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# The ω-carboxyl group of 7-ketocholesteryl-9-carboxynonanoate mediates the binding of oxLDL to CD36 receptor and enhances caveolin-1 expression in macrophages

Jingda Li[a,b], Chengjie Yu[a], Renjun Wang[a], Jianrong Xu[c], Yan Chi[a], Jianzhong Qin[a], Qingping Liu[a,*]

[a] Key Laboratory of Carbohydrate and Lipid Metabolism Research, College of Life Science and Technology, Dalian University, 10-Xuefu Avenue, Dalian Economical and Technological Development Zone, Liaoning 116622, China
[b] School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, Liaoning, China
[c] Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

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ABSTRACT

CD36 signal transduction modulates the uptake of oxidized low-density lipoprotein (oxLDL) and foam cell formation. We previously observed that 7-ketocholesteryl-9-carboxynonanoate (oxLig-1), the lipid moiety of oxLDL, activates the CD36-Src-JNK/ERK1/2 signalling pathway. In this study, we assessed the role of the ω-carboxyl group in the binding of oxLig-1 to CD36 and investigated whether the binding of the ω-carboxyl group to CD36 triggers CD36-mediated signalling, thereby resulting in the upregulation of caveolin-1 expression. Our results showed that oxLig-1 bound to CD36 and that the ω-carboxyl group was critical for this binding. Furthermore, immunoprecipitation and Western blot analyses showed that interaction between the ω-carboxyl group of oxLig-1 and CD36 triggered intracellular Src-JNK/ERK1/2 signal transduction. Moreover, the binding of the ω-carboxyl group to CD36 induced caveolin-1 expression and translocation to the membrane in macrophages. Additionally, inhibitors of Src, JNK and ERK and siRNA targeting CD36 and NF-κB significantly suppressed the enhanced caveolin-1 expression induced by oxLig-1. In conclusion, these observations suggest that oxLig-1 is a critical epitope of oxLDL that mediates the binding of oxLDL to CD36 and activates downstream Src-JNK/ERK1/2-NF-κB signal transduction, resulting in upregulation of caveolin-1 expression in macrophages.

## 1. Introduction

Atherosclerosis (AS), the leading cause of cardiovascular and cerebral vascular diseases, is the most important source of morbidity and mortality worldwide (Yu et al., 2013). Lipid metabolism disorders and chronic inflammation are the major characteristics of atherosclerosis (Chistiakov et al., 2016; Collot-Teixeira et al., 2007). Oxidized low-density lipoprotein (oxLDL) is a key factor that is intimately involved in the early development of atherosclerotic lesions through the formation of macrophage-derived foam cells (Chistiakov et al., 2016; Nicholson, 2004). In the early stages of atherosclerosis, several scavenger receptors (SRs), including CD36, SR-A1 and lectin-like oxLDL receptor-1 (LOX-1), mediate oxLDL uptake. After internalization, oxLDL is catabolized in macrophages. Acyl coenzyme A:cholesterol acyltransferase-1 (ACAT1) and neutral cholesteryl ester hydrolase (nCEH) regulate cholesterol esterification and accumulation. Simultaneously, macrophages initiate reverse cholesterol transport (RCT), an atheroprotective process, to assist in the efflux of excess cholesterol from macrophages via ATP-binding cassette transporters A1 (ABCA1), ABCG1 and scavenger receptor BI (SR-BI) (Chistiakov et al., 2016). Under atherogenic conditions, the disruption of the balance between cholesterol influx and efflux leads to excessive CE accumulation as lipid droplets in macrophages, thereby contributing to the formation of foam cells (Yu et al., 2013).

Among scavenger receptors on the surface of macrophages, CD36 has been identified as a major scavenger receptor for the binding and uptake of oxLDL (Nicholson, 2004). Mounting evidence has revealed that CD36 signal transduction, triggered by oxLDL, plays an important role in AS progress (Park, 2014). Previous studies have reported that the interaction between CD36 and oxLDL induces the phosphorylation of Src family non-receptor tyrosine kinases, such as Fyn and Lyn, and subsequent activation of the mitogen-activated protein (MAP) kinases c-Jun N-terminal kinase (JNK) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), leading to the formation of foam cells

* Corresponding author at: Key Laboratory of Carbohydrate and Lipid Metabolism Research, College of Life Science and Technology, Dalian University, Dalian 116622, China.
*E-mail address:* qingpingliu40@126.com (Q. Liu).

