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PPARg phosphorylation mediated by JNK MAPK: a potential role in macrophage-derived foam cell formation[1]

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Key words

peroxisome proliferator-activated receptor γ; phosphorylation; macrophages; LDL lipoprotein

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Abstract

Aim: To investigate whether oxidized low-density lipoprotein (ox-LDL) modulates peroxisome proliferator-activated receptor γ (PPARγ) activity through phosphorylation in macrophages, and the effect of PPARγ phosphorylation on macrophages-derived foam cell formation. **Methods**: After exposing the cultured THP-1 cells to ox-LDL in the presence or absence of different mitogen-activated protein kinase (MAPK) inhibitors, PPARγ and phosphorylated PPARγ protein levels were detected by Western blot. MAPK activity was analyzed using MAP Kinase Assay Kit. Intracellular cholesterol accumulation was assessed by Oil red O staining and cholesterol oxidase enzymatic method. The mRNA level of PPARγ target gene was determined by reverse transcription-polymerase chain reaction (RT-PCR). **Results**: ox-LDL evaluated PPARγ phosphorylation status and subsequently decreased PPARγ target gene expression in a dose-dependent manner. ox-LDL also induced MAPK activation. Treatment of THP-1 cells with c-Jun N-terminal kinase-, but not p38- or extracellular signal-regulated kinase-MAPK inhibitor, significantly suppressed PPARγ phosphorylation induced by ox-LDL, which in turn inhibited foam cell formation. **Conclusion**: In addition to its ligand-dependent activation, ox-LDL modulates PPARγ activity through phosphorylation, which is mediated by MAPK activation. PPARγ phosphorylation mediated by MAPK facilitates foam cell formation from macrophages exposed to ox-LDL.

Introduction

Massive clustering of macrophage-derived foam cells in the subendothelial spaces of arterial walls is one of the characteristic features of the early stages of atherosclerotic lesions[1]. Macrophages take up oxidized low-density lipoprotein (ox-LDL) through the scavenger receptor pathways and transform into foam cells[2]. Foam cells produce various bioactive molecules, such as cytokines and growth factors, and are believed to play an important role in the development and progression of atherosclerosis[1].

Multiple intracellular signal pathways, including peroxisome proliferator-activated receptor γ (PPARγ), have been reported to be involved in macrophage-derived foam cell formation[3]. PPARγ is a member of a nuclear hormone superfamily that heterodimerizes with the retinoid X receptor. These proteins are transcriptional regulators of genes that encode proteins involved in adipogenesis and lipid metabolism[4]. 15-deoxy-Δ12,14 prostaglandin J_2 (15d-PGJ_2) and the thiazolidinedione (TZD) class of antidiabetic drugs is nature and synthesis ligand of PPARγ, respectively[5]. Components of ox-LDL, including 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE also activate PPARγ and subsequently induce the expression of the CD36 scavenger receptor, a key mediator for uptake of ox-LDL in macrophage[6]. This observation suggested that PPARγ ligand might promote the formation of foam cells. But Chinetti *et al* have shown that the treatment of human macrophages with PPARγ agonists did not facilitate foam cell formation because they induced the expression of ATP-binding cassette transporter, class A1 (ABCA1), a transporter that controls apoAI-mediated cholesterol efflux from macrophages. These effects are likely to

be caused by the enhanced expression of liver-x-receptor alpha (LXRα), an oxysterol-activated nuclear receptor that induces ABCA1 transcription. In fact, Chinetti *et al* showed that PPARγ activators increased apoAI-induced cholesterol efflux from macrophage-derived foam cells[7]. Thus, the effects of ox-LDL uptake in response to increased macrophage CD36 expression following PPARγ activation is balanced by LXRα activation and ABCA1-mediated cholesterol efflux. Because previous studies implicated PPARγ in both proatherogenic and antiatherogenic pathways mediated by components of ox-LDL and synthesis PPARγ agonist, respectively, we hypothesized that, in addition to activating PPARγ in a ligand-dependent manner, other components of ox-LDL might have a negative regulatory effect on PPARγ activity through unidentified mechanisms.

Several studies have shown that PPARγ is a phosphoprotein. Multiple kinase pathways, such as cAMP-dependent protein kinase (PKA), AMP-activated protein kinase (AMPK), and mitogen-activated protein kinase (MAPK), have been implicated in the regulation of PPARγ phosphorylation[8]. Phosphorylation significantly inhibits both ligand-independent and ligand-dependent transcriptional activation by PPARγ[9]. The implications of the post-translational modification of PPARγ activity through phosphorylation might be the pathway by which various growth factors and cytokines could affect the transcription of numerous genes involved in lipid metabolism as well as lipid homeostasis in the macrophage-derived foam cells induced by ox-LDL.

