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Original Article

Oxidized low-density lipoprotein activates adipophilin through ERK1/2 signal pathway in RAW264.7 cells

Qingnan Liu[1,3], Zhibing Dai[2,4], Zhiqiang Liu[1], Xiaohui Liu[1], Chaoke Tang[1], Zuo Wang[1], Guanghui Yi[1], Lushan Liu[1], Zhisheng Jiang[1], Yongzong Yang[1], and Zhonghua Yuan[1*]

[1]Institute of Cardiovascular Disease, Key Laboratory for Arteriosclerology of Hunan Province, University of South China, Hengyang 421001, China
[2]Institute of Pathobiology, University of South China, Hengyang 421001, China
[3]Department of Basic Nursing, YiYang Medical College, Yiyang 413000, China
[4]Department of B type Ultrasonic Diagnosis, Central Hospital of Yiyang, Yiyang 413000, China
*Correspondence address. Tel: +86-734-8282276; E-mail: yzh5555@163.com

It has been reported that oxidized low-density lipoprotein (Ox-LDL) can increase the expression of adipophilin. However, the detailed mechanisms are not fully understood. The aim of this study was to investigate the mechanism of Ox-LDL on adipophilin expression and the intracellular lipid droplet accumulation. A mouse macrophage-like cell line, RAW264.7, was used throughout, and it was found that Ox-LDL induced adipophilin expression in a dose-dependent manner. Moreover, Ox-LDL induced peroxisome proliferator-activated receptor-γ (PPARγ) expression and PPARγ-specific inhibitor T0070907 abrogated Ox-LDL-induced adipophilin expression, but specific agonist GW1929 not. Furthermore, Ox-LDL induced phosphorylation of ERK1/2, and ERK1/2-specific inhibition by PD98059 suppressed the Ox-LDL-induced PPARγ and adipophilin expression. The results showed that ERK1/2 or PPARγ-specific inhibition decreased the amounts of intracellular lipid droplets. Meanwhile, the PPARγ-specific agonist increased intracellular lipid droplets. These results suggested that Ox-LDL-induced increase in adipophilin level via ERK1/2 activation is one of the mechanisms of inducing greater amounts of intracellular lipid droplets in RAW264.7 cells, which indicated that adipophilin is involved in atherosclerotic progression.

Keywords adipophilin; ERK1/2; atherosclerosis; PPARγ

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Introduction

Atherosclerosis, a complex physiopathological process, is initiated by an infiltration of low-density lipoproteins (LDL) into the subendothelial spaces where they accumulate and become modified mainly by oxidation [1–3]. Macrophages, which are derived from monocytes in these areas, take up oxidized LDL (Ox-LDL) through scavenger receptor pathways resulting in lipid droplet accumulation and foam cell formation. Foam cells are well known to play an important role in the development and progression of atherosclerosis through the production of various bioactive molecules and proteins such as growth factors and cytokines, also including adipose differentiation-related protein (ADRP) or adipophilin. Adipophilin is a 50 kDa protein first identified by Serrero *et al*. [4], who showed that adipophilin mRNA is expressed most strongly in adipose tissue and is induced very early during adipocyte differentiation. Its expression has now been found in diverse cell types (such as fibroblasts, endothelial cells, and epithelial cells) in culture and in particular is associated with lipid fractions in the cells [5,6]. Adipophilin was also localized to specific types of cells in tissues, such as lactating mammary epithelial cells, adrenal cortex cells, Sertoli and Leydig cells of the male reproductive system, and steatotic hepatocytes in alcoholic liver [6]. Lipid storage is facilitated by production of large amounts of lipid vesicle coating proteins such as adipophilin and perilipin, etc. [7]. These data suggested that adipophilin may be a specific marker for lipid accumulation in the cells. Despite its observed overexpression in lipid-loaded macrophages [8–10], little data exist on the mechanism of adipophilin in lipid storage and cholesterol efflux in the cells and its relevance to atherosclerosis.

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily and form heterodimers with a retinoid X receptor (RXR) [11]. Three distinct PPARs, termed α, γ, and δ, have been identified. PPARs are characterized by distinct tissue distribution

patterns and metabolic functions. PPARα is highly expressed in tissues that demonstrate high catabolic rates for fatty acids, such as liver, heart, kidney, and muscle, whereas PPARγ is highly expressed in adipose tissue, where it plays a major regulatory role in adipocyte differentiation, and the expression of genes involved in lipid metabolism [11–13]. PPARδ shows a widespread tissue distribution, but its physiological role remains to be fully elucidated [14]. Recently, Ox-LDL has been reported to activate PPARγ in macrophages [15,16], moreover, PPARγ is induced in monocytes and/or macrophages following exposure to Ox-LDL and is expressed at high levels in the foam cells of atherosclerotic lesions [17,18]. Thus some researchers speculated that it is possibly caused by the action of oxidized metabolites of linoleic acid, including 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), and oxidized phospholipids, which are included in Ox-LDL [15,19]. It is suggested that PPARγ may be important for gene expression during atherogenesis. Furthermore, there is a peroxisome proliferator-activated receptor response element (PPRE) in the promotor gene of adipophilin. However, the detailed mechanism involved in the role of PPARγ in Ox-LDL-mediated acceleration of atherogenesis is not fully understood.

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that can phosphorylate their target proteins. Three major subfamilies have been: extracellular signal regulated kinases 1/2 (ERK1/2), p38 MAPKs, and Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs). MAPKs are activated by a family of MAPK kinases (MKKs) and they are important mediators for signal transduction from the cell surface to the nucleus [20,21]. JNKs and p38 mediate signals in response to cytokines and environmental stress, whereas the ERK subtypes are classically recognized as key transducers in the signaling cascade that mediates cell proliferation in response to growth factors such as platelet-derived growth factor and endothelial growth factor. It is becoming increasingly clear that the ERK pathway, like those of p38 and JNK, is activated by environmental stresses, including reactive oxygen species such as H_2O_2 [22].

