sPLA2-IIA Augments Oxidized LDL-Induced MCP-1 Expression in Vitro Through Activation of Akt

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Key Words
Lv-GFP-sPLA2-IIA • Atherosclerosis • Oxidized LDL • MCP-1 • Akt

Abstract
Background/Aims: Group IIA secretory phospholipase A2 (sPLA2-IIA) has an important role in atherosclerosis. In this study, we explored whether sPLA2-IIA overexpression could promote atherosclerosis in normal environment alone or with other inflammatory factors.

Methods: Human aortic smooth muscle cells (HASMCs) were transduced with Lv-GFP-sPLA2-IIA, a plasmid containing sPLA2-IIA coupled with green fluorescent protein (GFP). Cells were incubated in the presence or absence of oxidized low-density lipoprotein (LDL), sPLA2 inhibitor LY315920 or PI3K/Akt inhibitor LY294002. The mRNA expression and protein secretion of monocyte chemoattractant protein-1 (MCP-1) were assessed by quantitative real-time polymerase chain reaction (QRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. Phosphorylation of Akt was examined by western blotting.

Results: Lv-GFP-sPLA2-IIA-transduced HASMCs remained fluorescent during 72 h of the study period with infection ratio of around 80%. The mRNA expression and protein secretion of MCP-1 was not altered in groups of HASMCs, Lv-GFP transduced and Lv-GFP-sPLA2-IIA-transduced HASMCs (p>0.05), but was significantly increased in the presence of oxidized LDL especially in Lv-GFP-sPLA2-IIA transduction group (p<0.01). However, with the addition of LY315920, this enhancement was notably decreased (p<0.05). This enhancement was also markedly abolished by co-incubation with LY294002, paralleled with suppressed Akt phosphorylation.

Conclusions: Overexpression of sPLA2-IIA does not alter MCP-1 level at baseline, but could enhance the atherogenic effect of oxidized LDL in HASMCs, at least partly due to activation of Akt. These findings may provide a strategy for treatment of inflammatory cardiovascular diseases.

Y. Guo and B. Li contributed equally to the study.
Introduction

The secretory phospholipase A2 (sPLA2) family is comprised of a group of low molecular mass enzymes [1], and sPLA2-IIA has long been regarded as an inflammatory protein associated with infection and cardiovascular diseases [2, 3], due to its ability to induce low-density lipoprotein (LDL) modification, foam cell formation, and activation of various immune mechanisms [4]. Since there is little sPLA2-IIA expression in non-plaque areas of the vessel wall, sPLA2-IIA has been extensively studied to be a marker of high cardiovascular risks. High content and activity of the enzymes are associated with recurrent adverse events, such as vascular stenosis, acute coronary syndrome, myocardial infarction and even coronary death after a successful surgery [5-7]. In addition, elevated serum sPLA2-IIA served as an accurate predictor of long-term outcome, including all-cause mortality and readmission for heart failure, in 964 present post-AMI patients [8]. Thus, sPLA2-IIA is regarded to play an important role in promotion, progression, and prognosis of atherosclerosis [9].

LDL oxidation in the vascular wall is the main characteristic of atherosclerosis [10]. Oxidized LDL contributes to both initiation and progression of inflammatory disease, such as atherosclerosis [11-14]. Smooth muscle cells (SMCs) are important for the development and stability of atherosclerosis lesions [15]. SMCs are the main cell type in intimal thickenings and play a vital role in human atherosclerosis, as monocyte-derived macrophages and SMCs accumulate excess lipids. Monocyte chemotactic protein 1 (MCP-1) can recruit circulating monocytes and T-cells to the site of activation, and contribute to vascular inflammation [16-20]. Moreover, MCP-1 production can be induced by oxidized LDL [21]. However, hydrolysis of LDL phospholipids by sPLA2 is observed in addition to other modifications such as oxidation, aggregation, or proteolysis during lesion progression [22]. It has been demonstrated that phospholipase A2-modified LDL activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and increases cell survival in monocytes [23]. Moreover, sPLA2-IIA transforms oxidized LDL into a form, which is more atherogenic [24]. The above evidence suggests a central link between systemic as well as local inflammatory processes and lipid metabolism, and this link could be sPLA2-IIA.

