

Mechanism of LPA-induced lipid uptake in macrophages

**A Thesis Presented for the
Master of Science**

Degree

The University of Tennessee, Knoxville

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ABSTRACT

Cardiovascular disease, currently the leading cause of mortality throughout the developed countries, is mainly caused by atherosclerosis, which is recognized as a chronic inflammatory disease. Atherosclerotic plaques are characterized by accumulations of lipid in arterial walls together with infiltration of macrophages. These macrophages differentiate from monocytes which transform into foam cells through phagocytizing various forms of lipid, are believed to be the main component of early atherosclerotic lesions.

Lysophosphatidic acid (LPA), a potent bioactive lipid, regulates a broad range of cellular functions in various cell types. In our findings, LPA-induced macrophages may enhance the lipid uptake effect in both J774A.1-mouse macrophage cell lines and bone-marrow derived macrophages (BMDMs) in a time and dose dependent manner.

To date, LPA activated signaling pathways in macrophage and possible intracellular molecules mediating LPA-triggered foam cell formation wait to be explored. According to our results, we found: 1) LPA receptor 2 and 5 were predominantly expressed than other receptors in macrophages. 2) Protein Kinase D (PKD) underwent activation both on the Ser744/748 activation loop and auto phosphorylation site Ser916 from 2 minutes to 5 minutes upon the addition of LPA. 3) During the early period of LPA treatment, phospho-ERK, phospho-JNK and

phospho-P38 all showed signs of short term inhibition - which indicates that MAPK activity had been transiently inhibited in macrophages by LPA. 4) NF-kappaB, a key transcriptional regulator in macrophages, showed no obvious patterns of activation in our investigation through observing that neither IKK nor IKB units were activated upon LPA induction. And this finding excludes the possibility that NF-kappaB pathway is involved in LPA-induced macrophage activation. 5) Our northern blot analysis demonstrated that, SR-B1 and CD36 – two scavenger receptors that are critical in lipid accumulation of macrophages – were up-regulated by LPA.

Collectively, all emerging data leads us to hypothesize that LPA, through its receptors to activate intracellular pathways, mediates downstream gene expression level, then expedites foam cell formation, which may contribute to atherosclerotic lesion formation.

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Chapter1 Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in most developed countries, which proximately accounts for 16.7 million deaths each year worldwide [1]. Atherosclerosis – underlies coronary artery disease and is the most common form of cardiovascular disorders. Currently atherosclerosis is widely accepted as a slow progressive chronic inflammatory disease, which is characterized by the formation of atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, migrated smooth muscle cells (SMCs), foam cells, endothelial cells (ECs), and leukocytes [2]. Macrophages play an important role in plaque formation. Their precursor form - monocytes, together with platelets and T-lymphocytes, interact with ECs and SMCs in multiple ways to contribute to the development of atherosclerotic lesions [3].

Structure of Normal Arteries

Normal arteries have a well-developed tri-laminar structure which includes intima – the innermost layer, media – lies between intima and outer layer of blood vessels, and adventitia –outermost layer of the artery. The intima layer is crucial in understanding the pathogenesis of atherosclerosis. Though many studies described the intima layer as consisting of a single layer of ECs with little underlying subendothelial connective tissues, the real structure of this layer in adults shows more complexity and heterogeneity [4]. The major cell type located

in the media layer is smooth muscle cells, interleaved with layers of elastin-rich extracellular matrix. The adventitia layer exhibits a loose array of cells - such as fibroblasts and mast cells, and receives little attention in atherosclerosis studies [4].

Inflammation in Atherosclerosis

Based on a strong clinical relationship between hypercholesterolemia and atheroma, the regulation of cholesterol and low density lipoprotein (LDL) seemed to be both valuable and dominant in evaluating cardiovascular risk factors during early investigations [5]. In the 1970s and 1980s, attention migrated to growth factors and smooth muscles due to an evolving knowledge on vascular biology. Finally in the past decade, investigations became more and more appreciative of the prominent role of inflammation in cardiovascular complications [2].

Inflammation has been closely linked with the formation, progression and rupture of atherosclerotic plaques [6]. Numerous studies show that either various kinds of inflammatory cytokines or inflammatory cells participate in disease regulation. The initiation of plaques requires activated endothelial cells to express adhesion molecules, which attract flowing blood monocytes to the endothelial layer. Many studies suggest adhesion plays a key role in monocyte/macrophage recruitment. And these adhesion molecules include VACM-1, ICAM-1, P-selectin and E-selectin [7]. After monocytes are recruited beneath the endothelial layer of the arterial wall, they continue to be stimulated by macrophage colony stimulating

factor (M-CSF) and other differentiation stimuli. Then these monocytes are differentiated into two major types of macrophages – M1 and M2 macrophages [1]. Fully matured macrophages stably express various kinds of scavenger-receptors (SRs), and these receptors are likely to be responsible for accumulating different forms of LDL. Lipid-laden macrophages - the so called “foam cells”, then undergo apoptosis and pile beneath the arterial wall (intima layer). The suppressed clearance of these apoptotic cells (efferocytosis) leads to the formation of the early plaques. Subsequently, smooth muscle cells located in the media migrate into the top intima layer and multiply to produce a tough, fibrous matrix to glue onto the fatty streak. However, these seemingly tough fibrous caps can be easily digested by inflammatory cytokines secreted from foam cells. Once the cap breaks down, blood coagulation initiator – tissue factor released out from the plaques, will cause the formation of a thrombus. As the clot grows bigger and bigger, the patient at this stage will experience heart attack or stroke due to the blockage of blood flow [8].

Macrophage Subtypes during Atherosclerosis

Induced by M-CSF and GM-CSF, monocytes can differentiate into two subtypes – M1 and M2 macrophages. The two type of macrophages play opposite roles during inflammation and both are present in atherosclerotic lesions. Ly6C is a cell differentiation antigen regulated by interferon gamma. In mice, monocytes which express a high level of Ly6C antigen will differentiate into M1 macrophages upon stimulation, and cells with a low level of Ly6C antigen will differentiate into M2

macrophages. M1 macrophages - classically activated by IFN- γ , produce a high level of cytokines such as IL-2, IL-23, IL-6, IL-1 and TNF- α . In contrast, monocytes with low Ly6C are usually activated in the presence of IL-4, IL-13, and IL-1, and tend to produce a large amount of IL-10 and express scavenger receptors. Clinical evidence suggests that an imbalance of M1 (classically activated) and M2 (alternatively activated) macrophages may have a significant role in advancing atherosclerosis and impairing resolution *in vitro* [9]. Although the role of macrophages in atherosclerosis development has not been fully understood, it is clear that macrophages participate in several major processes including: infiltrating into intimal layer, increasing plaque size, pathologically processing lipids uptake, inhibiting cholesterol efflux to form “foam cells”, and secreting a broad range of cytokines in response to various stimuli to amplify the inflammatory process [10].

Scavenger Receptor - mediated Lipid Metabolism

The first scavenger receptors were identified through a combination of uptake assays using I¹²⁵-labeled acetylated LDL, then the work of identification was quickly extended to a broad range of structurally unrelated molecules [11-13]. Until 1997, a detailed framework of scavenger receptors' classification was brought up by Krieger and colleagues and is now becomes widely accepted. The proposed classification encompassed 8 different scavenger receptors from A to H. Most of which have shown significant roles in atherosclerosis development. (Please see table I)

For SR class A (SR-AI and AII), initial studies on these receptors showed that SR-AI and AII knock-out mice (Msr-/-) backcrossed with apoE-deficient (ApoE-/-) mice showed a significant decrease in aortic sinus atherosclerotic lesions, indicating that lipid uptake via SR-A is pro-atherosclerotic [14]; however, subsequent investigations of SR-A failed to support earlier observations – over expression of SR-A does not necessarily leads to more atherosclerotic lesion formation[15, 16]. Recent reports suggest that SR-As, account for the uptake of acetylated LDL (AcLDL) but not oxidized-LDL (oxLDL) uptake in a majority of macrophages [14]. Notably, the role played by SR-As may turn from an earlier pro-atherosclerotic one into an athero-protective one by recent findings that a higher SR-A expression level was found in animals with low atherosclerotic response compared to normal animals with a normal atherosclerotic response [17].

Class B scavenger receptors were identified along with CD36 for up-taking oxLDL. Three members were included: SR-BI, SRBII, and SRBIII(CD36). Different from the SR-A family, CD 36 as a type III receptor which only shows 30% homology with the other two members in this family (SR-BI, SRBII), and traverses twice in cell membrane to form a heavily glycosylated extracellular loop with 2 short intracellular tails. Studies by Moore and Freeman's group and others indicate that CD 36 rather than SR-A played a major role in clearance the cholesterol from the body: contributing 60%-70% of cholesterol ester accumulation in macrophages when exposed to copper oxidized LDL [18-20]. Along with recent findings, CD36 activates signaling via TLR2 and TLR6 in response to lipoteichoic acid and diacylated macrophage-activity [21].

LOX-1 was identified as scavenger receptor class E, a lectin-like, type II transmembrane protein [22]. LOX-1 is also expressed on the macrophage cell surface and is a potential receptor for phagocytizing oxLDL, recent research suggests that overexpression of LOX-1 in Apoe^{-/-} mice enhances oxLDL uptake and accelerates intramyocardial vasculopathology[23]. However, the role of LOX-1 in the development of atherosclerosis still needs further confirmation and investigation.

Lysophosphatidic acid (LPA): Function, Receptors and Implication in Atherosclerosis

Lysophosphatidic acid (LPA; 1-acyl 2-hydroxyl glycerol 3-phosphate) is a bioactive lipid component with multiple biological functions by regulating a variety of diseases[24]. The earliest study of LPA's physiological role traced back to 1963 by Vogt[25]. After which, Tokumura and colleagues applied an animal model to prove that LPA exerts a strong physiological role in affecting the function of blood vessels *in vivo*[26]. Schumacher and Gerrard in 1979 discovered that LPA could stimulate platelets aggregation which was considered as among the first recognition of LPA function in thrombotic vascular disease [27, 28]. Recently, Chun's group and others found at least five LPA receptors (LPA₁₋₅), that links to multiple downstream effector pathways. By using these receptor-deletion mouse models, recent studies on LPA biological and physiological functions shed new light on the subject.

