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Original Article

OxLDL up-regulates Niemann–Pick type C1 expression through ERK1/2/COX-2/PPARα-signaling pathway in macrophages

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The Niemann–Pick type C1 (NPC1) is located mainly in the membranes of the late endosome/lysosome and controls the intracellular cholesterol trafficking from the late endosome/lysosome to the plasma membrane. It has been reported that oxidized low-density lipoprotein (oxLDL) can up-regulate NPC1 expression. However, the detailed mechanisms are not fully understood. In this study, we investigated the effect of oxLDL stimulation on NPC1 expression in THP-1 macrophages. Our results showed that oxLDL up-regulated NPC1 expression at both mRNA and protein levels in a dose-dependent and time-dependent manner. In addition, oxLDL also induced the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). Treatment with oxLDL significantly increased cyclooxygenase-2 (COX-2) mRNA and protein expression in the macrophages, and these increases were suppressed by the ERK1/2 inhibitor PD98059 or ERK1/2 small interfering RNA (siRNA) treatment. OxLDL up-regulated the expression of peroxisome proliferator-activated receptor α (PPARα) at the mRNA and protein levels, which could be abolished by COX-2 siRNA or COX-2 inhibitor NS398 treatment in these macrophages. OxLDL dramatically elevated cellular cholesterol efflux, which was abrogated by inhibiting ERK1/2 and/or COX-2. In addition, oxLDL-induced NPC1 expression and cellular cholesterol efflux were reversed by PPARα siRNA or GW6471, an antagonist of PPARα. Taken together, these results provide the evidence that oxLDL can up-regulate the expression of the NPC1 through ERK1/2/COX-2/PPARα-signaling pathway in macrophages.

Keywords Niemann–Pick type C1; oxLDL; ERK1/2; COX-2; PPARα; cholesterol

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Introduction

Atherosclerosis is a complex, chronic inflammatory disease of the vessel wall with lipid–laden lesions, which may block the normal blood flow and may be a major life-threatening condition in humans [1]. Accumulation of modified low-density lipoprotein (LDL), such as oxidized LDL (oxLDL), and recruitment of monocytes in the arterial subendothelial spaces are early events in atherosclerosis [2,3]. Macrophages, which are derived from monocytes in these areas, take up oxLDL through the scavenger receptor (SR) pathways and finally become foam cells [4]. Foam cells are well known to play a critical role in the initiation and progression of atherosclerosis. The mobilization of intracellular cholesterol from the late endosome/lysosome (LE/LY) to the plasma membrane and its efflux to extracellular acceptors are important mechanisms in the regulation of intracellular cholesterol levels, which constitute the first step of reverse cholesterol transport, a pathway of cholesterol transport from peripheral tissues to the liver [5,6].

The Niemann–Pick type C1 (NPC1) is an integral membrane protein, containing a sterol-sensing domain that participates in cholesterol trafficking from the LE/LY to the plasma membrane and back to the endoplasmic reticulum, and is therefore thought to protect from cholesterol intracellular accumulation [7,8]. NPC disease is a fatal autosomal recessive disorder. Ninety-five percent of cases were caused by mutations in the *NPC1* gene, the remaining 5% of cases were resulted from mutations in the *NPC2* gene [9]. The major biochemical manifestation of NPC1 deficiency is an abnormal accumulation of LDL-derived cholesterol in the intracellular LE and LY compartment [10]. In the brain, NPC1 deficiency leads to neuronal degeneration due to a defective movement of lysosomal cholesterol to other locations, in particular, the

plasma membrane [11]. Recent studies have demonstrated that NPC1 serves an atheroprotective role in mice through regulating the liver X receptor (LXR)-dependent cholesterol efflux and the mitigation of cholesterol-induced oxidative stress in macrophages [12]. Our previous study also reported that synthetic LXR agonists, T0901317, could inhibit atherosclerosis development in apoE$^{-/-}$ mice, which was related to the up-regulation of NPC1 expression [13].

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that can phosphorylate their target proteins. Three major subfamilies of structurally related MAPKs have been identified in mammalian cells, which are termed extracellular signal-regulated kinase 1/2 (ERKl/2), p38 MAPK, and Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs) [14]. MAPKs are activated by a family of MAPK kinases and play important role in regulating cell growth, migration, differentiation, and the production of several inflammatory genes, including cyclooxygenase-2 (COX-2) [15,16]. Especially, ERK1/2-dependent COX-2 expression in response to several stimuli can induce the production of prostaglandins (PGs) [17].

Peroxisome proliferator-activated receptor α (PPARα) is a member of the nuclear hormone receptor family. It is highly expressed in tissues that demonstrate high catabolic rates for fatty acids, such as the liver, heart, kidney, and muscle, and is also present in cells of the arterial wall, including monocytes and macrophages [18]. PPARα controls macrophage lipid homeostasis and cholesterol efflux, the first step of the reverse cholesterol transport pathway. Agonists of PPARα have been reported to improve atherosclerosis in LDL receptor-deficient mice [19], which suggests that PPARα activation inhibits the development and progression of atherosclerosis.