Several studies have shown that PPARγ agonist such as 15-deoxy-D12, 14-prostaglandin J2 (15d-PGJ2), and statins can induce the expression of adipophilin, and also can induce the phosphorylation of ERK1/2 which was related to atherosclerosis [23,24]. Furthermore, ERK1/2 is involved in the activation of PPARγ. TNF-α inhibited PPARγ transactivity partly by diminished PPARγ–PPRE (DNA) binding and ERK1/2-mediated phosphorylation of Ser(82) of PPARγ in cultured hepatic stellate cells [25]. Moreover, ghrelin triggered PPARγ activation through a concerted signaling cascade involving ERK1/2 and Akt kinases [26], hyperglycemia enhanced the adipogenic induction of lipid accumulation through an ERK1/2-activated PI3K/Akt-regulated PPARγ pathway [27]. And mice lacking macrophages were resistant to atherosclerosis [28,29]. In the present study, we used a mouse macrophage-like cell line, RAW264.7, to investigate a novel mechanism of adipophilin in lipid accumulation. We demonstrated that Ox-LDL-activated adipophilin through ERK1/2-PPARγ pathway in RAW264.7 cells, which might provide a basis for the prevention and treatment of atherosclerosis.

Materials and Methods

Materials

PD98059 was purchased from Promega (Madison, USA), and T0070907 and GW1929 were purchased from Cayman Chemical (Ann Arbor, USA) and Alexis Biochemicals (Lausen, Switzerland), respectively. Rabbit polyclonal anti-ERK1/2 (total-ERK), anti-phospho ERK1/2 (p-ERK), and anti-PPARγ antibodies were purchased from Abzoom (Dallas, USA), and rabbit polyclonal anti-adipophilin antibody was purchased from Bejing Biosynthesis Biotechnology Co., Ltd (Beijing, China). Mouse polyclonal anti-β-actin antibodies were purchased from Boster (Wuhan, China). Peroxidase-conjugated affinipure Goat anti-mouse IgG (H + L) and peroxidase-conjugated goat anti-rabbit IgG (H + L) were purchased from ZSGB-Bio (Beijing, China) and Multisciences (Hangzhou, China), respectively. A RevertAid first strand cDNA synthesis kit was purchased from Fermentas (Burllington, Canada). Pancreatin, EB, demethyl sulfoxide (DMSO), and oil red O were purchased from Amresco (Solon, USA). RPMI 1640 medium was purchased from Gibco (Grand Island, USA). TRIzol was purchased from Sangon (Shanghai, China). A total of 100 bp ladder marker was purchased from Generay Biotech (Shanghai, China). All primers were synthesized by the Shanghai bioengineering of Biological Engineering Service Co., Ltd. Agarose was purchased from Gene Tech Limited (Shanghai, China). Acetonitrile, isopropanol, and *n*-hexane were all chromatographic pure and purchased from Chemical Reagent Factory of Fu Chen Co. (Tianjin, China). A BCA kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). All other chemicals were of the best grade available from commercial sources.

Cell culture

A mouse macrophage-like cell line (RAW264.7) was purchased from Cell Bank in Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were maintained in RPMI 1640 medium containing 25 mmol/l HEPES buffer and 10% fetal calf serum at 37°C in 5% CO_2. When cells presented monolayer fusion and fused up to 70%, they were pretreated for 2 h with PD98059, T0070907, or GW1929, which was dissolved in

DMSO, and then were treated with 50 μg/ml of Ox-LDL or 10% BSA for 24 h. Cells were harvested and splited for adopophilin protein analysis. Total RNA was extracted according to manufacturer's instruction and dissolved in DEPC-treated water.

Lipoprotein preparation and modification of native LDL

To 100–200 ml of fresh human plasma were added citrate buffer solution containing 50 IU/ml heparin (pH 5.04), and then the solution was subject to serial ultracentrifugation. Briefly, plasma was centrifuged at 42,000 rpm at 8°C for 18 h, the upper milky liquid and light yellow liquid was carefully removed. The supernatants were collected and solid potassium bromide was added. The liquid was centrifuged at 42,000 rpm at 8°C for 20 h, the upper orange–yellow liquid was carefully collected, which was LDL ($d = 1.019$–1.063 g/ml). LDL was dialyzed against phosphate buffered saline (PBS) buffer containing 200 μM EDTA for 48 h. Ox-LDL was prepared by incubation of LDL with 10 μM $CuSO_4$ for 24 h at 37°C, followed by addition of 100 μM EDTA for 24 h, and then dialyzed in PBS buffer for 24 h at 4°C, changing the buffer every 8 h [30]. The concentration of protein was determined by the BCA protein assay kit.

Analysis of oxidation and electrophoretic mobility in LDL modified with $CuSO_4$

Following oxidative modification of LDL with $CuSO_4$ (10 μM, 24 h, 37°C) lipid peroxidation was assessed by the following procedures. The electrophoretic mobility of native or Ox-LDL was measured on 1% agarose gel [31]. The lipoprotein pattern was visualized by staining the film with a lipid-specific stain. Relative electrophoretic mobility was defined as the ratio of the distances migrated from the origin by modified LDL versus native LDL (**Fig. 1**).

Figure 1 Electrophoretic mobility of native and Ox-LDL was measured on 1% agarose gel The lipoprotein pattern was visualized by staining the film with a lipid-specific stain. Relative electrophoretic mobility was defined as the ratio of the distances migrated from the origin by modified LDL versus native LDL.

Total RNA extraction, cDNA synthesis, and RT–PCR analysis

RAW264.7 cells (2×10^6 cells/well) were incubated with or without the indicated treatment. Total RNA was extracted using TRIzol, isopropyl alcohol, and chloroform at various treatments of factor. For RT–PCR analyses, the first strand cDNA synthesis containing 2 μg of total RNA was primed with oligo(dT) and RevertAid first strand cDNA kit under optimized conditions according to the manufacturer's protocol. PCRs were performed using 2× Taq PCR master mix from TIANGEN (Beijing, China) and specific primers for mouse PPARγ, adipophilin, and GAPDH, which were designed as follows: PPARγ, 5′-CC GAAGAACCATCCGATTGA-3′ (forward) and 5′-CGGG AAGGACTTTATGTATGA-3′ (reverse); adipophilin, 5′-C CAAGGATTCTGTAGCCAGCA-3′ (forward) and 5′-AC AGTGGGACTCATCGGTGTC-3′ (reverse); GAPDH, 5′-C AGTCCATGCCATCACTGCCA-3′ (forward) and 5′-AGG TGGAGGAGTGGGTGTCGC-3′ (reverse). The quantitative results for PPARγ and adipophilin were normalized by the levels of GAPDH mRNA. The resulting products were separated on a 1.2% agarose gel and stained with ethidium bromide.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis

RAW264.7 cells (2×10^6 cells/well in 6-well plates) were immediately lysed in ice-cold lysis buffer containing 50 mM Tris, 150 mM sodium chloride, 2 mM ethylene diaminetera acetic acid (EDTA), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium fluoride, 1 mM sodium ortho-vanadate, 15 mM sodium pyrophosphate, and 10 mM β-glycerophosphate, and then centrifuged (10,000 rpm at 4°C for 10 min). Supernatants were used as sample proteins. Protein concentrations were determined by Coomassie brilliant blue. Samples were applied to 10% SDS gels, followed by electrophoretic transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, USA). Then the membrane was blocked at 4°C for 4–6 h and washed with Tris-buffered saline containing Tween 20 (TBS-T). For immunodetection, the blocked membrane was incubated at room temperature for 3 h with anti-PPARγ, anti-ERK1/2, anti-p-ERK1/2, anti-adipophilin, and anti-β-actin primary antibodies at a dilution of 1:1000. After washing three times with TBS-T, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies at a dilution of 1:3000, washed with TBS-T. The immunoreactive proteins were visualized by chemiluminescence using an ECL plus kit with exposure to X-ray film. Immunoreactive bands were quantified by using AlphaImager2200 analysis software,

and the data represent the protein variation after treatment of the factors.

Oil red O staining

RAW264.7 cells, measured for lipid accumulation through staining of neutral fats and cholesterol esters (CEs) with oil red O, were placed into 6-well plates with slides at a density of 4×10^5 cells/cm^2 and cultured for 24 h, then treated with 50 μg/ml Ox-LDL for 1, 6, 12, or 24 h, or pre-treated with drugs for 2 h followed with 50 μg/ml Ox-LDL for 24 h, after which the cells were rinsed with PBS and fixed with 10% formalin for 5 min at room temperature, and incubated in fresh formalin for at least 1 h. The cells were then rinsed again with 60% isopropanol and then incubated with fresh filtered oil red O solution (60% saturated oil red O/40% deionized water) for 20 min. For analysis, slides were then washed in isopropanol for 10 min, rinsed in tap water, counterstained with hematoxylin and mounted in glycerol/gelatin solution. And then images of cells were captured using a light microscope.

Intracellular total cholesterol and free cholesterol assay by high-performance liquid chromatogram

The RAW264.7 cells were treated with 50 μg/ml Ox-LDL for 24 h, or preincubated with 50 μM PD98059 or 2 μM GW1929 or 5 μM T0070907 for 2 h followed by 50 μg/ml Ox-LDL for 24 h. Then the total cholesterol (TC) or free cholesterol (FC) in cells was detected by high-performance liquid chromatogram (HPLC), respectively. In brief, stigmasterol was used as internal standard and standard curve was obtained. After treatment, the cells were washed three times with PBS and lysed in solution the same as used for proteins; then the samples were centrifuged (3000 rpm, 4°C, 10 min) and the supernatants were collected. After protein quantification with the BCA kit, proteins included in the remaining supernatants were precipitated with 7.2% trichloroacetic acid, the supernatants were collected for cholesterol test after being centrifuged (800 rpm, 4°C, 10 min). For obtaining the TC, 200 μl of potassium hydroxide solution (8.9 M) was added to 100 μl of supernatant to hydrolyze CE, or 200 μl of sodium hydroxide (1 M) was added for obtaining the FC. All of the samples were mixed with the internal standard solution, respectively, extracted with *n*-hexane and ethanol and oxidized with 2 M chromium trioxide oxidation. Then samples were dried in a vacuum and dissolved in 200 μl of acetonitrile-isopropanol (80:20) and determined by HPLC.

Statistical analysis

The data were analyzed by ANOVA and were expressed as the mean ± SE. Differences were considered significant when $P < 0.05$.

Results

Ox-LDL increases PPARγ and adipophilin expression, lipid droplet accumulation, and ERK1/2 activation in RAW264.7 cells

We first examined the effects of Ox-LDL on PPARγ and adipophilin expression in RAW264.7 cells. As shown in **Fig. 2(A,B)**, Ox-LDL increased PPARγ and adipophilin expression in a time-dependent manner. Both protein and mRNA levels of adipophilin and PPARγ were increased at 1 h, and these increases remained for up to 24 h. We also measured the intracellular lipid droplets along with increased time of Ox-LDL incubation. Ox-LDL also increased the amounts of intracellular lipid droplets in a time-dependent manner [**Fig. 2(C), Table 1**]. Moreover, Ox-LDL increased PPARγ and adipophilin proteins expression by 1.8- and 2.9-fold, respectively, until 24 h ($P < 0.01$, compared with control).

To confirm the effect of Ox-LDL on ERK1/2 activation, we carried out the immunoblotting of ERK1/2 and p-ERK1/2. As shown in **Fig. 2(A)**, ERK1/2 was not changed obviously along with the time of Ox-LDL incubation. On the other hand, p-ERK1/2 increased in half an hour, and up to 24 h. The data showed that ERK1/2 activation was increased by the time of Ox-LDL incubation. We also prepared 10% BSA as control of Ox-LDL incubation with the same condition medium. As shown in **Fig. 2(A)**, PPARγ and adipophilin expression and phosphorylation of ERK1/2 were similar to the control.

Ox-LDL-induced PPARγ and adipophilin expression and intracellular lipid droplet accumulation were mediated by ERK1/2 signals

Senokuchi and coworkers have previously reported that Ox-LDL can activate ERK1/2 [32], which is known to be involved in PPARγ expression induced by several stimuli in macrophages. Therefore, we speculated on the involvement of MAPK signals in Ox-LDL-induced PPARγ and adipophilin expression in RAW264.7 cells. Since Ox-LDL (50 μg/ml) increased the phosphorylation of ERK1/2 [**Fig. 2(A)**], we therefore examined the effect of ERK1/2-specific inhibitor PD98059 (50 μM) [33,34] on Ox-LDL-induced PPARγ protein and mRNA expression. The incubation of RAW264.7 cells with 50 μg/ml Ox-LDL for 24 h increased PPARγ protein and mRNA levels by 1.8- and 3.4-fold, respectively [**Fig. 2(A,B)**], which was blocked by PD98059 [**Fig. 3(A,B)**]. In addition, Ox-LDL-induced intracellular lipid droplet increase was also inhibited by PD98059 [**Fig. 3(C), Table 1**]. These results suggested that the expression of PPARγ and adipophilin were enhanced by 50 μg/ml Ox-LDL in the absence of PD98059. In contrast, the increases of PPARγ and