LPA₁ is the most widely expressed receptor in a variety of organs and tissues: brain, colon, small intestine, placenta, and heart etc. LPA₂ and LPA₃ showed a more narrow expression map compared to LPA₁. In mice, LPA₂ expression is found to be most abundant in embryonic brain, kidney, and testis; LPA₃ expression is restricted to lung, kidney and testis. LPA₄ and LPA₅ has not been fully explored and understood. Significant expression of LPA₄ has only been observed in the ovary [29]. LPA₅ has a broad, low level expression over many tissues and organs[30].

Oxidized LDL is one of the most likely contributors in the pathological process of atherosclerosis and thrombosis. As LPA has been found to be formed during mild oxidation of LDL and the active compound in various forms of oxidized LDL, it may play an important role in the development of atherosclerosis and thrombosis. Although no severe vascular defects have been reported from single deletion of LPA receptors in animal models, the importance of LPA has never been neglected by these years' publications. It has been shown that, LPA induces the expression adhesion molecules, which include E-selectin, VCAM-1 and ICAM-1 in ECs [31-33]. In smooth muscle cells, our previous data suggest that LPA has a strong effect in promoting smooth muscle cells proliferation and migration, a central step in atherosclerotic lesion development. In monocytes and macrophages, LPA either directly or indirectly modulates monocytic cell migration via up-regulating secretion of MCP-1 and IL-8 from ECs [34, 35]. Our study intended to prove that LPA induces oxidized LDL uptake by using multi-sourced murine macrophages, and also focused on investigating how LPA activates macrophages and what possible

mechanisms are involved in this process. The increased uptake of lipids eventually leads to expedition of foam cell formation – a major component of atherosclerotic lesion.

Chapter2 Experimental Methods and Materials

Cell Culture

J774A.1 (mouse macrophage cell line) and L929 (mouse fibroblast) cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.). Mouse aorta smooth muscle cells (MASMCs) were prepared from explants of excised aortas [36]. Bone-marrow derived macrophages (BMDMs) were harvested from the femur section of C57B/6 mice (Jackson Laboratory, Bar Harbor, Maine, U.S.A.). After four consecutive days' culture in lymphocyte medium, greater than 95% of bone-marrow progenitor cells were differentiated into macrophages [37]. Lymphocyte medium was acquired by adding 10% L929 conditioned medium into DMEM (Lonza, Walkersville, MD, U.S.A.) culture medium. While the L929 conditioned medium was prepared by collecting the supernatant out from DMEM cultured L929 cells and filtering through a 0.22 μ M filter (Millipore, Billerica, MA, U.S.A.). Mouse peritoneal macrophages (Peri-M ϕ) were collected according to previous reports[37]. Three percent Brewer thioglycolate medium (BD Biosciences, Sparks, MD, U.S.A.) was delivered into 6-8 week old C57B/6 mice by intra-peritoneal injection on day 0. After 4 days, the mice were euthanized and their abdominal section were flushed with 5 to 7ml ice-cold PBS to harvest the cells. The cells were firstly culture in regular DMEM medium for 2 hours, and then the supernatant was removed leaving the cells attached to the bottom of the dish for experimental purposes.

Immunofluorescence Staining

Two specific macrophage antibodies were applied for phenotypic identification - rat-anti-mouse CD11b and rat-anti-mouse F4/80 (AbD Serotec, Raleigh, NC, U.S.A.). Three different types of cells were included: J774A.1 (positive control), mouse SMCs (negative control) and bone-marrow derived macrophages. Cells were plated on microscope cover glasses (Fisher Scientific, Loughborough, U.K.) in 12-well plates (Corning, NY, U.S.A.), fixed with 4% ice-cold paraformaldehyde (Sigma Aldrich, St. Louis, MO, U.S.A.) at room temperature for 30 minutes, blocked against 5% goat serum (Invitrogen, Camarillo, CA, U.S.A.) plus 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO, U.S.A.) in PBS for 1 hour, then tested against two antibodies above accordingly, followed by the goat anti-rat IgG (Alexa-Fluro 488 labeling) (Invitrogen, Camarillo, CA, U.S.A.) for 2 hours at room temperature. Then each well was washed with PBST 4 times (5 minutes each) at room temperature; followed by 2 minutes incubation with DAPI (Invitrogen, Camarillo, CA, U.S.A.). Finally, the cover glasses were mounted with permanent aqueous mounting medium (Biogenex, Fremont, CA, U.S.A.) and observed under the microscope.

Flow Cytometric Analysis

Both J774A.1 and bone marrow derived macrophages were plated in 30mm culture dishes (Corning, NY, U.S.A.), pretreated with LPA (1-Acy-2-hydroxy-sn-glycerol-3-phosphate, Avanti Polar Lipids Alabaster, Alabama, U.S.A.) and incubated for various time points with Dil-labeled oxidized LDL (Biomedical Technologies, Inc. Stoughton, MA, U.S.A.). After two ice-cold PBS washes, the cells were detached from dishes through trypsinization. The cells were suspended in 1ml ice-cold PBS, followed by analysis with an Epics XL-MCL Flow Cytometer (Beckman, Brea, CA, U.S.A.). The excitation wavelength was at 488 nm and the emitted red fluorescence (FL2) was collected using a 530nm band pass filter.

Oil-red O Staining

M ϕ were plated on microscope cover glasses in 12 well plates, after LPA pretreatment, the cells were incubated with copper oxidized LDL (Biomedical Technologies, Inc. Stoughton, MA, U.S.A.) at 37 degrees in 5% CO₂, and observed under a microscope after oil-red O staining. Cells were rinsed twice with PBS, and then 10% formalin 30minutes was added for fixation. This was followed by four washes in ice-cold PBS. Each sample was incubated with 0.75ml Oil-red O working solution for 5 minutes at room temperature. Oil-red O was removed by rinsing under tap water until the water rinse off clear. Then 0.75ml hematoxylin (Sigma Aldrich, St. Louis, MO, U.S.A.) was added for 1minute at room temperature and

rinsed off under tap water. Last, the cover glass was mounted with permanent aqueous mounting medium (Biogenex, Fremont, CA, U.S.A.) and observed microscopically.

Northern Blot

Total RNA was extracted from cells in accordance with the manufacturer's instructions (Qiagen, Gaithersburg, MD, U.S.A.) and subjected to denaturing electrophoresis in formaldehyde/ agarose gels. After the sample blotted onto hybond-N (Amersham GE healthcare, Pittsburgh,PA) membranes, they were hybridized with ³²P-labeled cDNA probes over night at 65°C. The membranes were washed twice for 15 minutes each with washing buffer prior to final detection. The primer pairs used here are showed in table C. Primers were designed to produce probes around 800bp in length. In order to confirm the probe specificity, each included one unique restriction enzyme cutting site. Probe testing was performed before northern hybridization. Amplified DNA fragments were separate on 1.0% agarose gel and target fragments were recollected using a DNA purification kit (Qiagen, Gaithersburg, MD, U.S.A.). Purified DNA fragments were incubated with matched restriction cutting enzymes and buffer at 37°C over-night. Gel electrophoresis was used to check the length of each cut fragment to confirm that it matched our original design.

Western Blot

Cells were collected at various times following LPA treatment, rinsed with ice-cold PBS and lysed in PARP buffer [50 mM Tris · HCl (pH 6.8), 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors]. The lysate was sonicated at 4°C for 30 seconds. Samples were boiled with SDS-PAGE loading buffer (5X) for 10 minutes. An equal portion of lysates were quantified using Bio-photometer (Eppendorf, NY, U.S.A.) and samples were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, U.S.A.). The membranes were then probed with specific antibodies, and reactive bands were visualized using ECL-Plus (GE Healthcare).

Conventional PCR

The level of expression of the LPA receptor gene was measured using conventional Reverse-transcript PCR. Total RNA was extracted using Trizol (Invitrogen, Camarillo, CA, U.S.A.). The first strand of DNA was reverse transcribed using the Thermo Script Reverse Transcript system (Invitrogen, Camarillo, CA, U.S.A.). Then the cDNA was amplified with target primers using the following conditions: 5 minutes at 94°C; various repetitive cycles based on experimental needs for 30 seconds at 94°C, 30 seconds at 58°C, 45seconds at 72°C; and a final extension for 10 minutes at 72°C. The primer pairs are described in table II.

RT-PCR

The expression of mRNA was evaluated by real time PCR. Total RNA was isolated from J774A.1 cells using an RNeasy mini-prep kit (Qiagen, Gaithersburg, MD, U.S.A.). The first strand of cDNA was reverse transcribed using the Thermo Script Reverse Transcript system (Invitrogen, Camarillo, CA, U.S.A.). Then the cDNA products were amplified with primers using SYBR Green Master Mix (Invitrogen, Camarillo, CA, U.S.A.). The amplification conditions went as follows: 2 minutes at 50°C; 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C, 45seconds at 60°C; 15 seconds at 95°C; finally 15 seconds at 60°C. The primer pairs are showed in table II.

Data Analysis

All data were representative of a minimum of three experiments. Results were expressed as means $\pm$ S.D. (Standard Deviation) Comparisons between multiple groups were performed using one-way ANOVA with Dunnett's post hoc t-tests. A single comparison analysis was made using two-tailed unpaired Student t-tests. P values of ± 0.05 for ANOVA or t-tests were considered to be statistically significant.