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(Park, 2014; Rahaman et al., 2006). Moreover, OxLDL (via CD36) also induces the activation of nuclear factor-kappa B (NF-κB) and the secretion of inflammatory cytokines, thereby promoting apoptosis and atherosclerotic inflammation (Collot-Teixeira et al., 2007; Kunz et al., 2008; Park, 2014; Xie et al., 2011).

Caveolin-1, the main structural protein of caveolae, is expressed in most cell types involved in the development of AS (Gargalovic, 2002; Qin et al., 2016a). In macrophages, caveolin-1 is involved in intracellular cholesterol trafficking and cholesterol efflux (Frank et al., 2006; Gargalovic, 2002; Gu et al., 2014; Mukhamedova et al., 2008; Qin et al., 2016b; Wang et al., 2014). Previous studies have shown that loss of caveolin-1 in macrophages increases the accumulation of cholesteryl esters, indicating that caveolin-1 expression is associated with an enhancement of cholesterol efflux (Arakawa et al., 2000; Hu et al., 2010; Pavlides et al., 2014; Wang et al., 2014). Moreover, Caveolin-1 has also been implicated in the regulation of apoptosis and the clearance of apoptotic cells through phagocytosis in macrophages (Pavlides et al., 2014). These findings suggest an anti-atherogenic role for Caveolin-1 in macrophages.

In a previous study, we purified a negatively charged lipid moiety from oxLDL, 7-ketocholesteryl-9-carboxynonanoate (oxLig-1) (Kobayashi et al., 2001; Liu, 2002), and observed that oxLig-1 triggered the CD36-Fyn-JNK/ERK signalling pathway (Li et al., 2013). However, the underlying mechanisms, whereby ligand binding to the receptor triggers activation of signalling events, remain unclear. Additionally, because activation of the ERK/JNK-NF-κB pathway by oxLDL upregulates caveolin-1 expression (Wu et al., 2009), we hypothesized that oxLig-1, as a lipid moiety of oxLDL, can upregulate caveolin-1 expression through the CD36-Fyn-JNK/ERK-NF-κB pathway. To assess this hypothesis and further expand the results, we investigated the mechanism underlying oxLig-1 binding to CD36 and determined whether the binding of oxLig-1 to CD36 upregulates caveolin-1 expression through the downstream Fyn-JNK/ERK-NF-κB pathway.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

5-Cholesten-3b-ol-7-one (7-ketocholesterol), azelaic acid, azelaic acid monomethyl ester, cholest-5-en-7-one, 4-dimethylaminopyridine (DMAP) and N,N'-dicyclohexyl carbodiimide (DCC) were purchased from Sigma-Aldrich Chemical Company (USA). Antibodies were obtained from the following sources: anti-CD36 (sc-9154), anti-Fyn (sc-16), anti-p-Fyn (sc-377555), anti-Lyn (sc-15), anti-Lamin A (sc-20680), anti-Na+/K+-ATPase α (sc-28800) and anti-p-NF-κB p65 (sc-33020) from Santa Cruz; anti-β-actin (ab8226), anti-p-JNK (ab124956) and anti-LOX-1 (ab60178) (Abcam); anti-caveolin-1 (610407) from BD-Transduction; and anti-ERK1/2(A0229), anti-p-ERK1/2 (AP0472), anti-JNK (A0288) and anti-NF-κB p65 (A11204) from ABclonal Technology. Monoclonal anti-$\beta_2$-GPI, WB-CAL-1 and anti-apoB-100 antibodies were a kind gift from Dr Eiji Matsuura (Okayama University, Japan). The recombinant CD36 protein was obtained from Sino Biological, Inc. (China). Inhibitors of Src (AG1879), ERK (PD98059), JNK (SP600125) and p38 (SB203580) were purchased from Calbiochem (San Diego, CA, USA). SiRNA against CD36, LOX-1, and NF-κB and negative control siRNA were purchased from GenePharma (China). The BCA protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). Nuclear and Cytoplasmic Protein Extraction Kit, Membrane and Cytoplasmic Protein Extraction Kit and Modified BCA Protein Assay Kit were purchased from Sangon Biotech (China).