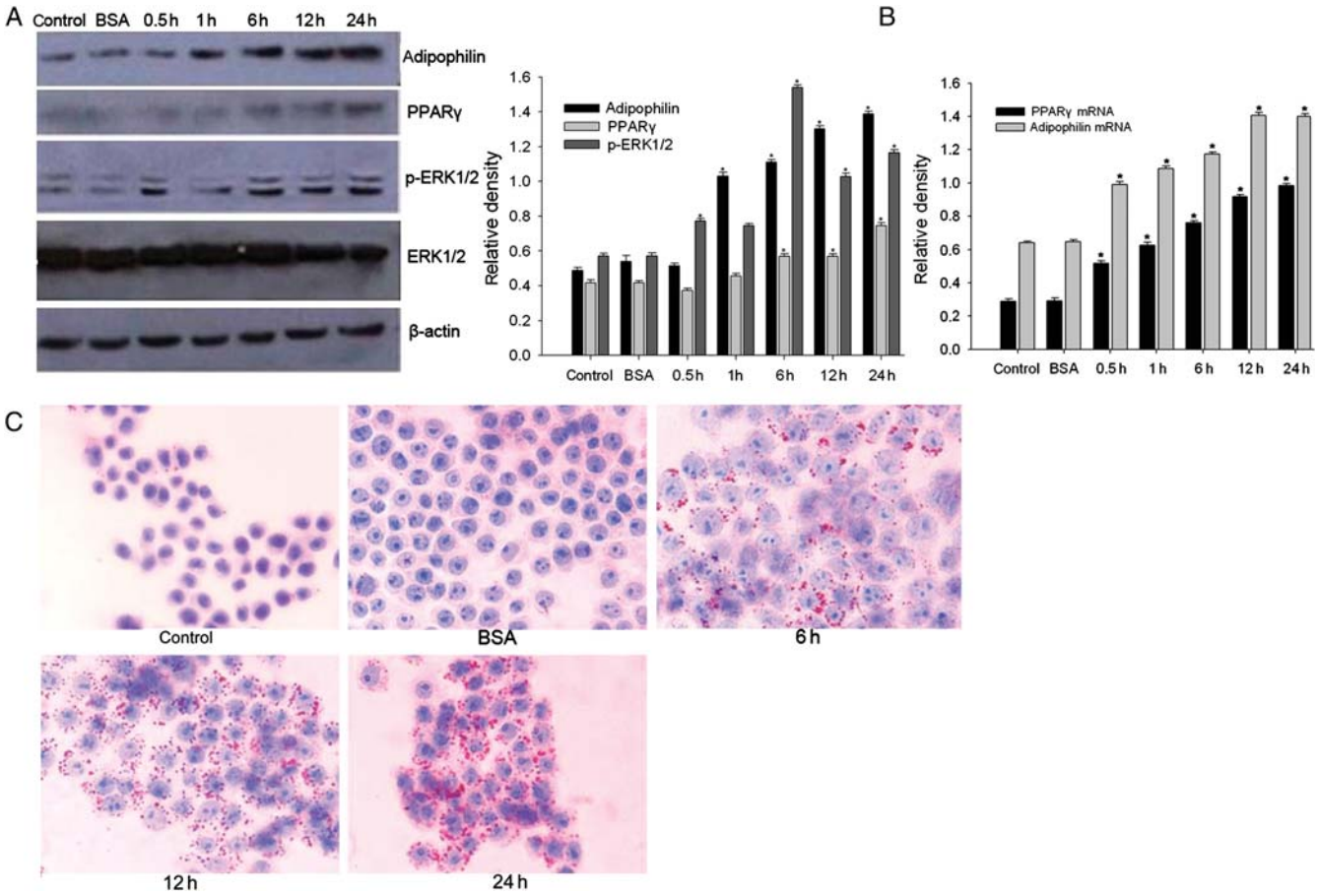

Figure 2 Ox-LDL increases PPARγ and adipophilin expression, and also increases phosphorylation of ERK1/2 and lipid droplet accumulation (A) RAW264.7 cells were incubated with 50 μg/ml Ox-LDL for 0, 0.5, 1, 6, 12, and 24 h or with 10% BSA for 24 h. Protein samples were immunoblotted with anti-PPARγ, anti-adipophilin, anti-p-ERK, anti-ERK1/2, or anti-β-actin antibodies by western blot analysis. (B) RAW264.7 cells were incubated with 50 μg/ml Ox-LDL for 0, 0.5, 1, 6, 12, and 24 h or with 10% BSA for 24 h. Total RNA was extracted with TRIzol. For RT–PCR analyses, the first strand cDNA synthesis containing 2 μg of total RNA was primed with oligo(dT) and the RevertAid first strand cDNA kit. PCRs were performed using 2× Taq PCR master mix and specific primers for mouse PPARγ, adipophilin, and GAPDH. Data are represented as the mean ± SE of seven separate experiments. $*P < 0.05$ versus control. (C) RAW264.7 cells were incubated with 50 μg/ml Ox-LDL for 0, 6, 12, and 24 h or 10% BSA for 24 h. Then the cells were stained with oil red O. Magnification, 100×.

Table 1 Effects of ERK1/2 inhibitor PD98059, PPARγ agonist GW1929, and antagonist T0070907 on intracellular lipid content in RAW264.7 cells

Group	TC (mg/g protein)	FC (mg/g protein)	CE/TC (%)
Control	161 ± 29	136 ± 21	15.4 ± 2.6
24 h Ox-LDL	251 ± 29*	165 ± 24*	34.2 ± 1.1*
PD98059 + 24 h Ox-LDL	205 ± 28*,#	165 ± 24*	28.2 ± 2.9*,#
GW1929 + 24 h Ox-LDL	296 ± 35*,#	165 ± 24*	38.3 ± 1.5*,#
T0070907 + 24 h Ox-LDL	200 ± 25*,#	165 ± 24*	29.3 ± 2.3*,#

Data are presented as the mean ± SE ($n = 3$). $*P < 0.05$ versus control, $^{\#}P < 0.05$ versus 24 h.

adipophilin expression induced by Ox-LDL were prevented by the presence of PD98059. These data showed that Ox-LDL-induced augmentation of PPARγ and adipophilin expression and intracellular lipid droplet accumulation are mediated by ERK1/2.