Chapter3 Results

1. Macrophage Characteristic Exploration

Macrophage Phenotypic Identification

In order to characterize the phenotypic characteristics of macrophages from multiple sources in our experiments, we used macrophage specific markers CD11b and F4/80 for phenotypic identification of three cell lines: The J774A.1 macrophage cell line was purchased from ATCC, Bone-marrow derived macrophages (BMDM) were isolated from mouse femurs, smooth muscle cells (SMCs) were isolated from mouse aorta. As demonstrated in the immunofluorescence data below (Fig.1) about 95% of the BMDM cell population were positively stained by the two markers compared to J774A.1 macrophages which served as a positive control. SMC, used as a negative control, exhibited no positive staining. This result suggested that isolated bone-marrow highly enriched for macrophages (95% purity level).

Comparison of LPA Receptor Expression Levels in J774A.1 Macrophages and SMC

LPA stimulates a plethora of different cellular responses through the activation of its family of cognate G protein coupled receptors. In order to understand which of the LPA receptors mediates LPA signaling pathway in macrophages, we performed RT-PCR and real-time PCR analyses to evaluate LPA receptor expression levels. The expression of LPA receptors in macrophages was compared with that in SMC. The summary of our PCR and real-time PCR results are shown in Fig. 2-3. The LPA₁₋₅ showed a heterogeneous expression pattern between two cell lines. For smooth muscle cells (included as a quality control), LPA₁, LPA₂, LPA₄ are dominantly expressed compared to LPA₃ and LPA₅. While in the J774A.1 macrophage cell line, only LPA₂ and LPA₅ are dominantly expressed on the cell surface. These data suggests that LPA₂ and LPA₅ may play important role in mediating LPA induced macrophage activation and function.

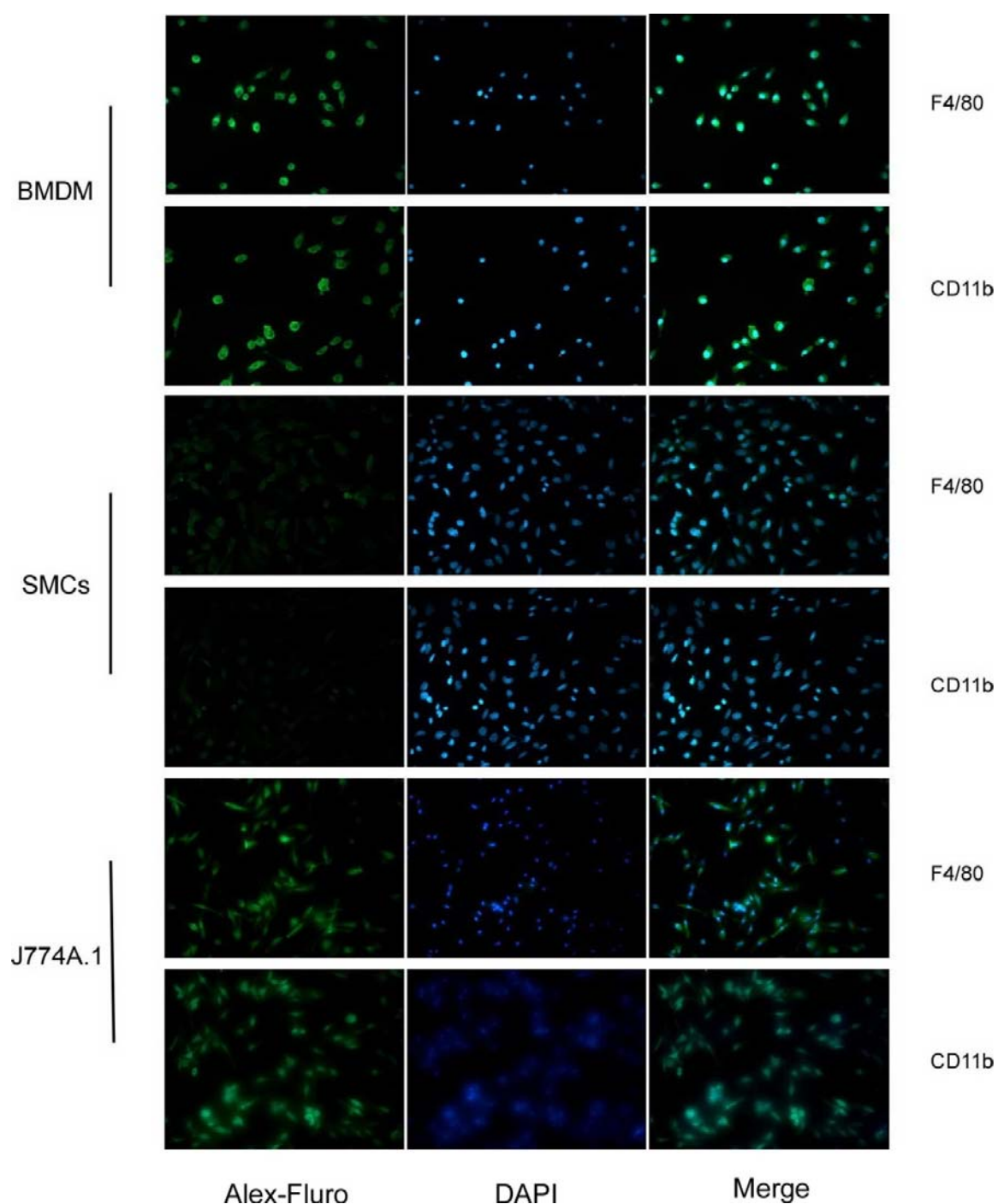


Figure 1 Phenotypic Identification

Macrophage specific markers were examined in three different cell lines. Cells were cultured on cover glass for at least 12 hours, then fixed with 4% paraformaldehyde. Incubated with CD11b or F4/80 followed by Goat anti Rat Alex-fluro 488, DAPI was added to stain the nuclear(Blue). (200X magnification).

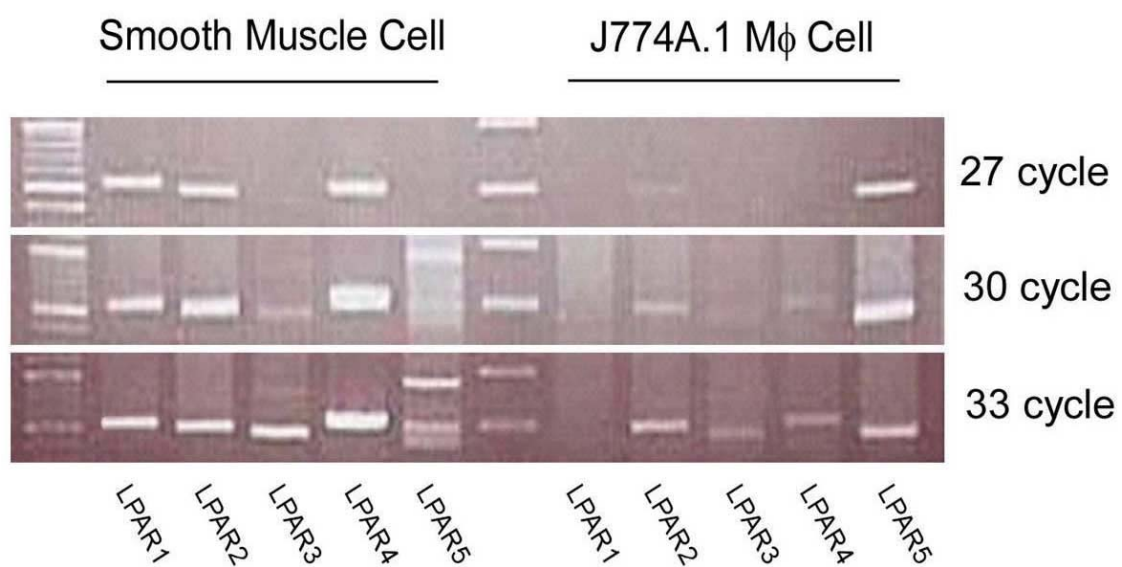


Figure 2 Reverse-transcript PCR Amplification

Total mRNA was extracted from J774A.1 mφ cells and SMCs, cDNA was obtained using the Invitrogen Thermo Reverse Transcript system. The oligo primers are described in table II.

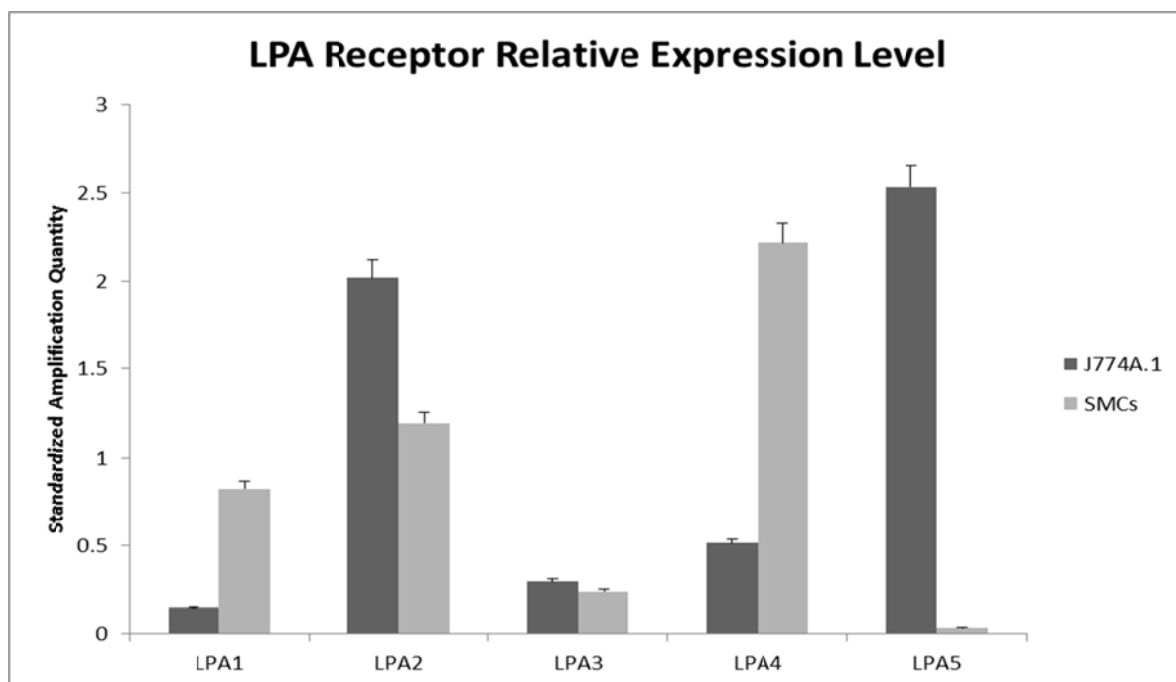


Figure 3 Real-time PCR Analysis

Oligo primers were designed to accommodate real time analysis. Real-time PCR primers are described in table II, SYBR Green dye was added to quantify the amplification products. Results were standardized for comparison.