More evidence indicated that oxLDL can induce the phosphorylation of ERK1/2 [20,21]. Statins have been reported to activate PPARα via ERK1/2-dependent COX-2 expression in macrophages [22]. Recently, Chinetti-Gbaguidi *et al.* [23] demonstrated that oxLDL strongly induced NPC1 expression and PPARα activators leading to a significant increase of NPC1 in human monocyte-derived macrophages. However, the detailed mechanisms of oxLDL-induced NPC1 expression are not fully understood. In this study, we try to explore the effects of oxLDL stimulation on the expression of NPC1, and the underlying molecular mechanisms. The results demonstrate that NPC1 could be induced by oxLDL in THP-1 macrophages and its expression might be involved in ERK1/2/COX-2/PPARα-signaling pathway.

Materials and Methods

Reagents

GW6471 and NS398 were purchased from Sigma (St Louis, USA). PD98059 was purchased from Biomol (Hamburg, Germany). Rabbit polyclonal anti-NPC1, anti-ERK1/2, anti-phospho ERK1/2 (anti-p-ERK1/2), anti-PPARα antibodies, mouse polyclonal anti-β-actin antibody, and horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Rabbit polyclonal anti-COX-2 antibody was purchased from Cayman Chemical (Ann Arbor, USA). TRIzol reagent (Invitrogen, Carlsbad, USA), BCA protein assay reagent (Pierce Chemical, Rockford, USA), ReverAid^{TM} first strand cDNA synthesis kit (Fermentas, Burlington, Canada), DyNAmoTM SYBR® Green qPCR kits (Finnzymes, Espoo, Finland), and immobilon-P transfer membranes (Millipore, Boston, USA) were obtained as indicated. All other chemicals were purchased from Sigma.

Cell culture

THP-1 cells, a human monocytic cell line, were purchased from the American Type Culture Collection (Manassas, USA) and seeded into six-well plates at 1.0×10^6 cells per well in RPMI1640 medium (Sigma) containing 10% fetal bovine serum, 20 IU/ml of penicillin, 20 μg/ml of streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2. The cells were differentiated into macrophages by the addition of 100 ng/ml phorbol 12-myristate 13-acetate for 72 h.

Isolation and oxidative modification of LDL

Human LDL ($d = 1.019-1.063$ g/ml) was isolated by sequential ultracentrifugation from the plasma of consenting normolipidemic participants, obtained after overnight fasting and dialyzed against phosphate buffered saline (PBS) at 4°C. The concentration of LDL proteins was determined by BCA protein assay reagent. Then, LDL was oxidized with CuSO4 at 37°C for 18 h. Then oxLDL was sterilized by filtration membrane and stored at 4°C as described previously [24].

RNA isolation and real-time quantitative polymerase chain reaction analysis

Total RNA from cells was extracted using TRIzol reagent in accordance with the manufacture's instructions. The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo (dT). Real-time quantitative polymerase chain reaction (PCR), using SYBR Green detection chemistry, was performed on Roche light cycler run 5.32 real-time PCR system (Roche Diagnostics, Mannheim, Germany). Specific primers for *NPC1*, *PPARα*, *COX-2*, and *β-actin* were designed as follows: *NPC1*, forward primer, 5′-TGAATGGGGTCTCCTTGGTC-3′ and reverse primer, 5′-CTCACTCGGCTTCCTTGTGTA -3′; *PPARα*, forward primer, 5′-GACTCAAGCTGGTGTATGACAAG T-3′ and reverse primer, 5′-CGTTGTGTAACCGCGAAA

T-3′; *COX-2*, forward primer, 5′-TTCAAATGAATTTGGA AATGCT-3′ and reverse primer, 5′-AGATCATCTCTGC CTGAGTATCTT-3′; and *β-actin*, forward primer, 5′-GT GGGGCGCCCCAGGCACCA-3′ and reverse primer, 5′-C TCCTTAATGTCACG CACGATTC-3′. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the ΔΔ Ct method and the expression of *β-actin* was used as the internal control.

Transfection of small interfering RNA

The small interfering RNA (siRNA) against ERK1/2, COX-2, PPARα, and an irrelevant 21-nucleotide siRNA duplex, which was used as a control, were purchased from Santa Cruz Biotechnology. THP-1 macrophages (2×10^6 cells/well) were transfected with the siRNA of COX-2, PPARα, or control, in the absence or presence of appropriate plasmids using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, real-time reverse transcription-PCR was performed.

Western blot analysis

Western blot analysis was performed as described previously [25,26]. In brief, cells were lysed with the lysis buffer containing 50 mM of Tris, 150 mM of sodium chloride, 2 mM of ethylene diamine tetraacetic acid, 1 mM of phenylmethyl-sulfonyl fluoride, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM of sodium fluoride, 1 mM of sodium orthovanadate, 15 mM of sodium pyrophosphate, and 10 mM of β-glycerophosphate, followed by centrifuging at 10,000 *g* at 4°C for 10 min. The supernatants were used as sample proteins. Protein concentrations were determined by the Micro BCA protein assay reagent, according to the manufacturer's protocol. Equal amounts of proteins (20 μg) were applied to 10% SDS polyacrylamide gels and then transferred to nitrocellulose membranes (Millipore). The membranes were incubated with the primary antibodies at a dilution of 1:1000 at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology). The proteins were visualized using a chemiluminescence method (electrochemiluminescence and western blotting detection system; Amersham Biosciences, Foster City, USA).