The present study was designed to study the role of PPARγ phosphorylation in macrophage-derived foam cell formation induced by ox-LDL. We found that ox-LDL evaluated PPARγ phosphorylation status during foam cell formation. ox-LDL-induced PPARγ phosphorylation was mediated by c-Jun N-terminal kinase (JNK)-MAPK activation. Treatment of JNK inhibitor suppressed PPARγ phosphorylation and subsequently prevented ox-LDL-induced foam cell formation. These observations demonstrate that PPARγ phosphorylation mediated by MAPK facilitates foam cell formation from macrophages exposed to ox-LDL.

Materials and methods

Cell culture The human monocytes line THP-1 was obtained from the cell bank in Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% (*v*/*v*) fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, and 12 mmol/L sodium carbonate. Cell cultures were maintained and incubated in a humidified atmosphere containing 5% (*v*/*v*) CO_2 at 37 °C. Differentiation of THP-1 monocytes into macrophages was induced by culturing the cells at a density of 1.0×10^6 cells/well in a 6-well plate in the presence of phorbol 12-myristate 13-acetate (PMA) 160 nmol/L for 24 h. Cells were then cultured for another 48 h without PMA, washed with serum-free medium or buffer to remove non-adherent cells, and then incubated with the respective stimuli for various periods in serum-free medium.

LDL isolation and oxidization Human LDL (1.019–1.063 g/mL) were prepared from different human healthy donors by density gradient ultracentrifugation in the presence of 1 mg/mL EDTA (pH 7.4). The isolated LDL was dialyzed to remove EDTA and filtered (0.22 μm pore size), and stored at 4 °C. The LDL was analyzed for protein content by the Bradford method, using bovine serum albumin as standard. The purity and charge of the lipoproteins were evaluated by examining electrophoretic mobility in an agarose gel. Oxidation of LDL was carried out with copper sulfate (final concentration of 10 μmol/L) at 37 °C for 12 h. The degree of oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARS). ox-LDL had TBARS of 18 nmol/mg. ox-LDL was then dialyzed against PBS containing EDTA 0.01% for 24 h at 4 °C and sterile filtered.

Oil red O staining In parallel experiments, THP-1-derived macrophages were plated at a density of 1.0×10^6 cells/well in a 6-well plate containing glass coverslips and incubated in serum-free RPMI-1640 medium in the presence of ox-LDL 100 μg/mL at 37 °C for 48 h. Cells were washed three times with PBS, fixed by 10% formalin in PBS for 1 h at room temperature, and then stained with 0.1 mL/mL Oil red O solution for 2 h, washed three times with water, and vaporized of all water (at 32 °C for 45 min). Cells were viewed *in situ* in 35-mm diameter tissue culture plates under a bright-field microscope in 100×fields using a microscope (Olympus IX70, Tokyo, Japan).

Measurements of free and total cholesterol THP-1-derived macrophages (5×10^5 cells/mL) were added to each well of a 24-well plate with ox-LDL (100 μg/mL). The incubation at 37 °C lasted for 48 h. The THP-1 cells were washed three times in PBS, then 1 mL isopropyl alcohol was added, and the cells were sonicated for 30 s. Total cholesterol and free cholesterol in extracts were determined by the cholesterol oxidase enzymatic method using a commercial kit by a Hitachi 7020 autoanalyser (Tokyo, Japan). Lipid-extracted cells were dissolved in 0.1% sodium dodecyl sulfate-0.1 mol/L NaOH for 30 min, and total cell protein was determined with a protein assay kit. Esterified cholesterol was calculated from (total cholesterol)-(free cholesterol) values. Results were expressed in mg/g protein.