2. LPA Enhances Macrophage Lipid Uptake Activity

Although Chang and others first reported that LPA would potentially induce oxLDL uptake in J774A.1 macrophage [38]; specific signaling cascades and the intracellular regulatory molecules involved in LPA induced foam cell formation are still unclear. The following questions are waiting for answers. 1) Which LPA receptors are involved? 2) Whether LPA elicits cellular protein kinase activation or whether the specific kinase activation leads to LPA-induced cellular activity? 3) Which genes regulated by LPA may contribute to LPA-induced lipid uptake in macrophages?

LPA Induces Macrophage DiI-oxLDL Uptake

Accumulation of oxLDL beneath arterial walls has been viewed as one of the initial risk factors for fatty streak formation [39]. In order to determine whether LPA plays a role in macrophage lipid uptake activity, we examined LPA's effect on macrophage uptake. J774A.1 were treated with 5 μ M LPA together with 15 μ g/mL DiI-oxLDL for 3 hours, then these cells were analyzed using flow cytometry to quantify the macrophage uptake activity. From the results below (Fig.4), we found that LPA significantly increases DiI-oxLDL uptake by J774A.1 macrophages.

LPA Induced ox-LDL Uptake in Macrophages in Both Time-dependent and Dose-dependent Manner

Dil (DiIc18) is a fluorescent lipophilic cationic indocarbocyanine dye that can be used to quantify oxidized LDL. In order to explore how LPA influences oxLDL uptake, we performed dose response (Fig.5 and Fig.7) and time course experiments (Fig.6 and Fig.7) in three different macrophage cell lines. Our data shown that 5 μ M is the optimal concentration for LPA to gain the maximum uptake effect, indicating that LPA-induced oxLDL-uptake effect is dose-dependent. In time course analysis, J774A.1 and BMDM showed similar peak uptakes times when LPA and Dil-oxLDL were added simultaneously. For peritoneal macrophages, however, the highest fluorescence intensity occurred at 2 hours LPA pre-treatment followed by 3 hours co-treatment with Dil-oxLDL. The inconsistency may be due to cell heterogeneity: peritoneal macrophages isolated from mouse abdominal sections showed less than 70% purity in macrophage population.

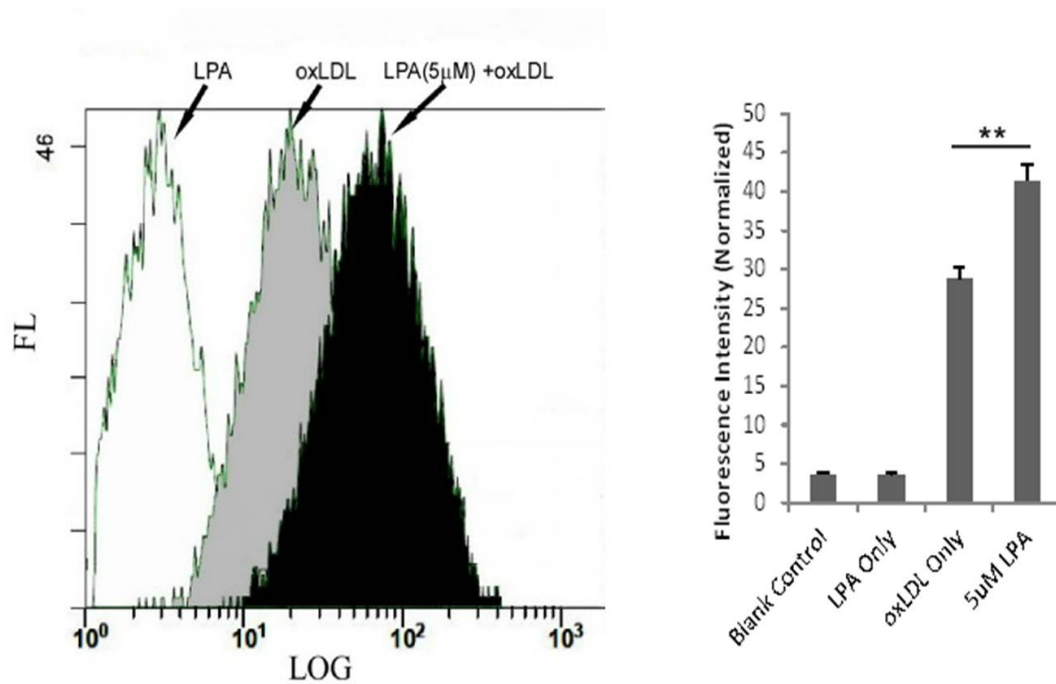


Figure 4 Flow Cytometric Analysis of LPA-induced Lipid Uptake in Macrophages.

J774A.1 macrophages were starved for 24hours, then were treated with 5μM LPA and 15μg/ml Dil-oxLDL for 3 hours. The horizontal axis in the left histogram represents the fluorescence intensity of Dil dye and the vertical axis indicates the cell quantity. The bar plot on the right showed the results of the LPA-induced uptake effect. The mean intensity value $\pm$ SD is shown. **p<0.01

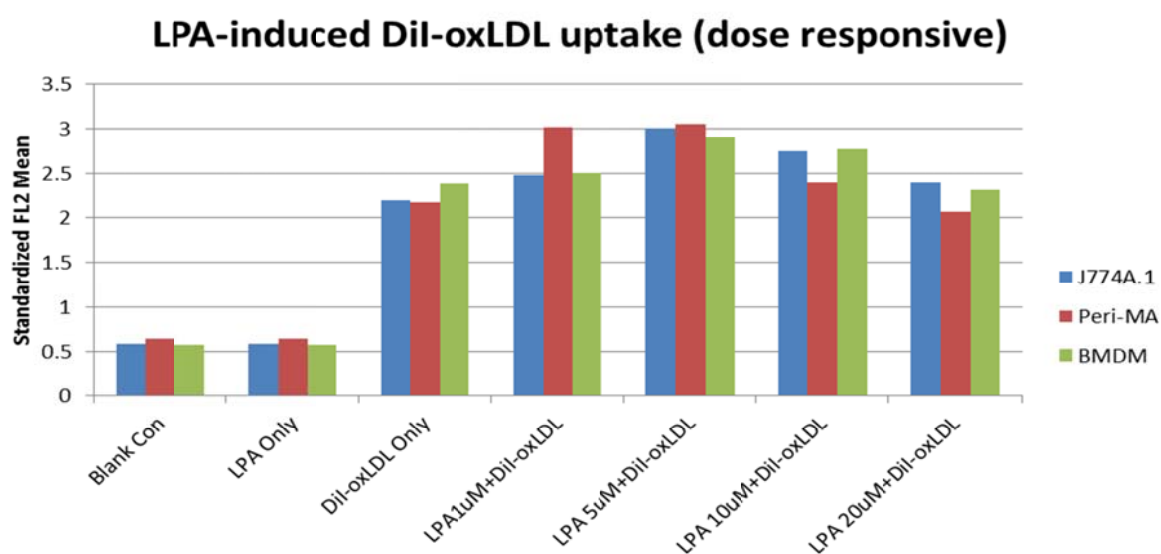


Figure 5 Dose-dependent Effect of LPA-induced oxLDL Uptake in Macrophages.

Flow cytometry analysis was performed to determine the LPA effect on oxLDL uptake in three different macrophage cell lines. Macrophage cells were starved for 24 hours before treatment, then were treated with various concentration of LPA and Dil-oxLDL at 15 μ g/ml simultaneously for 3 hours incubation, cells were then suspended in 1X PBS for analysis.

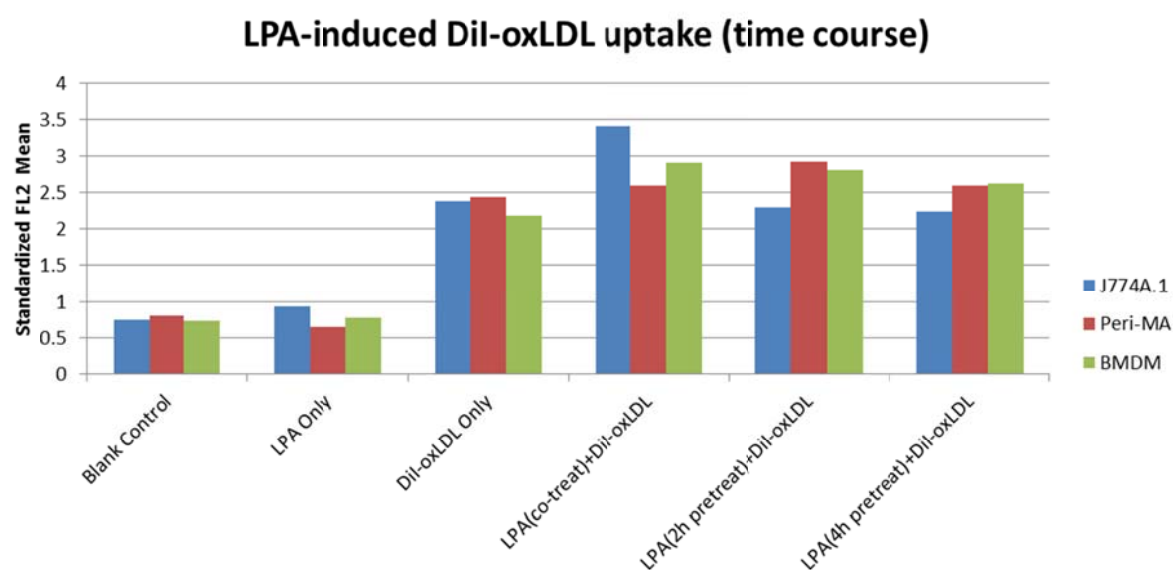


Figure 6 LPA-induced oxLDL Uptake in Macrophages in a Time-dependent Manner.