However, there is no report concerning the role of sPLA2-IIA in the atherogenic progress of oxidized LDL. Interestingly, treatment with pravastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor used to lower blood cholesterol and triglycerides, did not reduce sPLA2-IIA mass or sPLA2 activity levels, as compared to placebo [25]. Thus, the present study was designed to explore the role of sPLA2-IIA in the development of atherosclerosis induced by oxidized LDL in vitro.

Lentiviral plasmid containing sPLA2-IIA coupled with GFP was constructed for investigating the effects of sPLA2-IIA on human aortic smooth muscle cells (HASMCs). The effects of sPLA2-IIA on inducing the expression of MCP-1, a chemotactic factor contributing to monocyte adhesion to endothelial cells of the inflammatory arterial wall in early atherosclerosis, was evaluated. We also examined whether sPLA2-IIA could augment the effect of oxidized LDL on MCP-1 production, and whether sPLA2-IIA could activate phosphorylation of PI3K/Akt signaling during MCP-1 production.

Materials and Methods

Plasmids construction and lentivirus production

To obtain the lentivirus encoding a chimera of sPLA2-IIA and GFP at C-terminus, the lentiviral vectors carrying sPLA2-IIA and GFP reporter gene were constructed (purchased from Invitrogen Biotechnology Co. Ltd., Shanghai, China). Briefly, human sPLA2-IIA cDNA was synthesized according to the sequence (Gene bank accession number NM_000300.3). The amplified product was cloned into pDONR221 vector and then subcloned into pLenti6.3/V5-DEST using gateway homologous recombination. cDNA in pLenti6.3/V5-DEST-sPLA2-IIA plasmid was confirmed by sequencing. The sPLA2-IIA expression plasmid was transfected into HEK293A cells (Shanghai Institute of Cellular Biology, Chinese Academy, Shanghai, China).
for virus amplification. Concentrations of lentiviral particles were determined by TCID<sub>50</sub> method after being centrifugated at 2000 g and purified with a 0.45 µm filter.

**Lentivirus transduction and RT-PCR, ELISA**

To study sPLA2-IIA, a 50 multiplicity of infection (MOI) of the lentiviral particles encoding sPLA2-IIA coupled with GFP (Lv-GFP-sPLA2-IIA) or GFP only (Lv-GFP) were infected in HASMCs (Shanghai Institute of Cellular Biology, Chinese Academy, Shanghai, China), which were cultured under atmosphere of 5% CO<sub>2</sub> at 37°C in full medium, which is M231 medium supplemented with 10% fetal calf serum (FCS), 5% smooth muscle cell growth supplement (SMGS), 10 mg/ml streptomycin and 100 U/ml penicillin. Lv-GFP transduction served as a reference for infection efficiency. After infection for 6h, the lentivirus-containing medium was removed and replaced with fresh full medium. After 72 h incubation under atmosphere of 5% CO<sub>2</sub> at 37°C, infection efficiency was determined by counting GFP-positive and total cells in five representative high-power fields (Infection efficiency=GFP positive cells / Total cells × 100%).