PPARγ is involved in Ox-LDL-induced adipophilin expression and intracellular lipid droplet accumulation

We further examined whether Ox-LDL-induced-adipophilin expression measured by RT–PCR and western blot analysis and intracellular lipid accumulation detected by oil red O staining and HPLC are relating to PPARγ. After treatment of RAW264.7 cells with agonist GW1929 of PPARγ for 2 h, followed by 50 μg/ml Ox-LDL for 24 h, both protein and mRNA levels of adipophilin were increased by GW1929 [**Fig. 4(A,B)**]. We further examined the intracellular lipid accumulation. In contrast to some other data [35,36], which showed that the activation of PPARγ can impact the receptors related to lipid efflux and

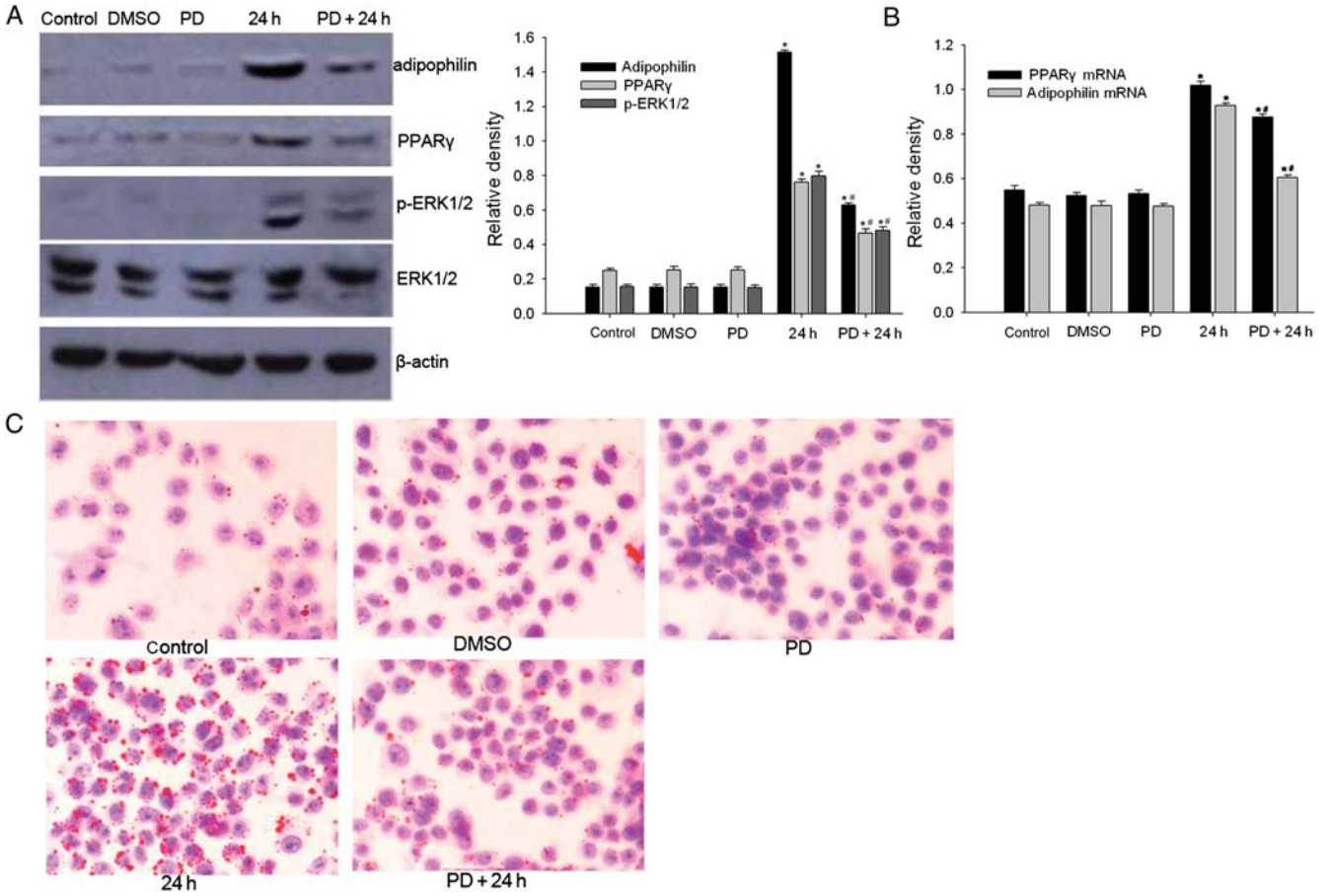

Figure 3 Ox-LDL-induced PPARγ, adipophilin expression, and intracellular lipid droplet accumulation were mediated by ERK1/2 signals (A) RAW264.7 cells were preincubated with 50 μM PD98059 (PD) for 2 h followed by 50 μg/ml Ox-LDL for 24 h. Cell proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with polyclonal antibodies anti-p-ERK, anti-ERK1/2, anti-PPARγ, anti-adipophilin, or anti-β-actin antibodies. (B) RAW264.7 cells were preincubated with 50 μM PD98059 (PD) for 2 h, and then with 50 μg/ml Ox-LDL for 24 h. PPARγ and adipophilin mRNA expression were evaluated by RT−PCR. Data are represented as the mean ± SE of five separate experiments. $^{*}P < 0.05$ versus control. Adipophilin, PPARγ, and p-ERK1/2 expression in the cells that were preincubated with 50 μM PD98059 (PD) for 2 h followed by 50 μg/ml Ox-LDL for 24 h were suppressed obviously. $^{\#}P < 0.05$ versus 24 h Ox-LDL. (C) RAW264.7 cells were preincubated with 50 μM PD98059 (PD) for 2 h, incubated with 50 μg/ml Ox-LDL, and then stained with oil red O. Magnification, 100×.

influx, there is no obviously impact on intracellular cholesterol. Ox-LDL increased the intracellular lipid accumulation, which was increased by agonist GW1929 of PPARγ [**Fig. 4(C), Table 1**].