Western blot analysis of PPARg/phosphorylated PPARg After treatment, cells were washed twice with PBS and then resuspended in 400 μL of cold buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF). After 15-min incubation on ice, 25 μL of 10% NP-40 was added to the cell suspension, which was subjected to a vortex for 10 s. The supernatant was removed after being spun for 30 s at 13 150×*g*. The pellet was resuspended in 100 μL of cold buffer C (20 mmol/L HEPES, pH 7.91, 400 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, freshly added 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 0.1 mmol/L P-aminobenzamidine, and 10 μg/mL aprotinin) and kept for 15 min at 4 °C. The mixture was spun for 5 min at 13 150×*g*. and the supernatant was collected as nuclear proteins. Nuclear proteins (500 μg) from each sample were incubated with an antibody to mouse PPARγ antibody (Sigma Chemical Co, St Louis, MO, USA). Immunoabsorbed proteins were separated by SDS-PAGE and transferred onto nylon-enhanced nitrocellulose membrane, then analyzed by Western blot for phosphorylated PPARγ (PPARγ-Pi) by incubation with anti-phosphoserine antibodies (Sigma). The nuclear proteins were also used to analyze PPARγ protein expression by SDS-PAGE/Western blot.

MAPK activity assay JNK, p38, and extracellular signal-regulated kinase (ERK) activities were detected using a stress-activated protein kinase (SAPK)/JNK, p38, and p44/42 MAP Kinase Assay Kit, respectively, according to the manufacturer's instructions (Cell Signaling, Beverly, USA). For the p38 and ERK assays, aliquots of 200 μg of protein were incubated with immobilized phospho-specific p38 or ERK MAPK monoclonal antibody. After washing with lysis and kinase buffer, pellets were suspended in kinase buffer with 200 μg ATP and 2 μg ATF-2 or Elk-1 fusion proteins and incubated at 30 °C for 30 min. For the JNK kinase assay, 250 μg of protein was incubated with 2 μg c-Jun fusion protein beads. After washing, pellets were suspended in kinase buffer with 100 μg ATP and incubated at 30 °C for 30 min. The reaction was terminated with SDS sample buffer, and boiled samples were analyzed by Western blotting using corresponding phospho-specific antibodies.

RT-PCR Total RNA was isolated using Trizol reagent. Total RNA content was determined by measuring the optical absorbance ratio at 260/280 nm after the sample was dissolved in diethylpirocarbonate-treated water. RNA was then stored at -70 °C before two-step RT-PCR protocol using 2 μg of total RNA. RNA was treated with DNase I, reverse transcribed, and amplified for ABCA1 and GAPDH using PCR enzymes and reagents according to the following conditions: 10 min 95 °C, then 34 cycles of 1 min 95 °C, 1 min 60 °C, and 1 min 72 °C, and then a final annealing step at 72 °C for 10 min. PCR amplification was performed using ABCA1 (306 bp) primers (forward: 5'-GCTGCTGAAGCCA-GGGCATGGG-3' and reverse: 5'-GTGGGGCAGTGGCCATA-CTCC-3') and GAPDH (697 bp) primers (forward: 5'-TCACCA-TCTTCCAGGAGCCGAG-3', reverse: 5'-TGTCGCTGTTGAA-GTCAGAG-3'). PCR products were separated on 1.5% agarose gel containing ethidium bromide. Densitometric quantitation of the intensity of GAPDH and ABCA1 products was determined using the "Quantity One" quantitation analysis software (Bio-Rad Laboratories, Hercules, CA, USA). The relative abundance of ABCA1 was expressed as the ratio of ABCA1 to GAPDH product.

Statistical analysis Data were expressed as mean±SD. Statistical significance of the data was evaluated by analysis of variance and *q* test. $P<0.05$ was considered significant. All experiments were performed a minimum of three times.

Results

ox-LDL increases PPARg phosphorylation After the cells were incubated with 15d-PGJ_2 (20 μmol/L), troglitazone (5 μmol/L) or ox-LDL (25, 50, 100 μg/mL) for 12 h, total and phosphorylated PPARγ were determined by Western blot. As shown in Figure 1A, no significant change of total and phosphorylated PPARγ was observed after incubation with 15d-PGJ_2 and troglitazone, which indicated nature or synthesis PPARγ ligand was not involved in transcriptional and post-transcriptional regulation of PPARγ. In contrast, when macrophages were incubated with ox-LDL, both total and phosphorylated PPARγ were increased in a dose-dependent manner. It is noteworthy that the ratio of phosphorylated/total PPARγ was also elevated d by ox-LDL. Thus, our results indicated that ox-LDL induced PPARγ phosphorylation in THP-1 cells. Previous studies have demonstrated that phosphorylation of PPARγ inhibited both its ligand-dependent and ligand-independent transcriptional activity[9]. Therefore, we subsequently studied the effects of these compounds on the expression of ABCA1, a well-known PPARγ target gene involved in cholesterol efflux. As shown in Figure 1B, treatment of THP-1 cells with the three compounds for 12 h all increased ABCA1 mRNA level. However, we found that the elevation of ABC1 mRNA induced by ox-LDL did not occur in a dose-dependent manner. Instead, the mRNA level of ABCA1 induced by higher concentration of ox-LDL was less than the lower (although it lacks statistical significance). These observations clearly demonstrated that PPARγ phosphorylation status was negatively correlated