To examine the transduction of sPLA2-IIA in HASMCs, semi-quantitive reverse transcription polymerase chain reaction (RT-PCR) was performed. Two pairs of oligonucleotide primer were designed according to the nucleotide sequence of cDNA of sPLA2-IIA and GAPDH. sPLA2-IIA sense: 5'-ATG AAG ACC CTC CTA CTG TTGG-3', 110bp, anti-sense: 5'-GCT TCC TTG CCT GGG ACTG-3'. As intra-reference GAPDH's sense: 5'-GAA GGT GAA GGT CGG AGTC -3', 226bp, anti-sense: 5'-GAA GAT GGT GAT GGG ATTTC -3'(synthesized by Invitrogen, Shanghai, China). Total cellular RNA was isolated from HASMCs using Trizol (Invitrogen, Shanghai, China) according to the manufacturer's instructions. To eliminate possible contaminating genomic DNA, RNA samples were treated with TURBO DNA-free™ kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. The reverse transcription (Biorad MyCycler; USA) was carried out at 42°C for 30 minutes using M-MLV Kit (Takara, Ohtsu, Japan). Reaction system is 20 µL including DNAase-treated RNA 0.5 µg, primer 0.5 µL (concentration 1μmol/L). Amplification was performed in 20 µL including 10 ng cDNA with 30 cycles and denaturation at 94°C 5 sec, annealing at 58°C 15 sec, extension at 72°C 15 sec. sPLA2-IIA expression was normalized to the expressed housekeeping gene GAPDH. Electrophoresis and image analysis system is from Biorad Geldoc XR, USA. Concentrations of sPLA2-IIA in medium were determined by a commercially available sPLA2-IIA ELISA kit (Cayman Chemical Co., Ann Arbor; USA) according to the manufacturer's instructions. ELISA value is from the ratio of sPLA2-IIA protein to total protein in cell lysate.

**MCP-1 assay**

After transduction, HASMCs and Lv-GFP / Lv-GFP-sPLA2-IIA transduced HASMCs were cultured in full medium. Co-incubations of Lv-GFP / Lv-GFP-sPLA2-IIA transduced HASMCs with oxidized LDL +/- LY294002 / LY315920 (Alexis, Lausen, Switzerland) were also performed for 6h. In these cells expression of MCP-1 genes was quantified using quantitative real-time polymerase chain reaction (QRT-PCR) (Takara, Ohtsu, Japan). Total RNA was isolated from cultured HASMCs using RNeasy Kit (Takara, Ohtsu, Japan) according to the manufacturer’s instructions. RNA samples were also treated with TURBO DNA-free™ kit (Ambion, Austin, TX, USA) according to the manufacturer’s recommendations. cDNA samples (10ng) were then amplified by QRT-PCR on a Light Cycler 480 (Roche Applied Science, Mannheim, Germany). The primers were designed as the following: MCP-1 upper strand 5'-CAA ATG CCC CAG TCA ATG TCT TGG GTG GGT GGA GTG-3'; lower strand 5'-GAT TCT GGG GTG GGA GTG-3'; GAPDH upper strand 5'-GTA AAC AGC CTC TCT TAA-3'; lower strand 5'-TGA CGG GAT CTC GCT GGT GGA AGAT-3'. Amplification was performed with 30 cycles of annealing at 58°C for 15 sec followed by a 15-sec extension at 72°C. Data were analyzed with Light Cycle software 4.0 (Roche Applied Science, USA). Fold changes in gene expression were determined using the relative comparison method with normalization to GAPDH. MCP-1 secretion to the supernatant during cell culture was analyzed with ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions. Optical density (OD) value was measured at 450 nm on a microtiter plate reader linked to a Biolinx software program (MR 7000; Dynex Technologies, Chantilly, VA, USA). MCP-1 concentrations in HASMCs of each group were calculated using a standard curve from different dilutions of human MCP-1.

