells treated with luteolin plus 3-MA displayed a higher frequency of TUNEL-positive staining (Fig. 3D and E).

Furthermore, the expression of pro-apoptotic markers was confirmed by Western blot analysis. As shown in Fig. 4, the expression of Bax, Bak, cleaved caspase-9, and cleaved caspase-3 were significantly up-regulated in the ox-LDL group than in the control group. Meanwhile, 25 μM luteolin significantly down-regulated the expression levels of Bax and Bak as well as reduced the cleaved caspase-9/pro-caspase-9 ratio and the cleaved caspase-3/pro-caspase-3 ratio, but these effects were inhibited by 3-MA. Collectively, these results further demonstrated that luteolin attenuated macrophage apoptosis through the induction of autophagy.

**Effects of luteolin on autophagy of ox-LDL-stimulated RAW264.7 cells**

Based on the data above, we investigated the effect of luteolin on autophagy activity. Autophagosomes were stained with MDC, and immunofluorescence staining for Beclin-1
expression was assessed. As shown in Fig. 5, MDC-labeled autophagic vacules were scarcely detected in ox-LDL-stimulated macrophages, whereas the cells treated with luteolin clearly showed numerous MDC-labeled fluorescent vacules with an increasing intensity, indicating that luteolin treatment in foam cells induced the formation of the MDC-labeled autophagosomes.

Beclin-1 plays an important role in autophagosome formation and it is considered as a marker for the activation of autophagosomes [20]. Immunofluorescence analysis showed a gradual increase in the number of Beclin-1-positive macrophages and staining intensity in the ox-LDL group compared with corresponding control group. Furthermore, higher Beclin-1 expression was observed in the luteolin treatment group. However, in the group pretreated with autophagy inhibitor, autophagosomes and Beclin-1 expression did not increase. In addition, Western blot analysis confirmed that Beclin-1 expression increased in the luteolin treatment group, and this increase was suppressed by 3-MA treatment. The autophagy-related protein light chain 3 (LC3) is the key biological marker to identify autophagy; upon autophagy activation, LC3-I is lipidated and converted to LC3-II. The ratio of LC3-II/LC3-I is widely used as a marker of autophagy activation [21]. Therefore, we investigated the expression of LC3 using Western blot to detect autophagy in this study. The results showed...
that the conversion of LC3-I to LC3-II increased gradually in RAW264.7 cells exposed to ox-LDL compared with control cells, and luteolin significantly increased the LC3-II/LC3-I ratio whereas 3-MA inhibited LC3 processing. These results suggest that the autophagy pathway is activated by luteolin and impedes foam cell formation and apoptosis in ox-LDL-stimulated macrophages.

**Discussion**

Apoptosis of lipid-containing macrophages in advanced atherosclerotic lesions promotes an inflammatory response, lesional necrosis and the enlargement of the lipid core, all of which contribute to atherosclerotic lesion instability, rupture, and acute thrombosis [22-24]. Thus, therapeutic approaches to reduce foam cell formation and apoptosis may illuminate an efficient strategy for the prevention and treatment of acute cardiovascular events. In the present study, we demonstrated that luteolin dramatically ameliorated macrophage lipid accumulation and upregulated ABCA1 expression. It was also observed that luteolin reduced the rate of ox-LDL-induced apoptosis and downregulated the expression of the pro-apoptotic proteins Bax, Bak, cleaved caspase-9, and cleaved caspase-3. However, the beneficial effects of luteolin on RAW264.7 macrophage-derived foam cells were abolished by 3-MA, indicating the involvement of autophagy in luteolin to promote cholesterol efflux and inhibit lipid accumulation and apoptosis.

In recent years, natural medicines (especially plant flavonoids) with cardiovascular protective functions have gained much attention and have been proven to reduce the risk of cardiovascular disease *in vitro* and *in vivo* based on different animal models [22]. Our previous studies have revealed that luteolin exhibits anti-inflammatory and anti-apoptotic effects in macrophages [7, 8]. However, whether luteolin could inhibit ox-LDL-induced macrophage lipid accumulation and apoptosis was unknown. Our results in the present work showed that luteolin remarkably restrained ox-LDL-induced lipid accumulation and macrophage apoptosis by activating autophagy.