Flow cytometric analysis was performed for three different sources of macrophages. They were starved for 24 hours, then treated at the indicated time with 5 μ M LPA followed by 3 hours incubation with 15 μ g/ml Dil-oxLDL. Cells were then suspended in 1X PBS for analysis.

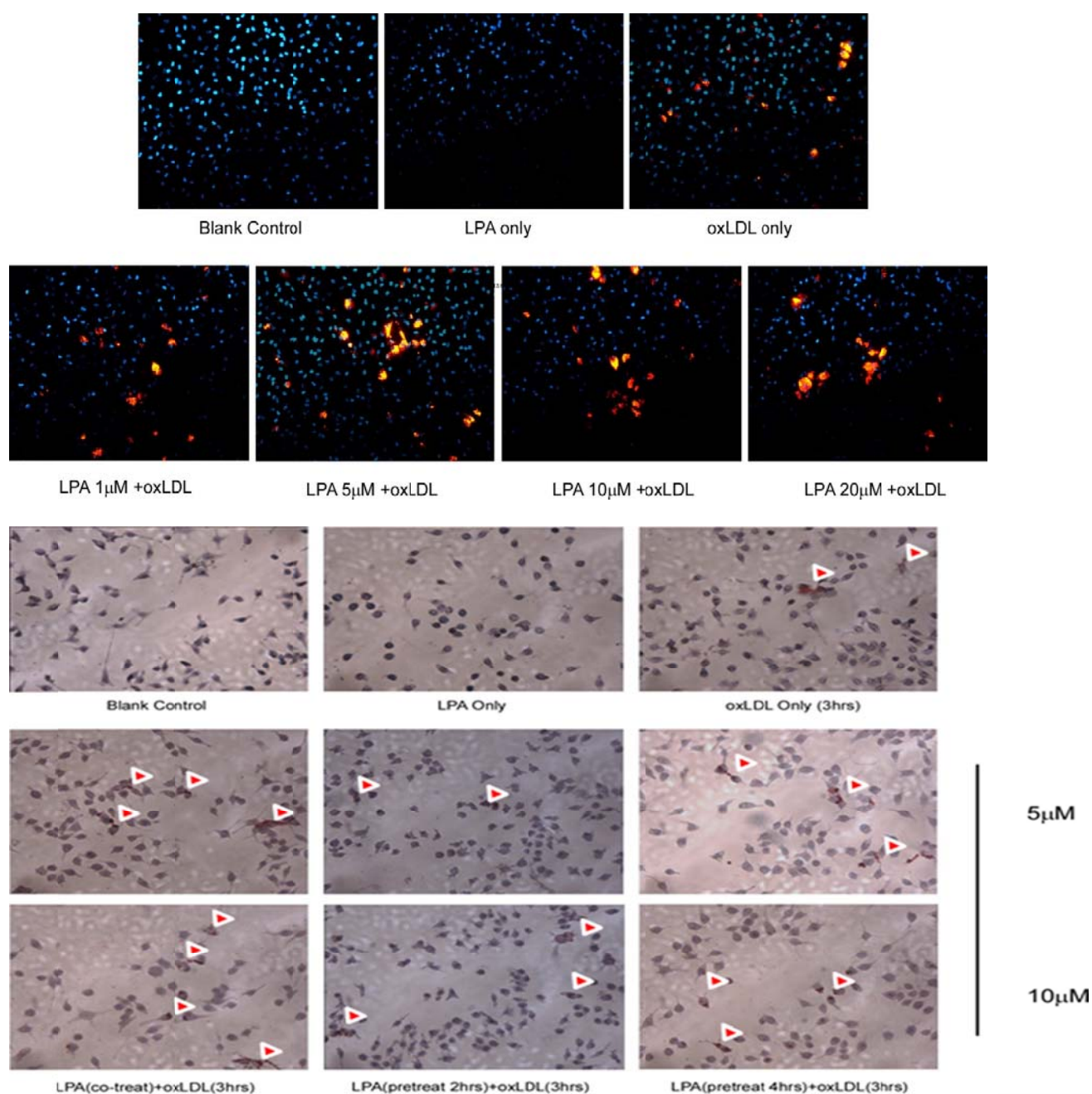


Figure 7 Dose and Time Dependent Uptake Assay.

Cells plated on glass cover slips were starved for 24 hours, and then treated at indicated time or concentration of LPA. Cells positively stained were quantified in two plots and analyzed using student's t-test.

3. LPA Triggers the Inhibition of MAPKs

It is well known that macrophages are key components in the inflammatory process of atherosclerosis, and macrophages are the first inflammatory cells to invade atherosclerotic lesions [1]. Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases which regulate a series of cellular functions including cell proliferation, survival, death, and inflammation. The major three members of MAPKs are: ERKs, JNKs and p38s. They are regulated through a phosphorylation cascade. We sought to explore whether the MAPK pathway was involved in LPA-induced cellular activity of macrophages, and whether MAPK pathway contributed to the lipid uptake in macrophages. To get a broad view of kinase activation, we examined MAPK activity through a complete time course by applying two sources of macrophage cells, bone-marrow derived macrophages and the J774A.1 cell line. Cells were starved for 24 hours prior to LPA treatment. LPA induction of MAPK activity in SMCs served as a positive control. We observed an interesting phenomenon that at the very early time points (around 2 to 15 minutes) of LPA stimulation, the activities of MAPK families were inhibited in both J774A.1 and BMDMs (Fig. 8). To date, LPA induction of MAPK activation in various cell types has been well documented. Our group showed that LPA induced MAPK activation in SMCs [40]. The discovery of inhibitory effects of LPA on MAPK activation in macrophages may present the first evidence of LPA-stimulated MAPK inhibition. The inhibitory effect of intracellular MAPKs may influence macrophage cellular function in response to LPA stimulation.

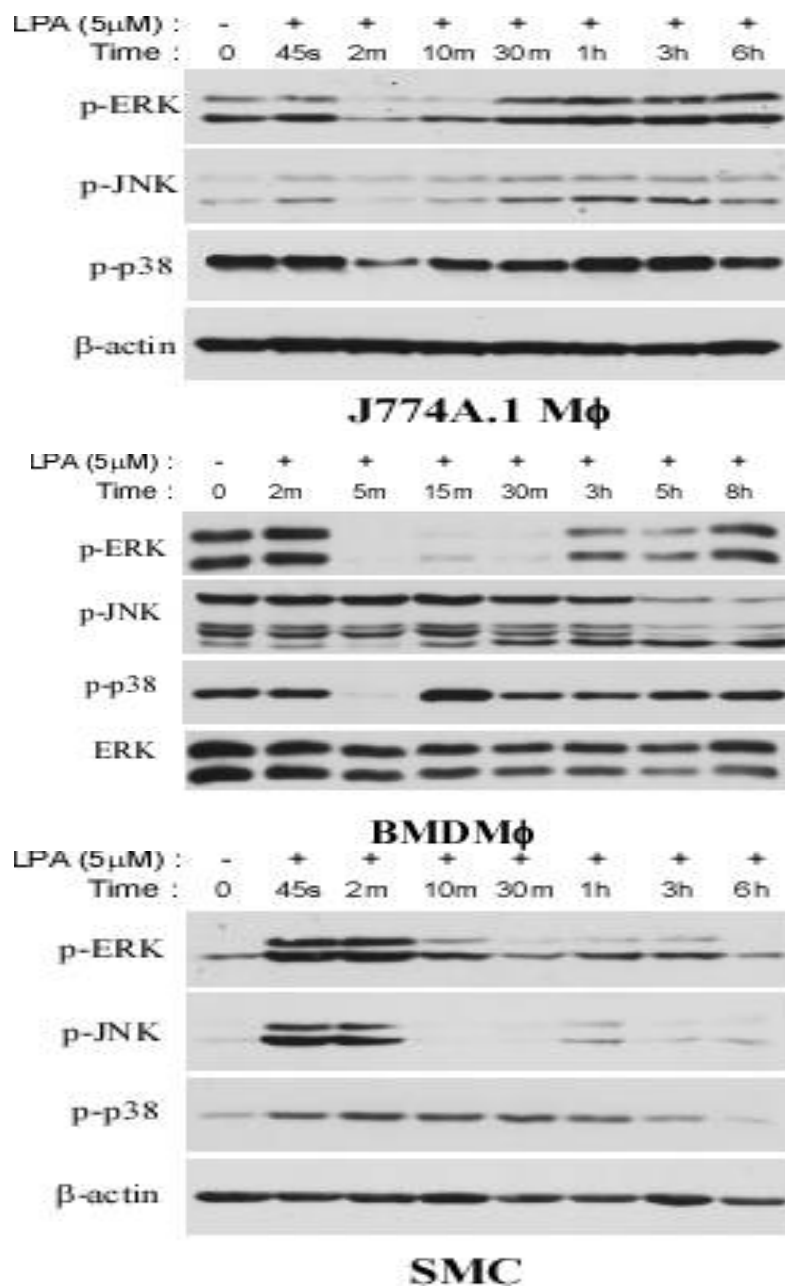


Figure 8 MAPK Inhibition in LPA-induced Macrophages.