**Akt examination**

To verify the role of Akt, the specific inhibitor LY294002 (Alexis, Lausen, Switzerland) was added into the culturing medium for 6h co-incubation. Western blot analysis was conducted to evaluate phosphorylated Akt.
and total Akt in order to investigate if this pathway is involved in the proinflammatory effect of sPLA2-IIA. Aliquot of protein extracted from the cells (40 μg) was mixed with 0.25 volume of loading buffer (250 mmol/l Tris-HCl, pH 6.8, 10% (w/v) SDS, 25% (v/v) glycerol, 0.2% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol), boiled for 5 min at 95°C and then subjected to 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech UK Ltd, Bucks, UK). The membrane was incubated with polyclonal antibodies against phospho-Akt (Ser473, 1:1000) or total-Akt (1:1000) (Cell Signaling Technology, Beverly, MA, USA) at 4°C with gentle agitation overnight. Peroxidase-conjugated AffiniPure Donkey Anti-rabbit IgG (H+L) secondary antibody (1:5000, Promega, Madison, WI, USA) was used after washing. The immunostains were visualized by ECL Western Blotting Detection Reagent (Amersham BioSciences, Piscataway, NJ, USA), and then exposed to Hyperfilm. The relative intensities of the bands were detected on Biomax MR X-ray film (Kodak, Nanjing, China).

Statistical analysis
All of the experiments were repeated at least three times. The data are expressed as mean ± the standard error of the mean (SEM). Comparisons were performed using the software SPSS v 13.0. Unpaired Student t-test was adopted for comparisons between two groups, and one-way ANOVA followed by Fisher’s post hoc test was used for multiple comparisons; p < 0.05 was considered statistically significant.

Results
Establishment of Lv-GFP- sPLA2-IIA transduced HASMC
To explore the role of sPLA2-IIA in atherosclerosis, we used lentiviral vector encoding a GFP reporter +/- sPLA2-IIA to promote the overexpression of these genes. Subsequently, GFP was used to successfully sort the infected cells. Fluorescent microscopy was adopted to examine the efficiency of Lv-GFP-sPLA2-IIA transduction. At MOI of 50:1, more than 80% of the cells were GFP positive (Fig. 1A, 1B). GFP fluorescence reached a peak level at 72 h and declined gradually. No fluorescence was seen in non-transduced cells. Infection of HASMCs with Lv-GFP-sPLA2-IIA resulted in a robust expression of sPLA2-IIA mRNA at 72 h (Fig. 1C, 1D). No significant sPLA2-IIA expression was determined in HASMCs infected with the same concentration of Lv-GFP, although they showed that green infection intensity was ~80% of HASMCs. Repeated experimental results are corresponding.

Fig. 1. Infection of HASMCs with Lv-GFP-sPLA2-IIA resulted in a robust expression of sPLA2-IIA mRNA at 72 h. (1A) The cultured Lv-GFP-sPLA2-IIA transduced HASMCs under visible light. (1B) Under fluorescence microscopy more than 80% of the cells were GFP positive. (1C) Semi-quantitative RT-PCR. lane 1, 2, 3 represent HASMCs, Lv-GFP transduced HASMCs and Lv-GFP-sPLA2-IIA transduced HASMCs, respectively. Lv-GFP-sPLA2-IIA transduced HASMCs showed the strongest expression of sPLA2-IIA mRNA indicating successful transduction. (1D) Transduction with Lv-GFP-sPLA2-IIA resulted in a robust expression of sPLA2-IIA mRNA compared to the other 2 groups (** p<0.01).
Elevated sPLA2-IIA secretion in medium of Lv-GFP- sPLA2-IIA transduced HASMCs

ELISA assay was performed to explore whether the sPLA2-IIA production was increased in the medium. The result indicated that sPLA2-IIA level was notably elevated in the medium of Lv-GFP-sPLA2-IIA transduced HASMCs (p<0.01, respectively) compared with HASMCs and Lv-GFP transduced HASMCs. Further experiments were performed at different infection efficiencies. As shown in Fig. 2B, expression of sPLA2-IIA in the medium was positively related to transfection efficiency, verified by ELISA. As the infection efficiency increased to 50% and 80%, sPLA2-IIA levels were also notably increased compared to 30% transduction efficiency group (p < 0.05 and p < 0.01, respectively).