To further confirm the effects of PPARγ on adipophilin expression induced by Ox-LDL, we examined the role of the PPARγ antagonist T0070907 on Ox-LDL-induced adipophilin expression and lipid accumulation in RAW264.7 cells, which were preincubated with the drug for 2 h, and then were treated using 50 μg/ml Ox-LDL. As shown in **Fig. 5(A,B)**, the antagonist attenuated the adipophilin expression and intracellular lipid accumulation [**Fig. 5(C), Table 1**].

Discussion

Macrophages contribute to the formation of arterial lesions by accumulating excessive amounts of lipids, mainly CE, through the accumulation of CE by a variety of mechanisms, including acyl-CoA:cholesterol acyltransferase 1 (ACAT1), the enzyme responsible for the esterification of intracellular FC. Thus, elimination of accumulated CE from macrophage foam cells represents a promising therapeutic approach to prevent atherosclerotic lesions.

ADRP or adipophilin is a 50 kDa protein, which was first identified by Serrero *et al*. [4], who showed that adipophilin mRNA is expressed most strongly in adipose tissue and is induced very early during adipocyte differentiation. Its expression has now been found in diverse cell types in culture and specific cell types in tissues and in particular, adipophilin is associated with the lipid fractions in the cell [5,6]. Lipid storage is facilitated by the production of large amounts of lipid vesicle coating proteins such as adipophilin and perilipin, etc. [7], thus preventing lipid efflux from macrophages [37]. These data suggest that adipophilin may be a specific marker for lipid accumulation in the cells. Despite its

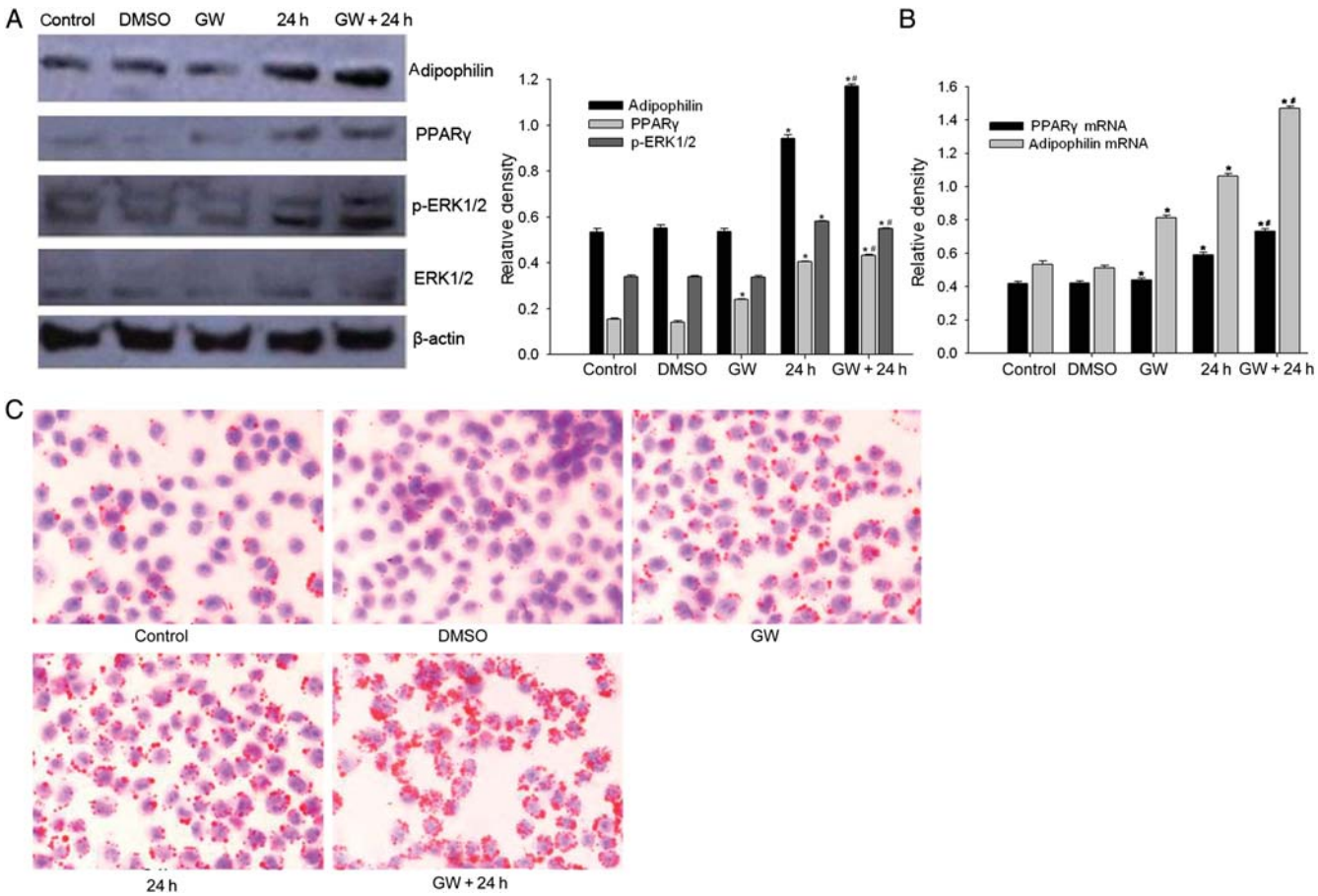

Figure 4 Effects of GW1929, agonist of PPARγ, on adipophilin expression and lipid accumulation in RAW264.7 cells (A) RAW264.7 cells were preincubated with 2 μM GW1929 (GW) for 2 h followed by 50 μg/ml Ox-LDL for 24 h. Protein samples were immunoblotted with anti-p-ERK, anti-ERK1/2, anti-PPARγ, anti-adipophilin, or anti-β-actin antibodies. (B) RAW264.7 cells were preincubated with 2 μM GW1929 (GW) for 2 h, and then with 50 μg/ml Ox-LDL for 24 h. PPARγ and adipophilin mRNA expression were evaluated by RT–PCR. Data are represented as the mean ± SE of five separate experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus 24 h Ox-LDL. (C) RAW264.7 cells were preincubated with 2 μM GW1929 (GW) for 2 h, incubated with 50 μg/ml Ox-LDL, and then stained with oil red O. Magnification, 100×.