This figure shows a temporary inhibition of p-ERK, p-JNK and p-38 in J774A.1 cells (upper panel) and bone-marrow derived macrophages (middle panel). In contrast to smooth muscle cells (lower panel), macrophages showed LPA-induced dynamic activation of p-ERK, p-JNK and p-p38. All cells were starved over night, after treatment with LPA for various time periods. Cells were collected and cell lysates were separated in 10% SDS-PAGE gels and detected in western blots with specific antibodies.

4. LPA Induces Protein Kinase D (PKD) Activation

Protein kinase D (PKD), occupies a unique position in early signal transduction pathways initiated by DAG, which elicits cellular responses through a variety of effectors [41]. The PKD family consists of three isoforms: PKD1, PKD2 and PKD3. This protein kinase family is also calcium/calmodulin-dependent protein kinase group of serine/threonine kinases [42] . We sought to examine whether PKD is activated in LPA-stimulated macrophages and whether PKD activity induced by LPA is effective on lipid uptake in macrophages. We first detected the expression of PKD isoforms in macrophages using specific PKD1, PKD2 and PKD3 antibodies. We found that all three PKD isoforms were expressed in macrophages (Fig.9). Next, we examined LPA-induced PKD activation and the dynamics of PKD phosphorylation in two sources of macrophages. As shown in Fig.10, both PKD1 activation site (Ser916) and PKD1/2 activation loop (Ser744/748) were phosphorylated in the early time periods in LPA-induced J774A.1 and BMDMs. The function of PKD in macrophages has not been determined. Our data suggest the possibility that activation of PKD may contribute to LPA-induced lipid uptake in macrophages.

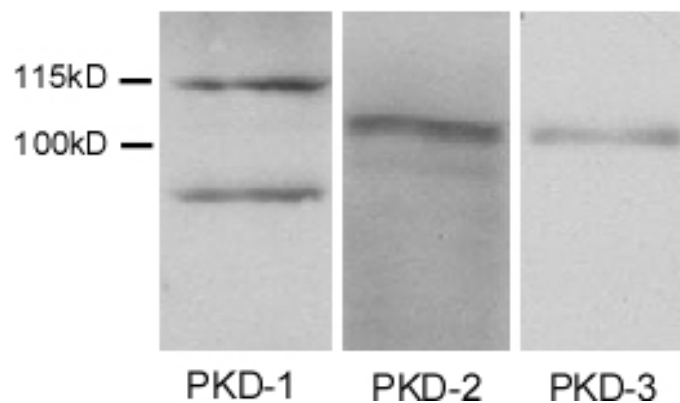


Figure 9 Expression Levels of Three PKD Isoforms in J774 A.1 Macrophages.

Cells were starved over-night and collected without any treatment. Western blot analysis showed that all three isoforms of PKD are present in J774A.1 macrophages. The molecular weights of the three PKD isoforms are as follows: PKD1 is 115 kD, PKD2 is 105 kD, PKD3 is 100 kD.

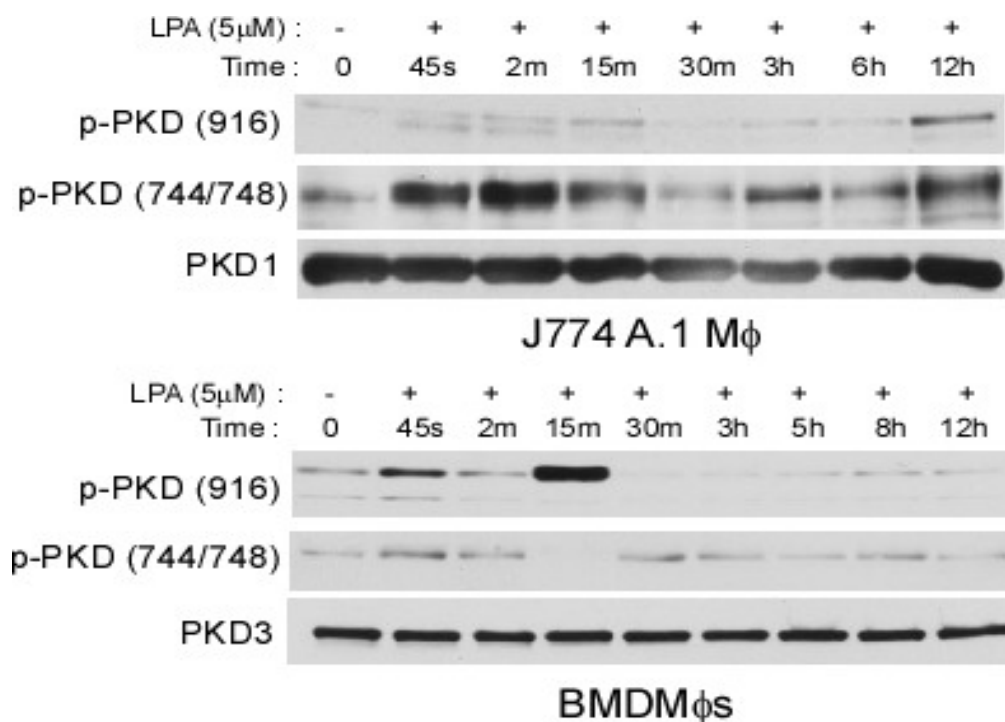


Figure 10 PKD Phosphorylation in LPA-induced J774A.1 and BMDMs.

All samples were starved over-night. Cell lysates were collected at various time points after LPA treatment,. Both 916 and 744/748 PKD were phosphorylated by LPA stimulation, PKD1 and PKD3 in served as loading controls.

5. LPA Up-regulates the Expression of Macrophage Scavenger Receptors

Classically, scavenger receptor class As and Bs have been considered to be major factors responsible for cholesterol accumulation in macrophages [1]. Three members are included in the Class A scavenger receptor family: SR-AI, SR-AII and SR-AIII. SR-AI which is dominantly expressed in mouse macrophages, accounts for 80% of modified LDL uptake[10]. In the Class B family, both SR-BI and CD36 are crucial for oxidized LDL metabolism in macrophages. Thus, we examined LPA-induced macrophage scavenger receptor mRNA expression using Northern blot analysis. Target probes designed around 800bp were used. Cells were starved for 16 hours prior to LPA stimulation. Total mRNA samples were collected at various time points after LPA treatment. Our data showed that both CD36 and SR-BI mRNA expression level were up-regulated in response to LPA in a time-dependent manner, while SR-AI remained at pre-stimulation levels (Fig.12). The results strongly suggest that the augmenting lipid uptake by LPA may be mediated through up-regulation of SR-B1 and CD36 but not SR-AI.

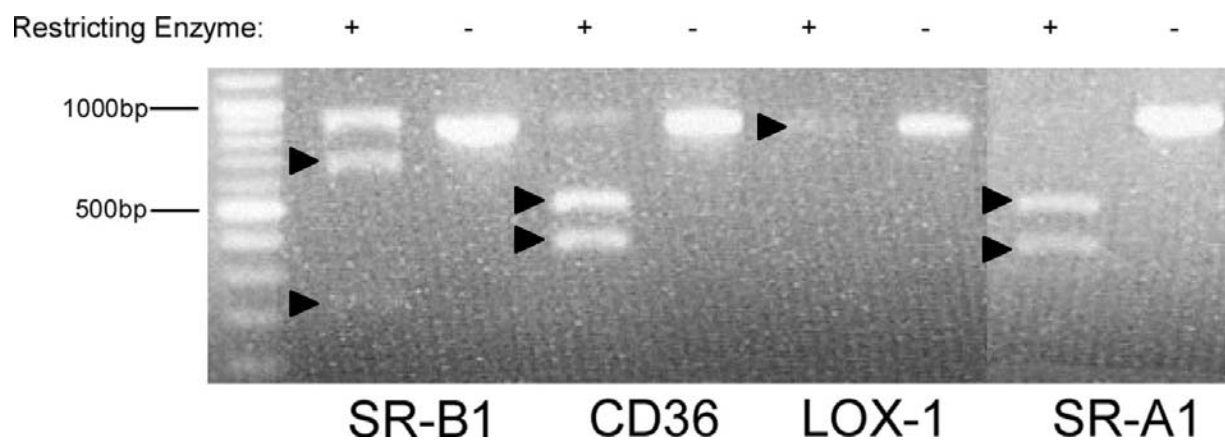


Figure 11 Probe Testing using Restriction Enzyme Cutting.

Four unique site restriction enzymes were applied to PCR products of four scavenger receptors. The length of four restriction fragments should be: SR-B1 (666bp,223bp EcorRI); CD36 (508bp,383bp BbsI); LOX-1 (824bp,35bp MslII); SR-A1 (529bp,398bp PstI).