Cellular cholesterol efflux experiments

Cells were cultured as indicated above. Then, they were incubated with 0.2 μCi/ml [^{3}H]cholesterol. After 72 h, cells were subsequently washed with PBS and incubated overnight in RPMI 1640 medium containing 0.1% (w/v) bovine serum albumin (BSA) to allow the equilibration of [^{3}H]cholesterol in all cellular pools. Equilibrated [^{3}H]cholesterol-labeled cells were washed with PBS and incubated in 2 ml of efflux medium containing RPMI 1640 and 0.1% BSA with 25 μg/ml human plasma apolipoprotein A-I (apoA-I). A 150 μl of sample of efflux medium was taken at the times designated and passed through a 0.45-μm filter to remove any floating cells. Monolayer of cells was washed twice in PBS, and cellular lipids were extracted with isopropanol. Medium and cell-associated [^{3}H]cholesterol was then measured by liquid scintillation counting. Percent efflux was calculated by the following equation: [total media counts/(total cellular counts + total media counts)] × 100%.

Statistical analysis

All the results are expressed as mean ± standard deviation from three independent experiments. The results were analyzed by one-way analysis of variance and Student's *t*-test, using SPSS 13.0 software. Statistical significance was obtained when P values were <0.05.

Results

OxLDL increases the expression of NPC1 and activates ERK1/2 in THP-1 macrophages

NPC1 belongs to a network of proteins mediating post-lysosomal cholesterol trafficking to the plasma membrane, a crucial process governing the balance between macrophage cholesterol import and export, with potential consequences in atherogenesis [27]. In this study, we first examined the effect of oxLDL on the expression of NPC1 in THP-1 macrophages by real-time quantitative PCR and western blot assays, respectively. As shown in **Fig. 1(A–D)**, oxLDL obviously up-regulated NPC1 expression at both transcriptional and translational levels in dose-dependent and time-dependent manners.

To confirm the effect of oxLDL on ERK1/2 activation, we carried out the immunoblotting of ERK1/2 and p-ERK1/2 in THP-1 macrophages. Our results showed that ERK1/2 was not changed obviously along with the time of oxLDL incubation, whereas p-ERK1/2 was increased at 6 h and this increase was maintained until 24 h [**Fig. 1(E)**]. These data suggest that oxLDL can activate ERK1/2 in THP-1 macrophages.

OxLDL-induced COX-2 expression is mediated by ERK1/2 signals

It has been reported that oxLDL can activate ERK1/2 [28], which is known to be involved in COX-2 expression induced by several stimuli in macrophages. Therefore, we examined whether oxLDL induced COX-2 expression in THP-1 macrophages. The results showed that when the cells were treated with 50 μg/ml of oxLDL for 24 h, the mRNA and protein levels of COX-2 were increased by 1.7 and 1.5 fold, respectively, which could be blocked by