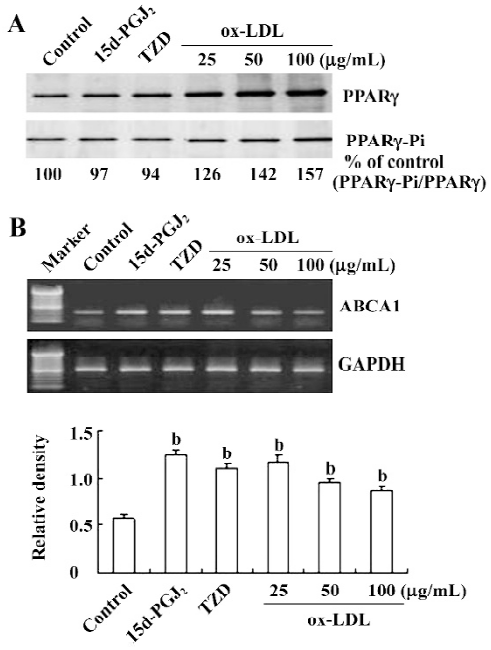

Figure 1. Effects of 15d-PGJ_2, troglitazone (TZD) and different concentrations of ox-LDL on total and phosphorylated PPARγ (PPARγ-Pi) protein level (A) and ABCA1 mRNA level (B) in THP-1 macrophages. n=3 independent experiments. Mean±SD. $^bP<0.05$ *vs* control.

with PPARγ target gene expression.

ox-LDL activates MAPK Recent investigations have demonstrated that the MAPK are activated by ox-LDL stimulation[10,11]. Because previous studies have demonstrated that PPARγ is phosphorylated by the MAPK family members[8], we hypothesized that ox-LDL-induced MAPK activation may regulate PPARγ phosphorylation in macrophage-derived foam cells. We first explored whether ox-LDL is able to activate MAPK in the human monocytic cell line THP-1. The application of ox-LDL resulted in increased activities of all three MAPK limbs. The increases of ERK- and p38-MAPK activity peaked at 24 h followed by failing to basal level at 48 h. In contrast, increased JNK activity maintained up to 72 h. The different kinetics, however, of the three MAPK suggested that they might play different roles in ox-LDL-induced macrophage foam cell formation (Figure 2).

ox-LDL-induced phosphorylation of PPARg is blocked by a JNK-MAPK inhibitor To investigate whether ox-LDL-induced MAPK activation regulates PPARγ phosphorylation in THP-1 macrophages, we used inhibitors that are selective for each MAPK cascade (PD98059, an inhibitor for ERK; SP600125 for JNK; SB203580 for p38) to evaluate their effects on PPARγ phosphorylation induced by ox-LDL. When ox-LDL-treated macrophages were incubated with

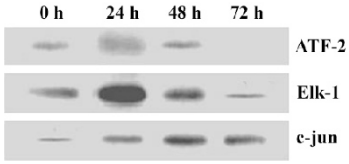

Figure 2. Effects of ox-LDL on different kinetics of p38-, ERK-, and JNK-MAPK in THP-1 cells.

PD98059 (20 μmol/L) or SB203580 (20 μmol/L) for 12 h, PPARγ phosphorylation status did not change. In contrast, treatment with SP600125 (20 μmol/L) significantly inhibited ox-LDL-induced PPARγ phosphorylation. These observations demonstrate that JNK may be predominantly responsible for ox-LDL-induced PPARγ phosphorylation during macrophage foam cell formation (Figure 3).

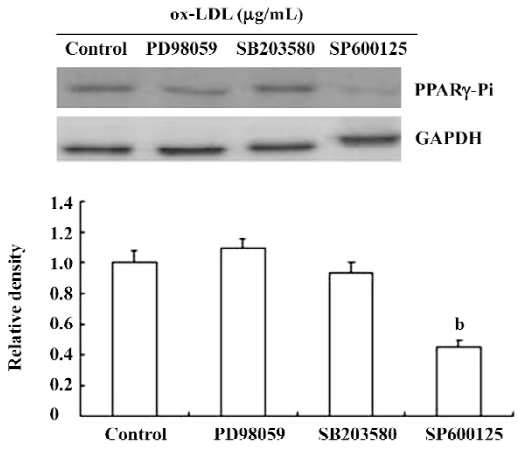

Figure 3. Effects of different MAPK inhibitors on phosphorylated PPARγ (PPARγ-Pi) induced by ox-LDL in THP-1 cells. n=3 independent experiments. Mean±SD. $^bP<0.05$ *vs* control.