sPLA2-IIA overexpression did not alter mRNA and protein expression of MCP-1

We next investigated the effect of sPLA2-IIA overexpression on MCP-1 expression produced by HASMCs. QRT-PCR and ELISA were performed to examine the mRNA and protein levels of MCP-1 of HASMCs, Lv-GFP and Lv-GFP-sPLA2-IIA transduced HASMCs, respectively. The results revealed that sPLA2-IIA overexpression could not affect MCP-1 mRNA and protein expression after 0h, 24 h, 48 h and 72 h of transduction (Fig. 3A and 3B, p > 0.05, respectively). MTT assay showed that cell viability was >90% in the cell cultures (data not shown).

sPLA2-IIA augments oxidized LDL-induced MCP-1 expression

To verify whether the secreted sPLA2-IIA was with enzymatic activity in the medium of Lv-GFP-sPLA2-IIA transduced HASMCs, sPLA2 inhibitor LY315920 (1 μmol/l) was also...
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Fig. 4. sPLA2-IIA augments oxidized LDL-induced MCP-1 expression. Without oxidized LDL there was no significant difference in MCP-1 level in the medium between Lv-GFP and Lv-GFP-sPLA2-IIA transduced HASMCs +/- LY315920 (p > 0.05). With the addition of oxidized LDL, MCP-1 was significantly higher in Lv-GFP transduced HASMCs +/- LY315920 than those of without oxidized LDL (*p < 0.05, respectively), however, MCP-1 level in Lv-GFP-sPLA2-IIA transduced HASMCs was further increased compared to those without oxidized LDL or even Lv-GFP transduced HASMCs group (** p < 0.01 and * p < 0.05, respectively). In addition, the increased MCP-1 level in Lv-GFP-sPLA2-IIA transduced HASMCs was significantly reduced (p < 0.05) by addition of LY315920.

Effect of sPLA2-IIA on Akt activation in the presence of oxidized LDL

In cell lysate of Lv-GFP and Lv-GFP-sPLA2-IIA transduced HASMCs, western blot showed a significant increase in phosphorylated Akt / total Akt (P-Akt / T-Akt) ratio after the addition of oxidized LDL (50 mg/L) compared to that without oxidized LDL (Fig. 5A, p < 0.05, p < 0.01 respectively). Moreover, Akt activation can be significantly suppressed by coinoculation with LY294002 (2μmol/l), a specific inhibitor for PI3K/Akt pathway (p < 0.05), paralleled with abolished MCP-1 expression (Fig. 5B). Co-incubation of Lv-GFP-sPLA2-IIA transduced HASMCs with oxidized LDL markedly elevated MCP-1 levels compared to that without oxidized LDL (Fig. 5B). This effect was abolished by addition of LY294002 (p < 0.05). Moreover, Fig. 5 indicates a significant difference between GFP and sPLA2-IIA in the condition with oxidized LDL (p < 0.05, p < 0.01 respectively), but not in the condition with oxidized LDL and LY294002.
Discussion

In the present study, we successfully established Lv-GFP-sPLA2-IIA transduced HASMCs overexpressing sPLA2-IIA. Overexpression of sPLA2-IIA with enzymatic activity per se did not alter MCP-1 expression at baseline, but with the addition of oxidized LDL, MCP-1 expression was greatly elevated in Lv-GFP-sPLA2-IIA transduced HASMCs. Meanwhile, intracellular Akt was activated, which can be suppressed by the inhibitor, LY294002. Akt activity was low without oxidized LDL addition or even with LY294002 in medium suggesting that PI3K/Akt pathway can be activated during oxidized LDL induced MCP-1 expression and sPLA2-IIA could greatly amplify the reaction.