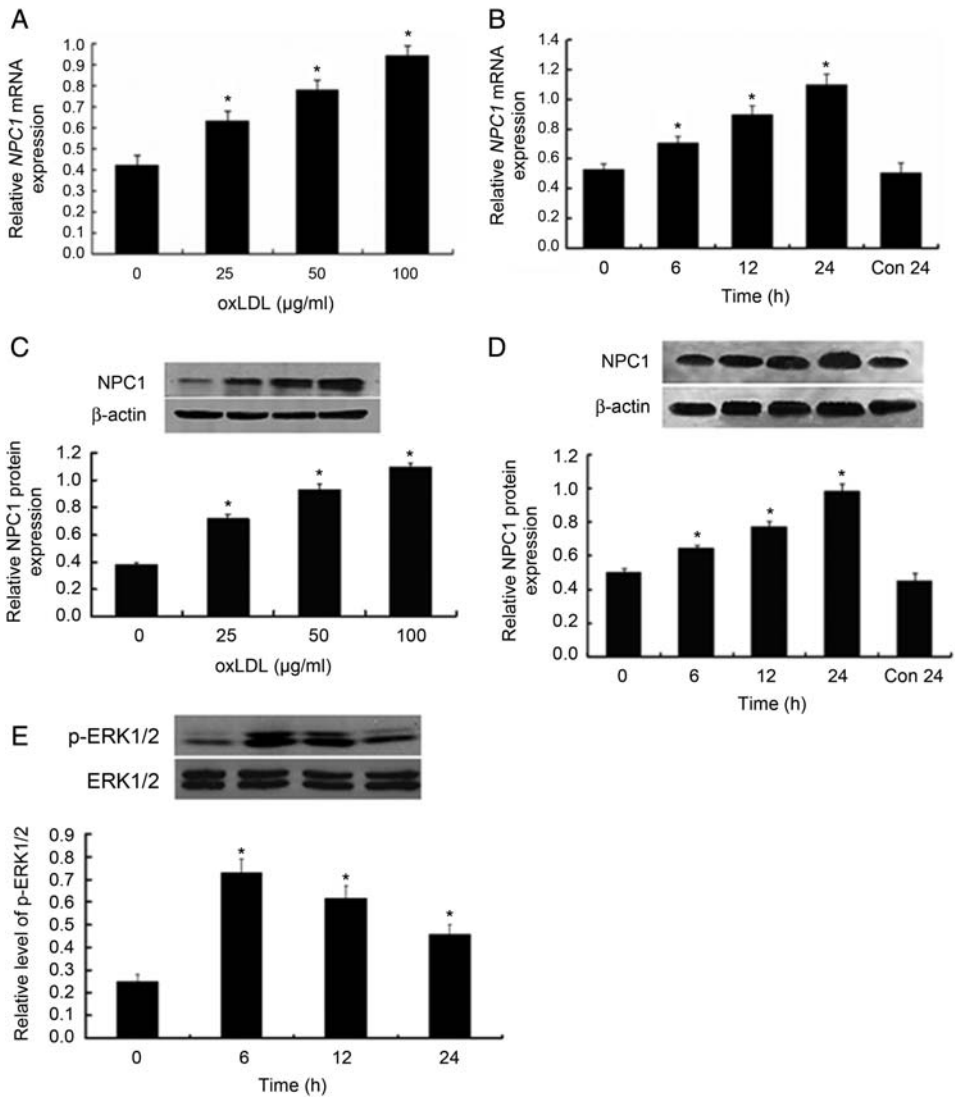

Figure 1 OxLDL induces NPC1 expression and ERK1/2 activation in THP-1 macrophages (A–D) In dose-dependent experiments, cells were treated with oxLDL at 0, 25, 50, and 100 μg/ml for 24 h, respectively. In addition, in time-dependent experiments, cells were treated with 50 μg/ml of oxLDL for 0, 6, 12, and 24 h, respectively. BSA (5 mg/ml) treatment for 24 h was used as the control (Con 24). (A, B) *NPC1* gene was measured by real-time quantitative PCR. (C, D) NPC1 protein expression was measured by western blot. *$P < 0.05$ vs. 0 μg/ml of oxLDL or Con 24. (E) Cells were incubated with 50 μg/ml of oxLDL for the indicated times. Protein samples were immunoblotted with anti-p-ERK1/2 or anti-ERK1/2 antibodies. *$P < 0.05$ vs. 0 h.

PD98059, an inhibitor of phosphorylation of ERK1/2 [**Fig. 2(A,B)**]. We further investigated the effect of ERK1/2 siRNA on ox-LDL-induced COX-2 expression. Western blot results showed ERK1/2 siRNA could down-regulate ERK1/2 protein expression by 78% [**Fig. 2(C)**] and significantly inhibited oxLDL-induced COX-2 mRNA and protein expression [**Fig. 2(D,E)**]. Collectively, all these results support the idea that oxLDL-induced COX-2 expression is mediated by ERK1/2.

COX-2 is involved in up-regulation of PPARα in response to oxLDL

We next analyzed whether oxLDL elevated PPARα expression in THP-1 macrophages. After 24-h incubation of cells with 50 μg/ml of oxLDL, both PPARα mRNA and protein were obviously increased as compared with the control group [**Fig. 3(A,B)**]. In contrast, oxLDL-induced up-regulation of PPARα was significantly prevented by NS398, an inhibitor of COX-2 [**Fig. 3(A,B)**]. To further confirm the involvement of COX-2 in oxLDL-induced PPARα expression, the effect of COX-2 siRNA was examined. As shown in **Fig. 3(C)**, treatment with COX-2 siRNA, the expression of COX-2 was suppressed by 86%. Compared with the control siRNA, COX-2 siRNA significantly suppressed oxLDL-induced PPARα mRNA and protein expression [**Fig. 3(D,E)**]. These results suggest that oxLDL-induced up-regulation of PPARα is mediated by ERK1/2-dependent COX-2 expression.

As PPARα controls cholesterol metabolism, we then examined the roles of ERK1/2 and COX-2 on cellular