Foam cell formation induced by ox-LDL is attenuated by inhibition of PPARg phosphorylation To determine the impact of MAPK activation and PPARγ phosphorylation on ox-LDL-induced foam cell formation, we used SP600125, a specific JNK inhibitor, to evaluate its effect on cholesterol accumulation by staining with Oil red O. Cholesterol accumulation was greatly increased in cells incubated with ox-LDL (100 μg/mL) for 48 h. When THP-1 cells were incubated with ox-LDL in the presence of different concentrations of SP600125 (5, 10, and 20 μmol/L), cholesterol accumulation decreased in a dose-dependent manner (Figure 4). These results were consistent with the morphological features identified by Oil red O staining. Thus, these data suggested that the JNK pathway was involved in PPARγ phosphorylation

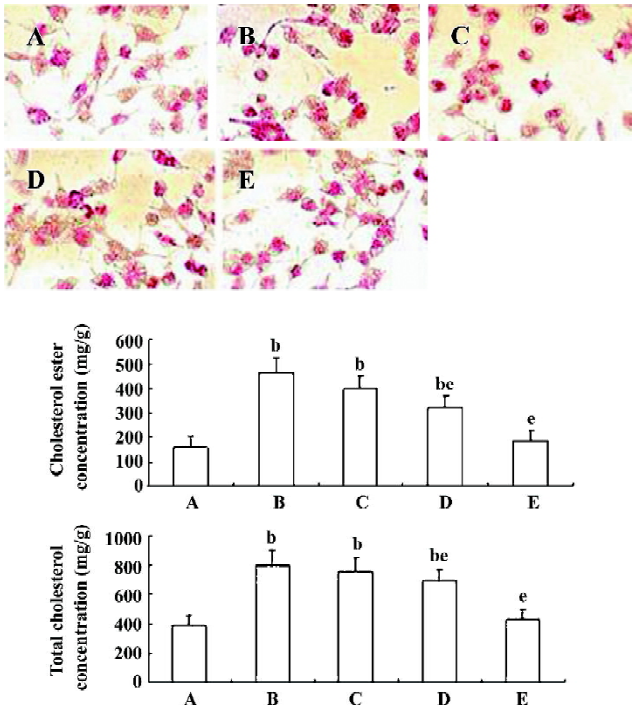

Figure 4. Effects of different concentrations of JNK inhibitor on ox-LDL-induced foam cell formation. (A) Control; (B) ox-LDL; (C) ox-LDL+SP600125 (5 μmol/L); (D) ox-LDL+SP600125 (10 μmol/L); (E) ox-LDL+SP600125 (20 μmol/L). n=3 independent experiments. Mean±SD. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* ox-LDL.

and macrophage foam cell formation induced by ox-LDL.

Discussion

In this study, we have observed an effect of ox-LDL on PPARγ phosphorylation in THP-1-derived macrophage. We found that ox-LDL evaluated PPARγ phosphorylation during foam cell formation. ox-LDL-induced PPARγ phosphorylation was mediated by MAPK activation. Using pharmaceutical inhibitors, we found that activation of the JNK pathway, but not the ERK or p38 pathway, was responsible for PPARγ phosphorylation in THP-1-derived macrophage. This data illustrated the complexity of regulation of PPARγ activity and provided a new insight into the mechanism of macrophage foam cell formation induced by ox-LDL.

In recent years the detection of PPARγ in lesion macrophages, coupled with its identification as the molecular target of antidiabetic agents, has raised significant interest in developing models of PPARγ function and its role as a therapeutic target for coronary artery disease[12]. In particular, TZD, synthesis ligands of PPARγ, are widely used in patients with diabetes, who also have a high risk of cardiovascular disease[13]. Several groups have evaluated the effects of TZD on the foam cell formation and showed that there was no significant difference in cholesterol accumulation in TZD-treated cells[14,15]. To determine the overall impact of TZD on the development of atherosclerosis, several groups have recently evaluated their effects *in vivo*[16,17]. Studies in LDL receptor-deficient or apolipoprotein E-deficient mice have consistently demonstrated protective effects of TZD on the development of diet-induced atherosclerosis. These observations indicated that PPARγ activation mediated by ligand-dependent manner was involved in antiatherogenic pathways. Therefore, the implication of PPARγ in proatherogenic pathway mediated by ox-LDL suggested that, in addition to activating PPARγ via 9-HODE and 13-HODE, another interaction might exist between ox-LDL and PPARγ, through which ox-LDL facilitates macrophage foam cell formation.

Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), have been shown to phosphorylate PPARγ via MAPK signaling pathway and to decrease PPARγ transcriptional activity[18]. The NH_2-terminal domain of PPARγ contains a consensus MAPK site in a region conserved between $PPAR\gamma_1$ and $PPAR\gamma_2$ isoforms[9]. PPARγ proteins migrate on immunoblots as closely spaced doublets, a pattern suggestive of phosphorylation. A putative MAPK site is phosphorylated by ERK and JNK kinase. Phosphorylation significantly inhibits both ligand-independent and ligand-dependent transcriptional activation by PPARγ[18]. This repression is mediated by MAPK phosphorylation of Ser82 on PPARγ. Mutation of the phosphorylated residue (Ser82) prevents PPARγ phosphorylation as well as the growth factor-mediated repression of PPARγ dependent transcription. Previously, Han *et al* showed that TGF-β decreased the expression of CD36 in THP-1-derived macrophage by phosphorylation of MAPK, subsequent MAPK phosphorylation of PPARγ, and decreased CD36 transcription by phosphorylated PPARγ[19]. Although phosphorylation of PPARγ has been implicated in macrophage lipid homeostasis, whether it is involved in ox-LDL-induced foam cell formation is unclear. Our study found that ox-LDL-induced MAPK activation led to phosphorylation and subsequent deactivation of PPARγ. This observation indicated that unknown component of ox-LDL might negative regulated PPARγ activity through MAPK-mediated phosphorylation pathway, which in turn promote macrophage foam cell formation. In contrast to the prevailing notion that ox-LDL is a positive regulator for PPARγ, our results demonstrated that ox-LDL also inhibited PPARγ transcriptional activity via phosphorylation pathway. We assume that the consequence

of interaction between ox-LDL and PPARγ may depend on the stage of macrophage-derived foam cell formation. Feature studies are needed to clarify this assumption.

MAPK play an important role in many cellular processes, such as proliferation, apoptosis, and adaptation to changes in the extracellular environment[20]. At least three major groups of MAPK have been identified in mammalian cells so far: (i) ERK, (ii) JNK or SAPK, and (iii) p38 MAPK. The ERK pathway is preferentially activated by growth-related stimuli, while the JNK and p38 pathways are often linked with cellular stress. MAPK can, however, be activated by oxidative stress in a variety of cells. Both ERK- and p38-MAPK members have been shown to be activated by ox-LDL in smooth muscle cells[10], and recently Zhao *et al* have reported a similar effect on p38-MAPK in the murine macrophage cell line, J774[21]. Napolitano *et al* reported that the activation of ERK-, but not p38-MAPK was involved in the induction of cholesterol esterification by acetylated LDL in human monocyte-derived macrophages[22]. These findings, therefore, suggested that MAPK might play an important role in the regulation of macrophage foam cell formation induced by modified LDL and the development of atherosclerosis. Consistent with previous studies, our results also demonstrated that ox-LDL induced activation of the p38-, JNK-, and ERK-MAPK. However, the three MAPK had different kinetics of activation. The different kinetics of the three MAPK suggest that their role may be different in macrophage foam cell formation.

Using pharmaceutical inhibitors, we demonstrated that the activation of JNK pathway, but not ERK or p38 pathway, was necessary and sufficient to phosphorylate PPARγ and subsequently facilitated macrophage foam cell formation. We do not know at present whether ox-LDL activates JNK directly, or if ox-LDL activates other cellular kinase pathways, such as PKA and AMPK, which in turn may activate MAPK. We still do not know which component of ox-LDL is responsible for MAPK activation and subsequent PPARγ phosphorylation. But it is clear that post-translational regulation of PPARγ via the phosphorylation pathway is crucial for macrophage foam cell formation induced by ox-LDL. A future challenge will be to clarify those problems in order to develop new strategies for the prevention and treatment of atherosclerosis through modulation of PPARγ phosphorylation status.

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