Given the potential role of sPLA2-IIA in inflammation, our results suggested that sPLA2-IIA alone might fail to provoke inflammation, but seemed to be able to enhance the atherogenic effect of oxidized LDL, a key pro-inflammatory mediator in the progression of vascular diseases such as atherosclerosis. Interestingly, an earlier study [26] reported that, except for an abnormality of the skin characterized by epidermal and adnexal hyperplasia, hyperkeratosis and nearly total alopecia, sPLA2-IIA-transgenic mice showed a nearly normal phenotype. Despite the high enzyme activities in plasma and most organs, no signs of an inflammatory response were detected in the skin or other tissues. However, sPLA2-IIA-transgenic mice were found to have increased susceptibility to atherosclerosis. Previous studies has verified that sPLA2-IIA hydrolyzes the sn-2 fatty acyl ester bond of...
glycerophospholipids to generate free fatty acidcs (FFAs) and lysophospholipids [27], which are not only inflammatory mediators but also the antecedent of strong inflammatory mediators such as platelet activating factor, and can induce strong inflammatory reaction via signal transduction resulting in atherosclerotic plaques formation [28]. Thus, it is conceivable that sPLA2-IIA might amplify inflammatory response by releasing FFA, lysophospholipids and arachidonic acid (AA) from LDL, including oxidized LDL.

The synthesis of sPLA2-IIA is regulated by pro-inflammatory cytokines responsible for the production of pro-inflammatory mediators and promoting atherogenesis, sPLA2 gene expression has been shown to be induced by various inflammatory stimuli such as endotoxin, IL-1β, TNF-α, and IL-6 [29, 30]. Previous studies demonstrated that sPLA2-IIA induces ICAM-1 expression [31]. Hence sPLA2-IIA may play an important role in the development of atherosclerosis accelerated by inflammatory cytokines. However, there is little evidence of sPLA2-IIA digesting the membrane of healthy HASMCs, although their ability to cleave the membrane of apoptotic cells and malignant or transformed cell lines has been reported [32, 33]. In the present study, MCP-1 expression and PI3K/Akt pathway activation induced by the combination of oxidized LDL and sPLA2-IIA could be retarded by administration of PI3K/Akt inhibitor LY294002, indicating that this pathway accounts for the sPLA2-IIA-enhanced atherogenic effect of oxidized LDL. Our data showed the importance of sPLA2-IIA in the pathogenesis of atherosclerosis, with emphasis on its effects in vascular SMCs. Our findings that sPLA2-IIA secreted by LV-GFP-sPLA2-IIA transduced cells failed to increase MCP-1 level is in contrast with a recent study by Leiquez et al. [34] that sPLA2-IIA from snake venom induces lipid body (LB) formation in macrophages through which atherosclerosis is facilitated. This might be due to the different localization and function of sPLA2-IIA in different cell types.

The limitation of the experiment is that sPLA2-IIA activity was not measured directly. However, by adding sPLA2-IIA inhibitor LY315920 in the medium of LV-GFP-sPLA2-IIA transduced HASMCs, MCP-1 product was examined with or without oxidized LDL. This strategy was thought to be one way to demonstrate the enzymatic activity of secreted sPLA2-IIA. Our observations focused on the effect of sPLA2-IIA on oxidized LDL induced MCP-1 expression. There are still many questions unclear, such as how sPLA2-IIA activates the Akt pathway, does the sPLA2-IIA-GFP fusion protein with enzymatic activity act on membrane phospholipids in activated HASMCs or on phospholipids in oxidized LDL. Additionally, since in present experiments, the cells used endogenously express some sPLA2-IIA, knockdown by the means of siRNA is merit in future studies.

In conclusion, besides modification of LDL, sPLA2-IIA still plays its atherosclerotic effect by augmenting oxidized LDL-induced MCP-1 expression via PI3K/Akt pathway activation. Our results indicated that sPLA2-IIA selectively activates proinflammatory functions in HASMCs, and this may concur with atherosclerosis progress. These findings might partially explain controversial reports regarding the proatherogenic effect of sPLA2-IIA. Meanwhile, our observation has raised some new questions which remain to be clarified.

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Disclosure Statement

None.
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