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Lysophosphatidic acid directly induces macrophage-derived foam cell formation by blocking the expression of SRBI

Linmu Chen [a], Jun Zhang [a], Xiao Deng [a], Yan Liu [a], Xi Yang [a, b], Qiong Wu [a], Chao Yu [a, c, *]

[a] Institute of Life Science, Chongqing Medical University, Chongqing, 400016, PR China
[b] College of Basic Medicine, Inner Mongolia Medical University, Hohhot, 010110, PR China
[c] College of Pharmacy, Chongqing Medical University, Chongqing, 400016, PR China

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ABSTRACT

The leading cause of morbidity and mortality is the result of cardiovascular disease, mainly atherosclerosis. The formation of macrophage foam cells by ingesting ox-LDL and focal retention in the subendothelial space are the hallmarks of the early atherosclerotic lesion. Lysophosphatidic acid (LPA), which is a low-molecular weight lysophospholipid enriched in oxidized LDL, exerts a range of effects on the cardiovascular system. Previous reports show that LPA increases the uptake of ox-LDL to promote the formation of foam cells. However, as the most active component of ox-LDL, there is no report showing whether LPA directly affects foam cell formation. The aim of this study was to investigate the effects of LPA on foam cell formation, as well as to elucidate the underlying mechanism. Oil red O staining and a Cholesterol/cholesteryl ester quantitation assay were used to evaluate foam cell formation in Raw264.7 macrophage cells. We utilized a Western blot and RT-PCR to investigate the relationship between LPA receptors and lipid transport related proteins. We found that LPA promoted foam cell formation, using 200 μM for 24 h. Meanwhile, the expression of the Scavenger receptor BI (SRBI), which promotes the efflux of free cholesterol, was decreased. Furthermore, the $LPA_{1/3}$ receptor antagonist Ki16425 significantly abolished the LPA effects, indicating that $LPA_{1/3}$ was involved in the foam cell formation and SRBI expression induced by LPA. Additionally, the LPA-induced foam cell formation was blocked with an AKT inhibitor. Our results suggest that LPA-enhanced foam cell formation is mediated by $LPA_{1/3}$ -AKT activation and subsequent SRBI expression.

1. Introduction

Atherosclerosis is a complicated chronic disease that is characterized by the accumulation of lipids within the arterial walls [1]. Monocytes/macrophages play key roles in both the initiation and progression of atherosclerosis [2]. An uncontrolled uptake of oxidized low-density lipoprotein (ox-LDL), excessive cholesterol esterification and/or impaired cholesterol release result in the accumulation of cholesterol ester (CE) stored as cytoplasmic lipid droplets and, subsequently, triggers the formation of foam cells [3]. Macrophage foam cell formation is associated with abnormalities in cellular cholesterol homeostasis in macrophages [4]. Macrophage pattern recognition receptors (PRRs) recognize oxidized low density lipoprotein (ox-LDL) and facilitate its internalization [5]. CD36 (a class B scavenger receptor) and SRA (a class A scavenger receptor) are the major scavenger receptors responsible for the binding and uptake of ox-LDL [6]. In contrast, the efflux of intracellular unesterified cholesterol to apoAI or HDL is mediated through the reverse cholesterol transport (RCT) process by ATP binding cassette (ABC) transporters, including ABCA1 and ABCG1 and class B scavenger receptor type I (SRBI) [7,8]. The up-regulation or down-regulation of the corresponding receptor by overexpression or interference significantly affects the formation of foam cells or artery plaque. Yu et al. [9] found that the inhibition of macrophage CD36 expression was attributed to the antiatherogenic properties of tamoxifen. Visfatin [10] upregulates CD36 and SRA expression and downregulates ABCA1 and ABCG1 expression and, subsequently, increases ox-LDL uptake and decreases cholesterol efflux to finally promote foam cell formation. The physiological importance of the SRBI receptor for reverse cholesterol transport is suggested by findings using

* Corresponding author. Institute of Life Sciences, Chongqing Medical University, No. 1 Yixueyuan Road, Yuzhong District, Chongqing, 400016, PR China.
E-mail address: yuchaom@163.com (C. Yu).

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hypercholesterolemic mice homozygous for a hypomorphic SRBI allele, which develop increase atherosclerosis, while mice that overexpress SRBI exhibit reduced atherosclerosis [11]. Taken together, lipid homeostasis plays a crucial role in the formation of foam cells.

Lysophosphatidic acid (LPA; 1-acyl-*sn*-glycerol 3-phosphate) is a bioactive lipid with a wide variety of biological activities. The action of LPA is mediated by its binding to and activation of surface GPCRs, including the Edg family of LPA receptors, namely, LPA_{1-3}, LPA_4 (GPR23), LPA_5 (GPR92), and LPA_6 (P2Y5) [12]. These receptors differ in their ability to alter downstream signaling pathways, including intracellular Rho and AKT [13]. The absolute concentration of circulating LPA varies considerably in different assays, but it is clear that LPA is detectable in plasma in the low μM range. However, the concentration of LPA in serum significantly increases during the development of atherosclerosis [14]. LPA accumulates during atherogenesis induced by perivascular collar placement in $Apoe^{-/-}$ mice and is increased in the lipid core region of human atherosclerotic plaques [15]. In addition, bioactive LPA is synthesized during LDL oxidation and accumulates in atherosclerotic lesions, which provides further evidence for the involvement of LPA in atherosclerosis [16]. What is the relationship between a high concentration of LPA in pathological conditions and atherosclerosis?