observed overexpression in lipid-loaded macrophages [8–10], little data exist on the mechanism of adipophilin in lipid storage and cholesterol efflux in these cells and its relevance to atherosclerosis. To elucidate the potential mechanism of adipophilin in atherosclerosis, our present study identified the induced expression of adipophilin protein and mRNA in a mouse macrophage-like cell line RAW264.7 cells stimulated with Ox-LDL. In this article, we reported a high expression level of adipophilin in RAW264.7 cells treated with Ox-LDL for 24 h compared with 0 h [**Fig. 2(A,B)**]. We also showed, for the first time to our knowledge, that Ox-LDL induced adipophilin overexpression in RAW264.7 cell, by which enhanced the accumulation of lipids, which was related to ERK1/2 [**Fig. 3(A-C)**]. Furthermore, adipophilin expression and the number of intracellular lipid droplets were not affected by the ERK1/2 antagonist alone in RAW264.7 cells [**Fig. 3(A-C)**]. Our data indicated that macrophage adipophilin expression is a consequence of lipid accumulation and contributes to further accumulation of lipids by inhibiting cellular cholesterol efflux.

The activation of MAPK-signaling pathways has been associated with the stimulatory effects of LDL, relevant to vascular pathology, in cultured cells implicated in macrovascular disease [38,39] and more recently in renal disease [40,41]. Native LDL and Ox-LDL have been demonstrated to activate ERK1/2, which are early mitogenic signals in mesangial cells [42]. It has been reported that PPARγ agonists improve endothelial function, inhibit the proliferation and migration of vascular smooth muscle cells, and inhibit the production of inflammatory cytokines and MMPs in macrophages [43], which is beneficial in preventing atherosclerosis. However, other reports indicated that moderate Ox-LDL induces PPARγ activation, and that oxidized phospholipids, which are components of Ox-LDL, are involved in Ox-LDL-induced PPARγ activation [15,19].

It has been reported that in murine myoblasts, treatment with ciglitazone, and GW1929, which are PPARγ agonists, leads to ERK1/2 phosphorylation in a time- and concentration-dependent manner [44], showing that ERK1/2 is related with PPARγ activation. In the present study,

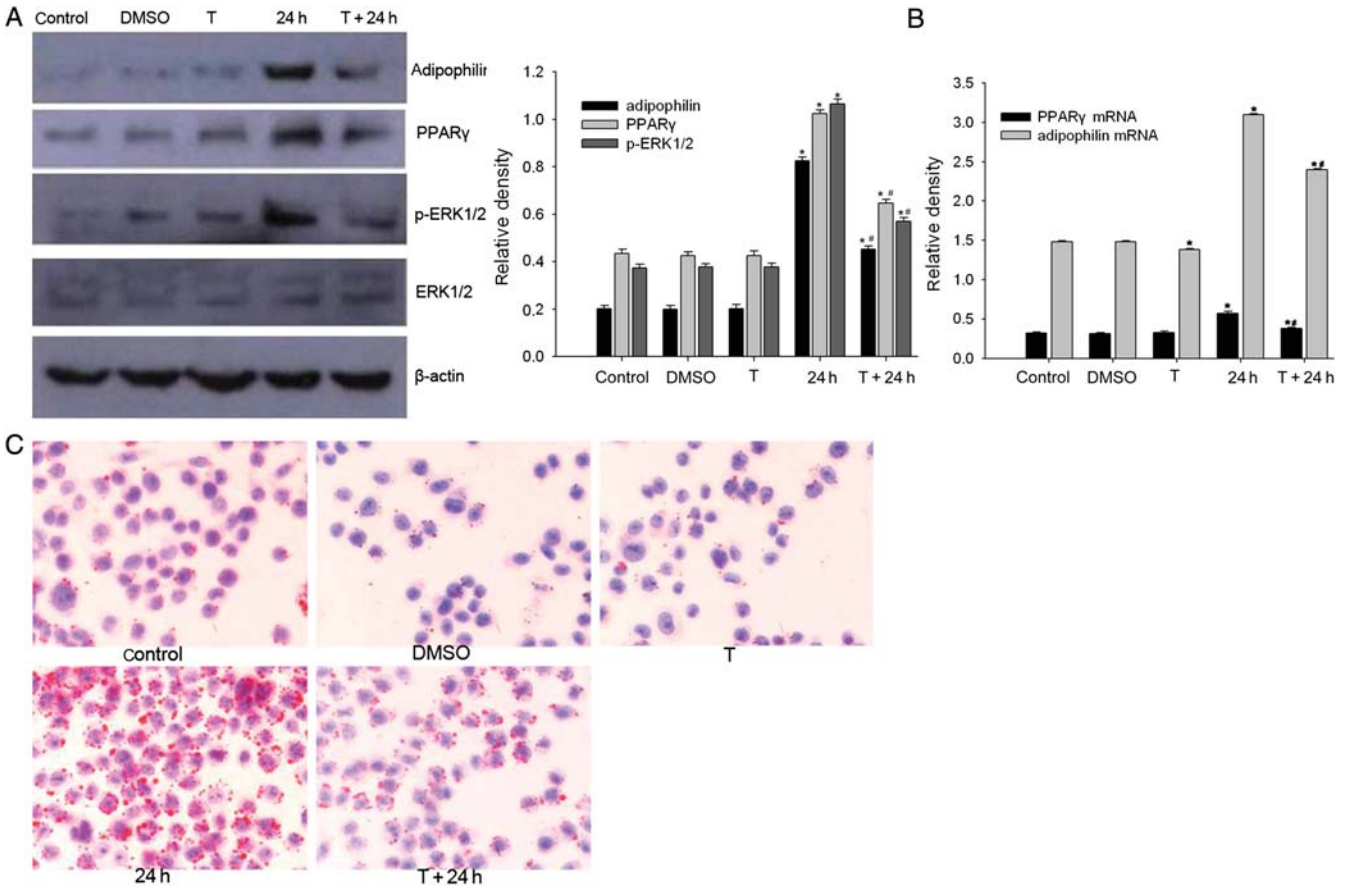

Figure 5 Blockade of PPARγ by T0070907, the antagonist of PPARγ, inhibits Ox-LDL-induced adipophilin protein and mRNA expression in RAW264.7 cells (A) RAW264.7 cells were preincubated with 5 μM T0070907 (T) for 2 h followed by 50 μg/ml Ox-LDL for 24 h. Protein samples were immunoblotted with anti-p-ERK, anti-ERK1/2, anti-PPARγ, anti-adipophilin, or anti-β-actin antibodies. (B) RAW264.7 cells were preincubated with 5 μM T0070907 (T) for 2 h, and then with 50 μg/ml Ox-LDL for 24 h. PPARγ and adipophilin mRNA expression were evaluated by RT−PCR. Data are represented as the mean ± SE of five separate experiments. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus 24 h Ox-LDL. (C) RAW264.7 cells were preincubated with 5 μM T0070907 (T) for 2 h, incubated with 50 μg/ml Ox-LDL, and then stained with oil red O. Magnification, 100×.