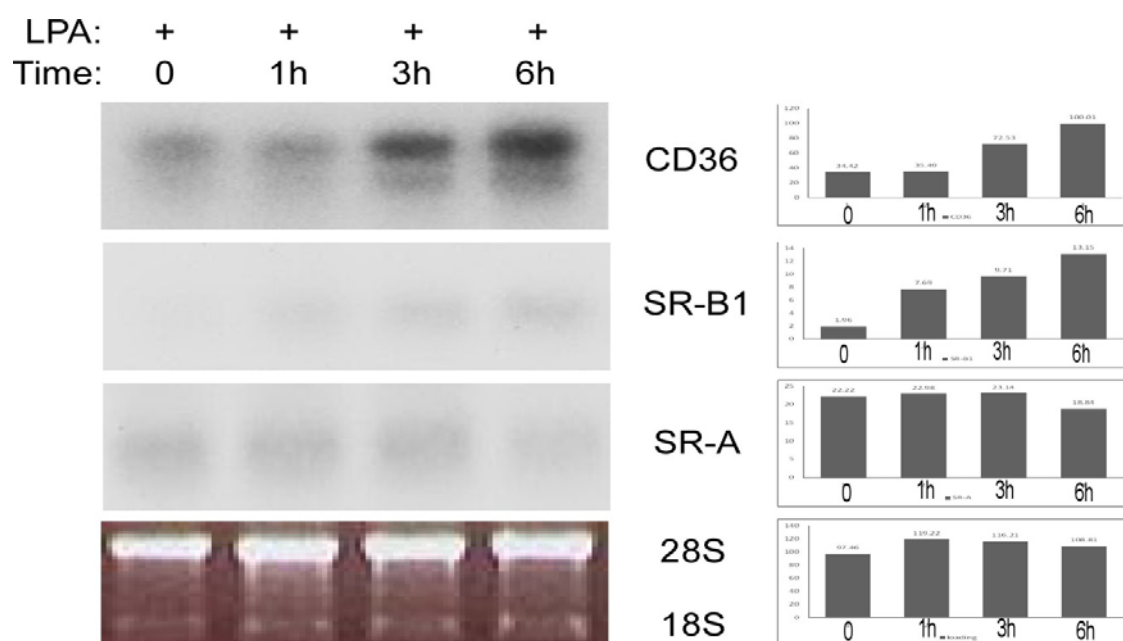


Figure 12 Northern Blot Analysis for Scavenger Receptor mRNA Expression Level.

Cells were starved for over-night before adding LPA. First-strand cDNA was acquired using a thermo-script reverse transcription kit. The results were quantified by densitometry and reflected histogram to the right.

6. NF- κ B Pathway is NOT Involved in LPA-induced Macrophage Activation

The intracellular NF-kappa B pathway has been established for at least a half century. As a pro-inflammatory nuclear transcription factor, NF- κ B regulates inflammatory responses in a variety of cell types. In 1996, NF-kappa B activation was found in human atherosclerotic lesions and NF-kappa B activation was detected in smooth muscle cells, macrophages and endothelial cells [43]. Years later, NF- κ B was found to be activated by hypercholesterolemia in the vessel walls of a pig model for atherosclerosis [44]. These findings link the NF- κ B transcription pathway to pathogenesis of cardiovascular disease.

NF-kappa B is a general term referring to a family of transcription factors which consists of 5 members: p65 (RelA), c-Rel, RelB, NF- κ B1 (p50 and its precursor protein p105), NF- κ B2 (p52 and its precursor p100). These proteins can be folded into various complexes of homodimers or heterodimers. The most common form, p65/p50 is widely referred as NF- κ B. In this canonical pathway, the activation of NF- κ B requires the phosphorylation of I κ B α by IKK α / β complex; which is also an essential step for NF- κ B activation cascade. The process for NF- κ B activation begins with the binding of a ligand to a cell surface receptor (TNF Receptor, G-coupled Receptor or Toll-like Receptor, etc), and in turn, these adaptors often recruit an IKK complex (α and β catalytic subunits). Activated IKK then phosphorylates I κ B at two serine residues, which leads to its K48 ubiquitination

and degradation by the proteasome. NF- κ B then enters the nucleus to stimulate the transcription of target genes.

The LPA-induced NF- κ B pathway has been reported recently [33, 45]. However, whether LPA triggers NF- κ B activation in macrophages is unknown. We examined whether NF- κ B is activated by LPA in macrophages using Western blot analysis. Cells were starved for 24 hours before LPA addition. Cell lysate were protected against protein degradation and dephosphorylation by adding protease/phosphatase inhibitors in the lysis buffer. After sonication, lysates were separated by 10% SDS-PAGE gels and transferred onto PVDF membranes for antibody incubation. We observed no phosphorylation of IKK α/β , IKK α , IKK β , IKB α , and IKB α . Also, no obvious phosphorylation occurred either on IKK and IKB α unit. These data indicate that NF- κ B is not involved in LPA-induced macrophage activation (Fig.13).

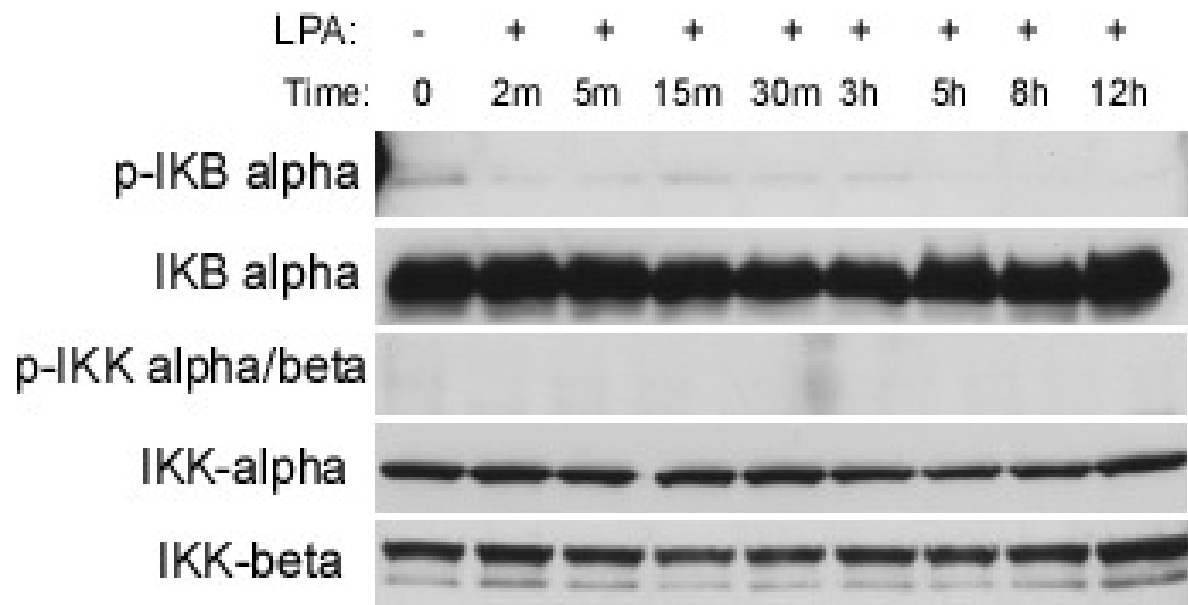


Figure 13 NF- κ B pathway Detection in LPA-induced J774A.1 Macrophages.

Time course of NF- κ B phosphorylation in J774A.1 macrophages is shown. IKK and IKB alpha phosphorylation levels are the two most important indicators of NF- κ B activation.

Chapter4 Discussion

Atherosclerosis, a chronic inflammatory cardiovascular disease, is characterized by the accumulation of lipids, macrophages, smooth muscle cells, leukocytes and fibrous tissues at the inner most layers (intima) of the arterial wall. Lysophosphatidic acid, a bioactive phospholipid, accumulates in high concentrations in atherosclerotic lesions. Recent investigations established solid evidences for LPA in promoting cardiovascular disease progression: LPA induced smooth muscle cells proliferation and migration and also induced macrophages and endothelial cells to secrete a variety of inflammatory cytokines and adhesion molecules[4]. Moreover, an *in vivo* study showed that LPA could potentially lead to hypertension and neo-intima formation in animal models, or expedite plaque formation in a mouse atherosclerosis model. All evidence to date indicates the crucial roles of LPA in cardiovascular disease.

Macrophages, one of the most important immune effector cells, serve as body guards in the human body. Recent findings suggest that the accumulation of cholesterol-laden macrophages in the intima layer of the arterial wall is the hallmark of early plaque formation. Understanding the mechanism of cellular response involved in macrophages is crucial to unveiling the progression of cardiovascular disease. Although LPA induced smooth muscle cell activation has been widely reported and explored, the links between LPA and macrophages were barely established and detailed mechanisms were rarely reported.

Therefore, it is important to investigate the physiological and pathological role of LPA in blood monocyte differentiated macrophages. Three different sources of cells were used in our study, including the J774A.1 cell line purchased from ATCC, BMDMs isolated from mouse femurs, and peritoneal macrophages isolated from thioglycolate-elicited mouse abdominal cavities. However, after a few experiments we found that only J774A.1 and BMDMs are stable macrophages with a high purity level, while peritoneal macrophages only showed less than 70% purity in macrophage population. Thus, we decided to use BMDMs and J774A.1 in our subsequent experiments. Through the uptake assays in both BMDMs and J774A.1 cells, our data showed an obvious increase in lipid accumulation in LPA treated macrophages compared to LPA only (negative control) and ox-LDL only (positive control) groups. We further showed that LPA-induced lipid uptake in macrophages is both time and dose dependent. In order to confirm our results from flow cytometric analysis, we used a variety of approaches such as Oil-red O staining and fluorescent microscopy. Our results indicate that LPA enhances lipid uptake in macrophage foam cell formation.

Recent studies on LPA-induced macrophage activities offer a promising direction toward understanding the indispensable roles of macrophages in cardiovascular disease: LPA enhances LPS-induced CXCL16 expression and T-lymphocyte migration[46] and LPA induces interleukin-1 β expression in macrophages[47]. However, a detailed receptor-related cell signaling pathway is still unclear and failed to be addressed in those reports. Our Western blot data clearly indicates that upon treatment with LPA, both BMDMs and J774A.1

macrophages exhibited an unexpected, transient inhibitory/deactivated pattern of MAPK activation. Though current knowledge seems to be far from fully explaining this phenomenon, the importance of MAPKs in the cellular inflammatory response reminds us that the properties of LPA-induced macrophages are totally different from other LPA-induced cell models - which promote inflammation through activating MAPK. Also, macrophages had a very high background of MAPK activation compared to vascular SMCs that showed an inducible response to LPA stimulation.