In previous studies, LPA was always regarded as an external factor to observe the uptake effect on ox-LDL in macrophages, which affects the formation of foam cells. However, there is no report about the effect of individual LPA on the transformation of foam cells from macrophages. As the most bioactive component of ox-LDL, we hypothesized that LPA might promote foam cell formation from macrophages through its receptors. Based on this theory, we used LPA and observe its effects on macrophages and explored the possible mechanism.

2. Materials and methods

2.1. Reagents

1-Oleoyl-lysophosphatidic acid (LPA) was purchased from Avanti (857130P, Alabaster, Alabama). Oil red stain powder and Ki16425 were purchased from Sigma (St. Louis, MO). MK-2206 was purchased from Selleck (Texas, UK). The rabbit monoclonal anti-scavenger receptor class A (SRA) antibody, CD36 antibody, HMGCR antibody, SRBI antibody, ABCA1 antibody and ABCG1 antibody were purchased from Abcam (Oxford, UK). The rabbit polyclonal anti-LPA_1 and LPA_3 antibody were purchased from Bioss (Beijing, China). The rabbit polyclonal anti-AKT and *p*-AKT antibody were purchased from Santa (California, USA). Fetal bovine serum (FBS) was purchased from BI (Israel).

2.2. Cell lines and culture

The murine Raw264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in DMEM supplemented with 10% FBS under a humidified atmosphere at 37°C and 5% CO_2.

2.3. Oil red O staining

The details of the staining procedure were previously described [17]. Briefly, the cells were fixed with 4% PFA followed by 3 washes with cold PBS. The cells were stained for 30 min at 37 °C with the Oil Red O working solution and were counterstained with hematoxylin. Images of the positively stained cells (red) were acquired by a light microscope (Leica Microsystems, Germany).

2.4. Cholesterol/cholesteryl ester quantitation

For analyses of the TC and FC, Raw264.7 macrophage cells were cultured in 6-well plates and incubated with the indicated concentrations of LPA. TC and FC were determined using the Cholesterol/cholesteryl ester quantitation Assay (PPLYGEN, Beijing, China), according to the manufacturer's protocol. The OD was measured at 570 nm by a VERS Amax Microplate Reader (Molecular Devices Corp, Sunnyvale, CA). The TC and FC results are presented in μg/mg protein. The CE was determined by subtracting the value of the FC from the TC.

2.5. CCK8 assay

The procedure was conducted as previously described [18]. Briefly, the Raw264.7 cells were plated in a 96-well plate at a density of 10,000 cells per well and were cultured overnight. The cells were then treated with gradient dilutions of LPA (0, 50, 100, and 200 μM) or 200 μM LPA (0, 12 h, 24 h, and 36 h) in 100 μl of medium. One hundred microliters of the CCK-8 solution was added to each well of the plate, and the plate was incubated at 37 °C for 4 h. The absorbance of each well was measured at 450 nm using a VERS Amax Microplate Reader (Molecular Devices Corp, Sunnyvale, CA).

2.6. Western blot analysis

A Western blot analysis was performed as previously described [18]. Briefly, the cell lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The concentrations of the protein samples were determined by a BCA protein assay kit. The proteins were separated by 6–10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were transferred to a polyvinylidenedifluoride membrane (Millipore). The blot was incubated overnight with antibodies. The blots were washed three times (10 min each) in TBST, followed by 60 min incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Beyotime). The specific proteins were visualized using an Enhanced Chemiluminescence kit (Millipore). The protein content was calculated by densitometry using ImageJ analysis software.

2.7. RNA isolation, and real-time PCR

The total cellular RNA was extracted from the cells using the TRIzol reagent (Takara, Japan). The total RNA (1 μg) was subjected to an RT reaction with the PrimeScript™ RT reagent Kit (Takara). Real-time PCR was carried out using an iCycleriQ real-time detection system with SYBR Premix Ex Taq™ II. The targeted genes were amplified with the following primers: 5′- TGGCCTTACTTGGGAT TGG -3′ (F:sense primer) and 5′- CCAGTGTATA TGTAGGCTCATCCA -3′ (R:anti-sense primer) for the CD36 gene; 5′- TGGTCCACCTGGTGCTCC -3′ (F) and 5′- ACCTC CAGGGAAGCCAATTT -3′ (R) for the SRA gene; 5′- TGGCAGGACGCAACCTCTAT -3′ (F) and 5′- TGACGGCTTCACAAAC CACA -3′ (R) for the HMGCR gene; 5′-CCCAGAGCAAAAAGCGACTC -3′ (F) and 5′- GGTCATC ATCACTTTGGTCC TTG -3′ (R) for the ABCA1 gene; 5′- AAGG CCTACTACCTGGCAAAGA -3′ (F) and 5′- GCAGTAGGCCACAGGGAACA -3′ (R) for the ABCG1 gene; 5′- GGCT GCTGTTTGCT GCG -3′ (F) and 5′- G CTGCTTGATGAGGGAGGG -3′(R) for the SRBI gene; 5′- CATGGTGGCAA TCTACGTCAA -3′ (F) and 5′- AGGCCAATCCA GCGAAGAA -3′ (R) for the LPA_1 gene; 5′- GTAC CTGAGCCCCCCATTG -3′ (F) and 5′- AAACCCATGCGGAA ACAACT -3′(R) for the LPA_3 gene and 5′- AAGA CCTCTATGCCAACAC -3′ (F) and 5′- CTGCTTGCTGATCCACAT -3′ (R) for the β-Actin gene (the internal standard gene).

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