we revealed that the effect of PPARγ and adipophilin expression by Ox-LDL was blocked by PD98059, a MEK inhibitor. We also indicated that Ox-LDL-induced PPARγ activation was also mediated by phosphorylation of ERK1/2 [**Fig. 3(A,B)**]. Furthermore, PD98059 pretreatment reduced the expression of PPARγ and adipophilin apparently compared with the 24 h Ox-LDL group in the present study. We demonstrated here, possibly for the first time, that Ox-LDL-induced adipophilin expression in RAW264.7 cells was mediated by the activation of ERK1/2. In addition, Ox-LDL-induced expression of PPARγ was inhibited by inhibition of ERK1/2, suggesting that Ox-LDL-induced expression of both PPARγ and adipophilin is mediated by ERK1/2 in macrophages. Therefore, Ox-LDL may cause PPARγ and adipophilin expression via an ERK1/2-dependent pathway. This observation indicates that the MAPK pathway operates upstream of PPARγ and is involved in PPARγ and adipophilin expression in RAW264.7 cells and intracellular lipid droplet accumulation by Ox-LDL. Interestingly, our recent report revealed that although inhibitors of ERK1/2 suppressed the Ox-LDL-induced increase in adipophilin level by 60% or more, they suppressed PPARγ expression by 30%. These results suggested that mechanisms other than PPARγ are involved in Ox-LDL-induced adipophilin expression, because it was reported that other members of PAT, including perilipin and Tip47 were overexpressed in atherosclerotic plaques [45,46], which could favor cholesterol accumulation.

Other investigators have reported that MAPK-signaling pathways influence PPARγ activation [27,47]. Therefore, Ox-LDL-induced ERK1/2 activation may activate PPARγ and adipophilin, thus resulting in the transcriptional activation of the adipophilin gene.

We found that the PPARγ expression induced by Ox-LDL and its agonist GW1929 increased the amount of intracellular lipid droplets and adipophilin expression. Moreover, the antagonist T0070907 of PPARγ decreased intracellular lipid droplets and adipophilin, suggesting that Ox-LDL-induced PPARγ expression mediated the

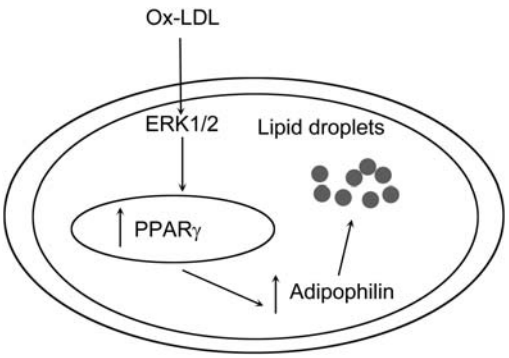

Figure 6 Schematic representation of the effects of Ox-LDL on PPARγ and adipophilin expression and intracellular lipid droplet accumulation in RAW264.7 cells The results of present study revealed the following scheme for the mechanism of the Ox-LDL-induced PPARγ and adipophilin expression and intracellular lipid droplet accumulation. When cells are treated with Ox-LDL, PPARγ, and adipophilin increased, which was the same as for lipid accumulation in cells. Furthermore, Ox-LDL also induces ERK1/2 phosphorylation. Phosphorylation of ERK1/2 increases PPARγ expression, and the increasing of PPARγ mediates the accumulation of intracellular lipid droplets and upregulation of adipophilin.

downstream production of adipophilin, therefore affecting intracellular lipids. These results suggest that ERK1/2-mediated increase in the PPARγ level is one of the mechanisms by which Ox-LDL induces expression of adipophilin. Most studies have reported that PPARα suppresses macrophage-derived foam cell formation, so it is benefit to depress the formation of atherosclerosis [48,49]. Moreover, activation of PPARγ can affect the receptors relating to lipid efflux and influx [35,36]; thus there is no obviously impact on intracellular cholesterol, suggesting that PPARγ acts as an inhibitor of atherosclerosis. In view of the above research, we took PPARγ to our study. According to our findings, it is possible that PPARγ may play an adverse role in controlling atherosclerotic progression through the activation of adipophilin in contrast, which was consistent with some other data [50,51]. In the present study, we revealed for the first time that the activities of PPARγ were increased in RAW264.7 cells incubated with Ox-LDL. Thus, Ox-LDL-induced activation of PPARγ fundamentally accelerates the uptake of Ox-LDL via increase in adipophilin expression. The study showed that Ox-LDL induced the phosphorylation of ERK1/2, and then the phosphorylation of ERK1/2 increased PPARγ expression. However, as shown in **Fig. 5(A)**, T0070907, an antagonist of PPARγ, could effectively inhibit the phosphorylation of ERK1/2, indicating that the antagonist and agonist of PPARγ may be capable of regulating the phosphorylation of ERK1/2, further regulating the expression of PPARγ; but the study used mouse macrophages and human Ox-LDL, and LDL is low in mouse. Thus, we should also pay attention to the limitation of the study.

In conclusion, we demonstrated that Ox-LDL-induced adipophilin expression was mediated by an increase in PPARγ, via ERK1/2 activation in a mouse macrophage-like cell line RAW264.7 (**Fig. 6**). In addition, inhibition of PPARγ and ERK1/2 suppressed Ox-LDL-induced adipophilin expression and intracellular lipid droplets. These unique signals of RAW264.7 cells induced by Ox-LDL may be one of the mechanisms of promoting progression of atherosclerosis. Thus, the involvement of the ERK1/2-PPARγ pathway in Ox-LDL-induced expression of adipophilin may be a novel therapeutic approach for atherosclerosis.

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