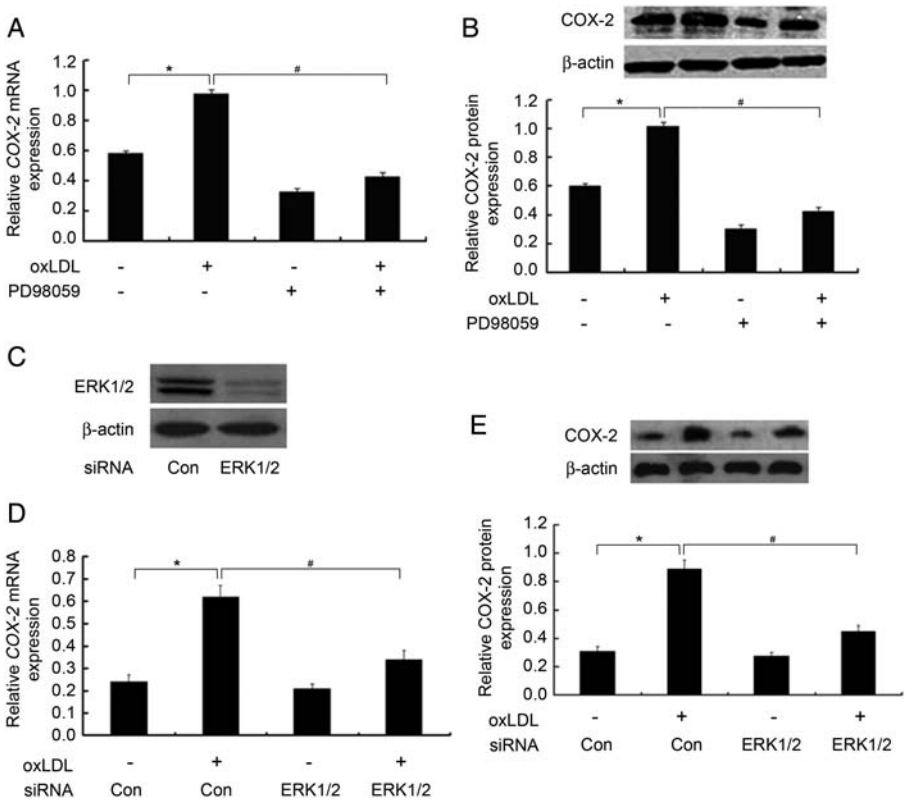

Figure 2 OxLDL-induced COX-2 expression is mediated by ERK1/2 in THP-1 macrophages (A, B) Cells were pre-incubated with 20 μM PD98059 for 1 h, and then incubated with 50 μg/ml oxLDL for 24 h. (A) The expression of mRNA for *COX-2* was evaluated by real-time quantitative PCR. (B) Protein samples from the cells were immunoblotted with anti-COX-2 or anti-β-actin antibodies. (C–E) Cells were transfected with ERK1/2 siRNA or control siRNA (Con), and then incubated with 50 μg/ml oxLDL for 24 h. (C) Protein samples were immunoblotted with anti-ERK1/2 or anti-β-actin antibodies. (D, E) The expression levels of COX-2 mRNA and protein were determined using real-time quantitative PCR and western blot assays. $^{*}P < 0.05$. $^{\#}P < 0.05$.

cholesterol efflux in THP-1 macrophages by liquid scintillation counting method. Treatment of cells with 50 μg/ml of oxLDL for 24 h strongly increased cellular cholesterol efflux, whereas this increase was inhibited by PD98059, NS398, or combination of both inhibitors [**Fig. 3(F)**]. In addition, ERK1/2 siRNA and COX-2 siRNA also had such effect [**Fig. 3(G)**].

Blockade of PPARα inhibits up-regulation of NPC1 by oxLDL and reduces cholesterol efflux

To determine whether PPARα was implicated in oxLDL-induced NPC1 expression, we treated THP-1 macrophages with PPARα antagonist GW6471 and PPARα siRNA, respectively. GW6471 stimulation significantly prevented oxLDL-induced up-regulation of NPC1 mRNA and protein [**Fig. 4(A,B)**]. PPARα siRNA transfection down-regulated PPARα protein expression by 82% [**Fig. 4(C)**] and suppressed oxLDL-induced NPC1 expression as well [**Fig. 4(D,E)**]. At the same time, cellular cholesterol efflux was markedly decreased in cells treated by oxLDL in combination with GW6471 compared with those treated by oxLDL alone [**Fig. 4(F)**]. Similar results were obtained using PPARα siRNA [**Fig. 4(G)**].

Discussion

In atherosclerosis, cellular cholesterol accumulates in lipid-engorged macrophage foam cells and this drives lipid deposition in the atherosclerotic plaques. The control of macrophage cholesterol homeostasis is of great importance in the prevention of atherosclerosis. Dysregulation of the balance of cholesterol influx and cholesterol efflux will lead to excessive accumulation of cholesterol in macrophages and their transformation into foam cells. Within the cells, modified LDL-derived cholesterol esters are hydrolyzed in lysosomes, and cholesterol is probably initially transported to the plasma membrane where it integrates the cell membrane [29]. Trafficking of cholesterol from the LE/LY to the plasma membrane is a protein-mediated process principally controlled by NPC1 [30]. The cholesterol in LE/LY is a priority as ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux [31]. Here, we provide evidence that oxLDL may significantly up-regulate