### 2.2. Preparation of human $\beta_2$-GPI

Native $\beta_2$-GPI was purified from healthy human plasma as previously described (Matsuura et al., 1992).

### 2.3. Preparation and oxidation of LDL

To obtain oxLDL, native LDL was oxidized using 5 μM $CuSO_4$ for 8 h at 37 °C as previously described (Liu, 2002). Briefly, LDL was isolated by ultracentrifugation from fresh normal human plasma. After ultracentrifugation, the protein concentration of LDL was determined using the BCA protein assay, and the LDL was then adjusted to 100 μg/mL of apoB equivalent with PBS buffer and oxidized with 5 M $CuSO_4$ in PBS buffer for 8 h at 37 °C. To terminate the oxidation, EDTA (final concentration of 1 mM) was added, and oxLDL was dialyzed extensively with PBS buffer to remove $CuSO_4$. Finally, oxLDL was concentrated using an ultrafiltration device (Amicon Mltra 100 K, Millipore, USA), and the protein concentration of oxLDL was determined using the BCA protein assay.

### 2.4. Synthesis and methylation of oxLig-1

The synthesis and methylation of oxLig-1 were performed as previously described (Kobayashi et al., 2001; Liu, 2002). The structure of the synthesized oxLig-1 (oxLig-1) and methylated oxLig-1 (me-oxLig-1) was evaluated through $^1$H- and $^{13}$C NMR spectroscopy and mass spectrometry (MS).

### 2.5. Cell culture and stimulation

J774A.1 macrophages were purchased from American Type Culture Collection (ATCC) and grown in DMEM medium (Invitrogen) supplemented with 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mmol/L sodium bicarbonate, 1 mmol/L sodium pyruvate and 10% (v/v) foetal bovine serum (FBS) at 37 °C in a 5% $CO_2$ atmosphere. For all stimulation experiments, the cells were starved in DMEM without FBS for 6 h and subsequently stimulated with oxLig-1 at the indicated concentrations and times. Synthesized oxLig-1 was dissolved in DMSO and added to J774A.1 cells in DMEM; the final DMSO concentration was 0.1%.

### 2.6. Molecular docking

To investigate the binding of oxLig-1 to CD36, molecular docking was performed using the Schrodinger Maestro 9.4 software package. Prior to docking, the crystal structure of CD36 was obtained from the Protein Data Bank (PDB ID: 5LGD), and standard protein preparation was performed as previously described (Zhuang et al., 2014). Briefly, all water molecules and the protein ligand were removed from the structure. Hydrogen atoms were added to the protein, and non-polar hydrogen atoms were then merged together. Next, a docking grid was generated based on the binding site of the original protein ligand and was subsequently used in the docking test. Following the docking test, the best conformations were generated based on the empirical glide gscore (kcal/mol). Molecular graphics for the best binding pose were generated using PyMOL.

### 2.7. Enzyme linked immunosorbent assay (ELISA)

The binding of oxLig-1 to CD36 was assessed by ELISA. A polystyrene plate (Immulon 1B) was coated with several lipids (indicated in the figure legends) at 4 °C overnight, and the plate was subsequently blocked with 1% gelatine. After washing with PBS, the plate was incubated with recombinant CD36 protein, which is the extracellular domain (Gly 30-Asn 439) of human CD36 fused to a polyhistidine tag at the C-terminus (20 μg/mL, 50 μL/well), for 1 h. The binding of the recombinant CD36 protein was detected after incubation with an anti-His antibody and subsequent incubation with an HRP-labelled goat anti-mouse IgG antibody. Finally, the samples were incubated with $H_2O_2$ and o-phenylenediamine for visualization. The reaction was terminated using 2 N sulphuric acid, and the absorbance was measured at 492 nm.