As part of our efforts to better understand the LPA-triggered signaling pathway in macrophages, we also examined some other related signal activation pathways such as PKD and NF- κ B. Previously our group identified that PKC and PKD were involved in LPA and growth factor induced smooth muscle cell and endothelial cell activation [40, 48, 49]. With similar motifs in classic and novel PKC isoforms, PKD shows its cysteine-rich, zinc finger-like N-terminal regulatory domain, which binds to DAG allowing many early cellular response to be activated. Phosphorylation of PKD1 (Ser916) and phosphorylation of PKD activation loop (Ser744/748) were established signs of PKD family kinase activation. Our results showed a significant PKD activation from 2 to 5 minutes in response to LPA in both J774A.1 and BMDM. To date, there is no report revealing these findings of LPA induction of PKD activation in macrophages. Our data also indicate that all three PKD isoforms are present in J774A.1 macrophages. In some cases, PKD is upstream of MAPK and the activation of PKD could provide insights toward explaining our earlier results on inhibited activation of MAPK.

Reportedly, NF- κ B is a key player in the inflammatory process of activated macrophages [50, 51]; however, our results revealed that LPA did not induce the activation of NF- κ B. No phosphorylation was observed on either IKK α / β subunits or IKB α . These data supports the notion that NF- κ B pathway is not involved in LPA-induced macrophage activation.

Another interesting finding was that the mRNA expression level of scavenger receptors – CD36 and SR-BI, are dramatically increased upon the induction of LPA in BMDMs. Though the function of scavenger receptors are diverse: mediating lipid accumulation and cholesterol efflux[52]; promoting inflammatory response [53] and cell migration[54] and enhancing adhesion molecule secretion[55]. Here we focused on the role of lipid accumulation in macrophages. Building upon previous results that showed LPA could expedite/enhance oxLDL uptake in the presence of LPA, the northern blot analysis on four different scavenger receptors was done in order to identify whether the mRNA expression level of these receptors changed over time during LPA treatment. As a result, we found that CD36 and SR-B1 are strongly induced upon LPA treatment in a time-dependent manner. Meanwhile, our densitometry quantification ruled out the possibility that SRA1 and LOX-1 were involved in LPA activation of macrophages.

In summary, our data suggests that lysophosphatidic acid (LPA), a bio-active lipid component, enhances lipid accumulation in monocyte-derived macrophages during early atherosclerotic plaque formation. In particular, LPA may work through its G-protein coupled receptors to initiate the intracellular kinase cascade. Two pieces of evidence were found: 1) LPA transiently inhibited MAPK activation; 2)

LPA shortly activated PKD phosphorylation. And next we excluded the possibility of NF- κ B as the downstream transcriptional factors in the LPA-induced macrophage activation. Lastly, we observed that scavenger receptors like CD36 and SR-B1 were up-regulated in the LPA-induced macrophages, and the two scavenger receptors may serve to boost the lipid uptake activity in macrophages. We believe that the results of our current study form a solid platform for our future investigation on more detailed cellular signaling pathways and transcriptional factors that mediate the LPA-induced macrophage activity which contribute to the development of vascular disease.

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Appendices

Table I Scavenger Receptor: ligands and expression profile

Class	SR	ligand relevant to atherosclerosis	Does not bind to	Expression profile (cell types)	Expression on monocytes and plaque macrophages in atherosclerosis
A	SR-AI/SR-AII	oxLDL, acLDL, malondialdehyde-LDL, maleylated LDL lysophosphatidylcholine phosphatidic acid cholesterol apo A-I, apo E (Brown et al. 2007; de Villiers et al. 1994; Loboda et al. 2006) glycated type IV collagen modified collagen type I, III and IV biglycan, decorin (ECM components) modified albumin, AGE-BSA beta-amyloid fibrils calreticulin, gp 96, HSP70 family members (reviewed in Pluddemann et al. 2007)	Native LDL, HDL oxidised phosphatidylcholine long-chain fatty acids apo-SAA native collagen I and III fibrillar collagen I	Myeloid cells (macrophages, subpopulations of DCs) (Horvai et al. 1995) mast cells (Brown et al. 2007) vascular endothelial cells (Loboda et al. 2006) and smooth muscle cells within plaques (Mietus-Snyder et al. 2000), induced by oxidative stress	Not on monocytes, upregulated during monocyte-to-macrophage differentiation (CSF-1 dependent) (de Villiers et al. 1994) on foam cells (Geng et al. 1995; Gough et al. 1999)
A	Macro	oxLDL > acLDL	Native LDL	Macrophage subpopulations (spleen marginal zone, medullary lymph nodes, resident peritoneal) (Karlsson et al. 2003)	
B	CD36	acLDL, oxLDL VLDL, LDL, HDL anionic phospholipids oxidised phosphatidylserine oxidised phosphatidylcholine long-chain fatty acids AGE-modified proteins thrombospondin-I beta-amyloid fibrils (reviewed in Silverstein and Febbraio 2009)	Phosphatidylserine	Macrophages, DCs, microglia, platelets microvascular endothelial cells hepatocytes, adipocytes cardiac & skeletal myocytes breast, kidney, gut specialized epithelia (reviewed in Silverstein and Febbraio 2009)	Upregulated with progression (PPARgamma dependent) (Nakagawa-Toyama et al. 2001; Tontonoz et al. 1998) upregulated during monocyte-to-macrophage differentiation (Huh et al. 1996) induced by atherogenic conditions including hypertension, glucose overload and oxidative stress (Geng et al. 1994; Griffin et al. 2001; Iwashima et al. 2000)

Table I Scavenger Receptor: ligands and expression profile

Class	SR	ligand relevant to atherosclerosis	Does not bind to	Expression profile (cell types)	Expression on monocytes and plaque macrophages in atherosclerosis
B	SR-BI	acLDL, oxLDL VLDL, LDL, HDL anionic phospholipids oxidised phospholipids oxidised phosphatidylcholine cholesterol		Macrophages hepatocytes adrenal gland epithelium (Terpstra et al. 2000)	CLA-I (human SR-BI) low in monocytes, increases with differentiation to macrophages (Chinetti et al. 2000; Hirano et al. 1999; Svensson et al. 2005)
E	LOX1	oxLDL Phosphatidylserine Fibronectin HSP70	acLDL native LDL	Endothelial cells induced in macrophages, smooth muscle cells (Chen et al. 2002)	
F	SREC-I/SREC-II	oxLDL, acLDL calreticulin, gp 96, Hsp70		Endothelial cells, macrophages	
G	SR- PSOX/CXCL16	oxLDL phosphatidylserine	acLDL native LDL	DCs, macrophages (Shimaoka et al. 2000)	
H	Stabilin 1/FEEL- 1	acLDL Apoptotic cells SPARC (Kzhyshkowska et al. 2005, 2006a,b,c; Park et al. 2009a,b)	Native LDL, HDL	Sinusoidal endothelial cells including spleen, liver, lymphnode (Kzhyshkowska et al. 2006a,b,c; Martens et al. 2006) monocytes/macrophages (Brocheriou et al. 2011; Kzhyshkowska et al. 2006a,b,c, 2009)	Upregulated by IL-4 and dexamethasone in human monocyte-derived macrophages (Kzhyshkowska et al. 2006a,b,c; Politz et al. 2002) Expressed on CD14(+)CD16(+) monocytes in patients with familial hypercholesterolemia (Mosig et al. 2009) Expressed on macrophages in human arterial and apoE null mice atherosclerotic tissues (Brocheriou et al. 2011)

Table II PCR Primer Pairs Sequence

Primer	Forward Sequence	Reverse Sequence
CXCL2	GCGCCCAGACAGAAGTCATAG	AGGGTCAAGGCAAACCTTTTGA
IL-1	TGGTTGGCTAACAGCACGTTT	TCGGTCACACGGCCTCTT
Hilpda	GTGCTGGGCATCATGTTGAC	CCTCCCAGAGACTCCATCACTCT
Lamin A	CGCACCGCTCTCATCAACT	TCAGTGAGCGCACCAGCTT
TNF	CAGCCGATGGGTTGTACCTT	GGCAGCCTTGTCCCTTGA
CD14	TCCCGCCCCACCAGAGC	CGGCCAGCGCAGAGCAC
FOS	GCATCGGCAGAAGGGGCAAAGTAG	AGGGGCTCCAGCTCTGTGACCAT
FOSB	AAATGCCCCGGCTCCTTCGTG	GGGGGTGCGTCTCGGCTGCTCT
TSP-1	CAGCATGGTCCTGGAAGTGA	CGGATGCTGTCCTGCAGAGT
TG-2	CCTGCCTGATGCTCTTGGAT	GCAGTCCCGACTACGGTTCTT
Cytohesin1	GGTCCTGCATGCGTTTGTG	AGGCTGGACGAGGTTTCAG
Angl-4	TGCATCCTGGGACGAGATG	CATGGCCGAGCTGTAGCA
C5AR1	AAGGTCTCTCCCCAGCATCA	TCCCTGCCCACTGAATCCT
CD36	GATCGGAACTGTGGGCTCATT	AAAGGCATTGGCTGGAAGAAC
SR-A	AAAATGGCCCCTCCGTTTCA	ATCCGCCTACACTCCCCTTCTC
SR-B1	CTGGGTGGCCTTGGGGTTGG	GGGGGCCGTGAAGCGATAC
LPAR1	AGCTGCCTCTACTTCCAGC	TTGCTGTGAACTCCAGCCAG
LPAR2	ATGGGCCAGTGCTACTACAACG	AGGGTGGAGTCCATCAGTG
LPAR3	GACAAGCGCATGGACTTT	CATGTCCTCGTCCTTGTACG
LPAR4	GTTGTATTCATCCTGGGTCT	AGCGACTCCATCCTTATATG
LPAR5	TGCTCTGACCTTGTTGTTCC	AGCAACCCATATACAGCCAGCG

Vita

Kan Xu was born in Urumqi, China in 1986. In 2005, he was admitted by Jilin University and started his 5-year training in Veterinary Medicine. In 2010, he successfully graduated from Jilin University in China and obtained his DVM degree. During 2011 and 2013, he started his graduate study in Comparative and Experimental Medicine at the University of Tennessee, Knoxville. And Kan Xu will graduate with a master degree in summer, 2013.