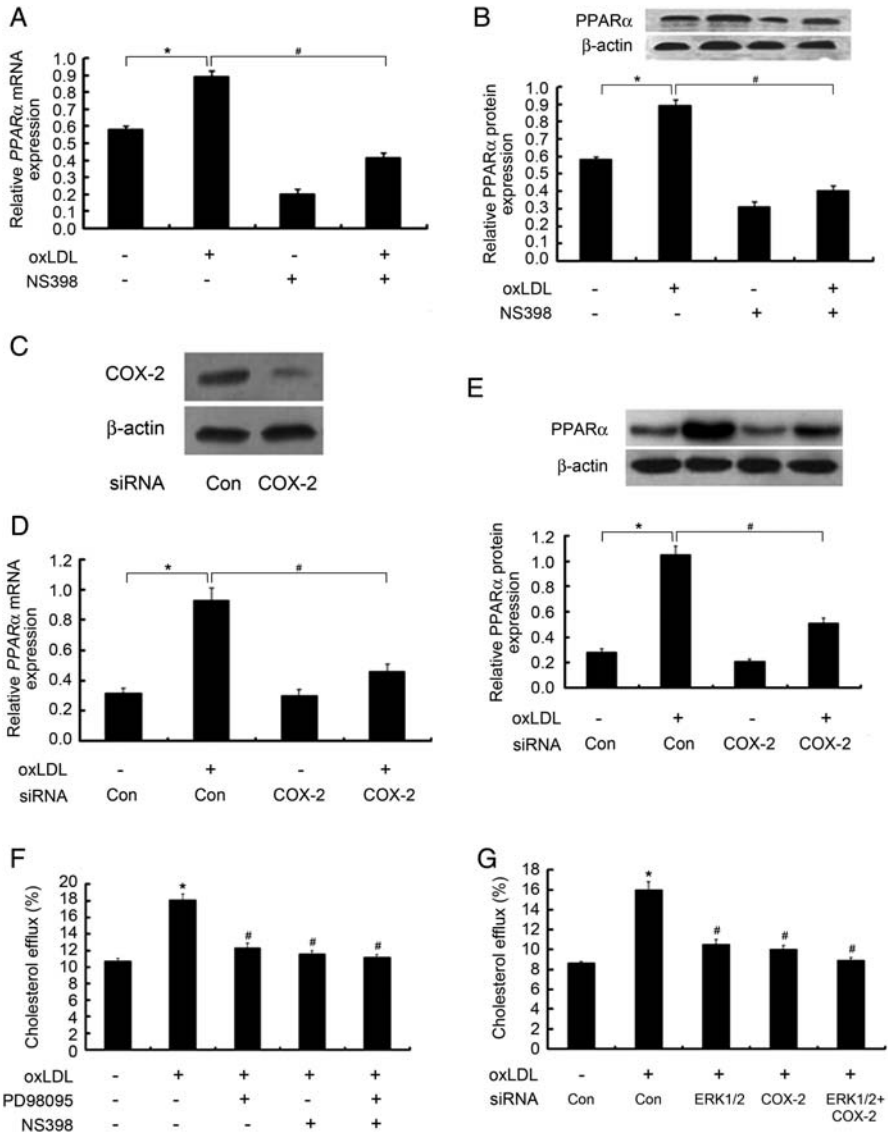

Figure 3 COX-2 is involved in oxLDL-induced PPARα expression in THP-1 macrophages (A, B) Cells were pretreated with 10 μM NS398 for 1 h, followed by treatment with 50 μg/ml of oxLDL for an additional 24 h. PPARα mRNA and protein expression was evaluated by real-time quantitative PCR and western blot assays. (C–E) After transfection of control or COX-2 siRNA, cells were incubated with 50 μg/ml oxLDL for 24 h. (C) Protein samples were immunoblotted with anti-COX-2 or anti-β-actin antibodies. (D, E) The expression of PPARα mRNA and protein were measured by real-time quantitative PCR and western blot, respectively. (F) Cells were treated with 20 μM PD98059, 10 μM NS398, or 20 μM PD98059 in combination with 10 μM NS398 for 1 h, and then incubated with 50 μg/ml of oxLDL for 24 h. Cellular cholesterol efflux was analyzed. $^{*}P < 0.05$ vs. untreated cells. $^{\#}P < 0.05$ vs. cells incubated with oxLDL alone. (G) Cells were transfected with control siRNA, ERK1/2 siRNA, COX-2 siRNA, or ERK1/2 siRNA plus COX-2 siRNA, and then incubated with 50 μg/ml of oxLDL for 24 h. Cellular cholesterol efflux was detected. $^{*}P < 0.05$ vs. cells incubated with control siRNA alone. $^{\#}P < 0.05$ vs. cells incubated with oxLDL plus control siRNA.

NPC1 expression and promote apoA-I-mediated cholesterol efflux in THP-1 macrophages.

OxLDL is widely believed to play an important role in the pathogenesis of atherosclerosis. The activation of MAPK-signaling pathways has been associated with the stimulatory effects of oxLDL, relevant to vascular pathology, in cultured cells implicated in macrovascular disease [32]. We found that oxLDL could activate ERK1/2 in THP-1 macrophages, which was also reported in endothelial cells and vascular smooth muscle cells [33,34]. Lectin-like oxLDL receptor-1 (LOX-1), which is an oxLDL receptor, has been shown to be associated with the activation of ERK1/2 induced by oxLDL in vascular smooth muscle cells [35]. Thus, it is possible that oxLDL activates ERK1/2 through LOX-1. However, besides LOX-1, other SRs such as SR-A, SR-BI, SR-C, CD68, and SR-EC also bind and internalize oxLDL in mammalian cells and mediate cell signaling. Although the main function of SRs is to regulate lipid transport, we also need to further investigate their role in cell signaling.