Autophagy, a conserved pathway that maintains cellular quality control, has a critical function in regulating lipoprotein metabolism and apoptosis in obesity and atherosclerosis [25]. Throughout this process, LC3-II and double-membrane autophagosomes are widely regarded as significant markers that can be used to detect the initiation of autophagy, while Beclin-1 is assumed to be a critical component that participates in autophagosome formation in mammals [9, 26].

ABCA1 is widely expressed in animal tissues where it may have multiple and diverse functions. Studies of animal models have shown that increased ABCA1 activity in arterial macrophages could prevent foam cell atherosclerotic lesion formation [27]. There is also evidence that ABCA1 is involved in processes other than modulating lipid homeostasis, such as promoting macrophage engulfment of apoptotic cells and generating microparticles [28, 29]. The ABCA1 pathway has therefore become an important new therapeutic target for treating atherosclerosis. However, the regulation of ABCA1 activity is highly complex and far from being completely understood. Several observations have demonstrated that autophagy facilitates intracellular lipid droplet degradation and cholesterol efflux to impede foam cell formation [30, 31]. Thus, current experimental evidence supports a role for autophagy-mediated efflux that favors ABCA1 expression [32, 33]. These observations are consistent with the possibility that autophagy plays a major role in the expression of ABCA1. Consistent with these results, we found that luteolin decreases the levels of total cholesterol and cholesterol esters and increases the cholesterol efflux mediated by ABCA1 in RAW264.7 cells exposed to ox-LDL. To further confirm that luteolin attenuates foam cell formation by activating autophagy, we examined the autophagosomal marker LC3-II and Beclin-1 expression. The results suggested that luteolin increased the LC3-II/LC3-I ratio and Beclin-1 expression. Our findings indicate that autophagy activity may play a crucial role in the regulation of lipid accumulation. With regards to autophagy and macrophage apoptosis in
the progression of atherosclerosis, Liao et al. [3] provided evidence that autophagy prevented lesional macrophage apoptosis in animal models. The results of the present study based on the fluorescence and Western blot data further confirmed that autophagy was enhanced in foam cells treated with luteolin. Furthermore, in RAW264.7 macrophage-derived foam cells, the activation of autophagy ameliorates cholesterol accumulation through an ABCA1-dependent pathway that mediates cholesterol efflux. Meanwhile, it is well documented that the accumulation of intracellular cholesterol is also crucial to induce macrophage apoptosis in vivo and in vitro [34, 35]. Similar to our previous study [14], we found that autophagy activation is protective against luteolin-induced apoptosis in ox-LDL-induced macrophages.

In this study, we found that after ox-LDL stimulation, the cellular autophagy markers are gradually increased, whereas the additional luteolin treatment intensifies autophagy. ox-LDL exposure has been shown to facilitate autophagosome marker protein expression in cultures probably by increasing oxidative stress and inflammation [36, 37]. However, the reported autophagic activities in ox-LDL-treated macrophages are currently in disagreement, as some reports considered that autophagy flux was suppressed in 100 μg/ml ox-LDL-treated macrophages [38, 39] while others insisted that ox-LDL could cause activation of autophagy [40]. Different cell culture types, the same cells treated with different concentrations of ox-LDL or the same cells exposed to ox-LDL for different amounts of time may have contributed to this phenomenon.

In conclusion, the results of the present study indicated that luteolin attenuates foam cell formation and suppresses macrophage apoptosis by activating autophagy. These findings provide new insights into the molecular mechanism of luteolin and its therapeutic potential in the treatment of atherosclerosis. However, whether luteolin could reduce foam cell formation and apoptosis through autophagy activation in human macrophages needs further investigation.

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Disclosure Statement

The authors have no conflict of interests.

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