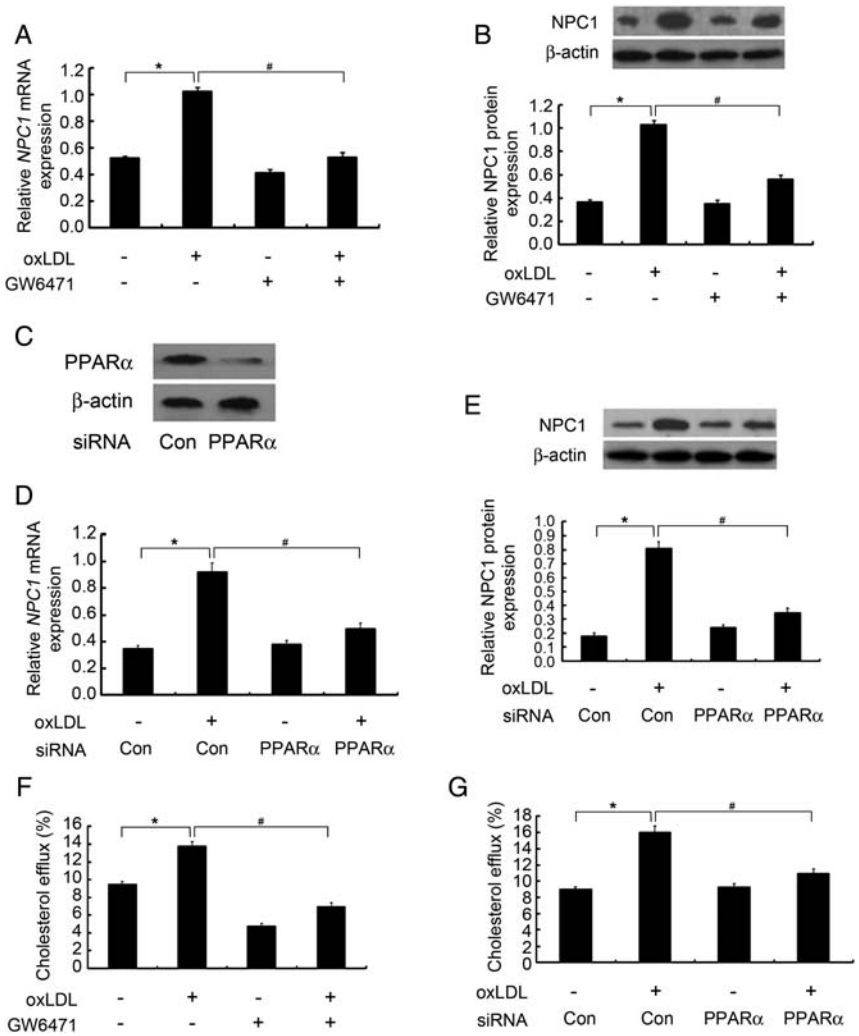

Figure 4 Blockade of PPARα inhibits oxLDL-induced up-regulation of NPC1 and reduces cholesterol efflux in THP-1 macrophages (A-B, F) Cells were pre-incubated with 0.5 μM GW6471 for 1 h, and then incubated with 50 μg/ml oxLDL for 24 h. (A, B) NPC1 mRNA and protein expression were determined using real-time quantitative PCR and western blot, respectively. (C–E, G) Cells were transfected with control or PPARα siRNA, and then incubated with 50 μg/ml of oxLDL for 24 h. (C) Protein samples were immunoblotted with anti-PPARα or anti-β-actin antibodies. (D) Real-time quantitative PCR was performed to determine the expression of *NPC1* mRNA. (E) Western blot assays using antibodies against NPC1 and β-actin were conducted. (F, G) Cellular cholesterol efflux was detected using liquid scintillation counting method under different treatment. $^{*}P < 0.05$, $^{\#}P < 0.05$.

Macrophages express COX-2 in response to a variety of cytokines as well as lipopolysaccharide [36,37]. In this study, our results also showed that oxLDL markedly increased COX-2 mRNA and protein levels, whereas the effect was blocked by ERK1/2 inhibition, suggesting that oxLDL-induced expression of COX-2 is mediated by ERK1/2. Numerous studies have demonstrated that the activity of ERK1/2 is linked to downstream activation of three transcription factors, which are known to recognize COX-2 promoter elements: activator protein 1, CCAAT/enhancer-binding protein-β, and cAMP response element-binding protein, and that ERK1/2 seems to participate in and be necessary for effective transcription factor activation [38–40]. One or more of these three transcription factors are probably involved in the increase of *COX-2* gene transcription after oxLDL treatment. Nevertheless, further studies are needed to clarify how oxLDL induces COX-2 expression.

In this study, we found that oxLDL could up-regulate PPARα expression in THP-1 macrophages and this was suppressed by inhibiting COX-2, suggesting that ERK1/2-dependent COX-2 expression was involved in oxLDL-induced up-regulation of PPARα. COX-2 is the enzyme that catalyzes the rate-limiting step in the synthesis of PGs, converting arachidonic acid into PGH2, which is then further metabolized to other PGs such as PGE2, PGD2, PGF2, and PGJ2, in inflammatory cells, transformed cells, and malignant tumor cells [41–43]. The most recently discovered PG 15-deoxy-$\Delta^{12,14}$-PG J2 (15d-PGJ2) is the end product of the dehydration of PGD2. It is well known that 15d-PGJ2 is a strong PPARγ activator [44]. However, recent studies have reported that 15d-PGJ2 is not a PPARα ligand

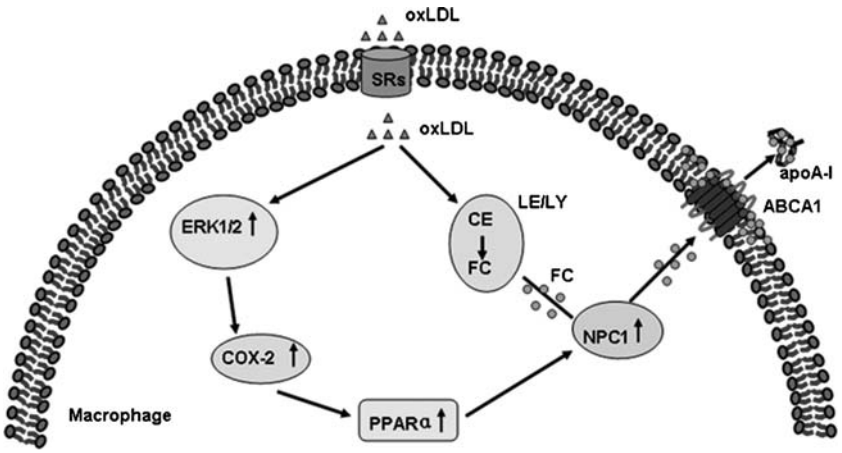

Figure 5 Schematic representation of oxLDL-induced NPC1 expression in macrophages Treatment of macrophages with oxLDL induces ERK1/2-dependent upregulation of COX-2, which subsequently increases PPARα expression. PPARα overexpression mediates the enhancement of NPC1 expression, resulting in significantly increase of intracellular cholesterol efflux through ABCA1 pathway ultimately. CE, cholesteryl ester; FC, free cholesterol.

but also activates PPARα [22,28,45]. Therefore, it is reasonable to presume that COX-2-mediated increase in 15d-PGJ2 level is involved in oxLDL-induced expression of PPARα. However, further study is thought to be necessary to support it.

PPARα regulates the expression of genes controlling lipid and cholesterol metabolism [46,47]. It was recently reported that PPARα activation resulted in the induction of *NPC1* gene and protein expression in human macrophages [23]. Here, our data revealed that oxLDL-mediated NPC1 expression was abrogated by PPARα antagonist and siRNA, respectively. PPARα has been shown to transcriptionally activate LXRα, a member of the nuclear receptor superfamily [48]. Furthermore, our recent studies demonstrated that LXRα can up-regulate NPC1 expression *in vitro* and *in vivo* [49,50]. Therefore, PPARα may regulate NPC1 expression via LXRα pathway after oxLDL treatment. Taken together, all of these data suggest that oxLDL up-regulates NPC1 expression in THP-1 macrophages through a mechanism underlying ERK1/2/COX-2/PPARα-signaling pathway. However, considerable intermediate mechanisms may exist in the whole signaling pathway. Therefore, we should also pay attention to the limitation of the study.

As NPC1 is associated with cellular cholesterol trafficking [51], we finally investigated the effect of ERK1/2, COX-2, and PPARα on cellular cholesterol efflux. Our results showed that oxLDL strongly elevated cellular cholesterol efflux, which was abrogated by inhibiting ERK1/2, COX-2, PPARα, or ERK1/2 in combination with COX-2. Several studies demonstrated that the enrichment of plasma membrane cholesterol as well as the induction of apoA-I-specific cholesterol efflux by PPARα was abolished in the presence of progesterone, which was caused by the progesterone that could block cholesterol mobilization from the LE/LY and mimic a phenotype comparable with the one observed in NPC-deficient cells [52,53]. In addition, our group revealed that NO-1886, an effective lipoprotein lipase activator, decreased cellular cholesterol content and promoted apoA-I-mediated cholesterol efflux by up-regulating NPC1 expression [49], and NPC1 siRNA resulted in a drastic reduction of cellular cholesterol efflux [50]. These observations indicate that the stimulation of post-lysosomal cholesterol mobilization to the plasma membrane by PPARα via NPC1 induction is a crucial upstream step for the stimulation of its efflux through ABCA1 pathway.

In conclusion, we demonstrated that oxLDL-induced up-regulation of NPC1 was mediated by the increase of PPARα, via ERK1/2-dependent COX-2 expression in macrophages, thus resulting in an increase of cellular cholesterol efflux through ABCA1 pathway in these cells (**Fig. 5**). These findings open new perspectives in targeting this signaling pathway as a potential novel approach for the treatment of atherosclerosis.

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