Lysophosphatidylserine has Bilateral Effects on Macrophages in the Pathogenesis of Atherosclerosis

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Aim: Lysophospholipids, particularly sphingosine 1-phosphate and lysophosphatidic acid, are known to be involved in the pathogenesis of atherosclerosis; however, the role of lysophosphatidylserine (LysoPS) in the onset of atherosclerotic diseases remains uncertain.

Methods: We investigated the effects of LysoPS on the uptake of oxidized low-density lipoprotein (oxLDL) and the modulation of inflammatory mediators and ER stress utilizing RAW264.7 cells and mouse peritoneal macrophages (MPMs).

Results: We found that LysoPS augmented cholesterol accumulation in both models. Consistent with these findings, LysoPS increased the expression of scavenger receptors (CD36, MSR1, LOX1 and TLR4). Regarding the involvement of these lipids in inflammation, LysoPS significantly decreased the expression of inflammatory mediators in lipopolysaccharide (LPS)-treated RAW264.7 cells and MPMs. LysoPS also attenuated ER stress in LPS-untreated RAW264.7 cells. The expression patterns of LysoPS receptors differed considerably among the LPS-untreated RAW264.7 cells, LPS-treated RAW264.7 cells and MPMs.

Conclusions: LysoPS may have proatherosclerotic properties in the setting of foam cell formation as well as antiatherosclerotic effects on inflammation in macrophages.


Key words: Atherosclerosis, Lysophosphatidylserine, Macrophage, Foam cell formation, Inflammation

Introduction

Atherosclerotic diseases are a frequent cause of disability and mortality worldwide¹, ². Therefore, the discovery of new biomarkers and therapeutic targets for atherosclerosis is eagerly anticipated, and many molecules have been proposed to be promising candidates.

Among candidate molecules, lysophospholipids (LPLs) have been suggested to act as potent lipid mediators in the field of vascular biology³-⁵. In particular, lysophosphatidic acid (LysoPA) and sphingosine 1-phosphate (S1P) have been demonstrated to have important roles in the pathogenesis of atherosclerotic diseases in several basic and clinical studies. In basic studies, for example, LysoPA causes the migration of smooth muscle cells and activated platelets, while S1P increases the integrity of blood vessels, protects epithelial cells and macrophages from apoptosis and induces eNOS in epithelial cells. In clinical studies, LysoPA has been found to be abundant in the lipid core region of human atherosclerotic plaques⁶, ⁷. Additionally, the plasma LysoPA levels are higher in patients with acute coronary syndrome⁸, whereas the plasma S1P levels and HDL-linked S1P levels are lower in those with ischemic heart disease.

Unlike LysoPA and S1P, the involvement of other minor LPLs, such as lysophosphatidylcholine (LysoPC), lysophosphatidylserine (LysoPS), lysophosphatidylinositol (LysoPI), lysophosphatidylglycerol (LysoPG) and lysophosphatidylethanolamine (LysoPE), in the pathogenesis of atherosclerotic diseases has not yet been elucidated. Among these LPLs,
LysoPS is thought to act as a potent lipid mediator, similar to LysoPA and S1P	extsuperscript{9}. For example, LysoPS stimulates the degranulation of mast cells	extsuperscript{10, 11}, suppresses the proliferation of T lymphocytes	extsuperscript{12} and induces apoptotic cell engulfment by macrophages	extsuperscript{13, 14}.

**Aim**

The biological effects of LysoPS on blood cells prompted us to investigate the possible involvement of LysoPS in the pathogenesis of atherosclerosis. The specific aim of this study was to investigate the involvement of LysoPS in the biological responses of macrophages, as macrophages have crucial functions in the development of atherosclerosis. Macrophages are thought to receive cholesterol from oxidized low-density lipoprotein (oxLDL) and subsequently become foam cells during the development of plaque lesions, thus playing important roles in the onset of inflammation causing plaque rupture	extsuperscript{15, 16}. Therefore, in the present study, we examined the effects of LysoPS on macrophages with regard to cholesterol accumulation and inflammation utilizing RAW 264.7 cells and mouse peritoneal macrophages.

**Methods**

**Materials**

1-Oleoyl (18:1) LysoPS and stearoyl (18:0) LysoPS (Avanti Polar Lipids, Alabaster, AL) were dissolved in methanol. Just before use, the methanol was evaporated, and the reagents were resolved in PBS containing 1% fatty acid-free BSA (Sigma-Aldrich Co., St. Louis, MO). Lipopolysaccharide (LPS, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added at a final concentration of 100 ng/mL.

**Cultures of RAW 264.7 Cells and Mouse Peritoneal Macrophages**

RAW 264.7 cells (ATCC, Manassas, VA) were seeded in DMEM (Sigma Aldrich Co.) containing 10% fetal bovine serum (FBS, Gibco BRL, Eggstein, Germany) and 1% penicillin/streptomycin (Gibco, Grand Island, NY). For the experiments regarding the uptake of cholesterol (Fig. 1), expression of scavenger receptors (Fig. 3) and splicing of XBP-1 (Fig. 5), RAW 264.7 cells were seeded at a density of 1.5 × 10⁶ cells/well on 6-well plates (Corning, Inc., NY), and the experiments were performed after 24 hours, as described below. For the experiments concerning the modulation of inflammation (Fig. 4) and expression of LysoPS receptors (Fig. 6), RAW 264.7 cells were seeded at a density of 2 × 10⁶ cells/well on 6-well plates (Corning, Inc.), and the analyses described below were performed after 24 hours.

Mouse peritoneal macrophages (MPMs) were prepared as described in a previous paper	extsuperscript{17}. Briefly, thioglycolate (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) was injected into the peritoneal space in C57BL6J mice obtained from CLEA Japan four days prior to harvesting cells from the mice. MPMs were collected using PBS and seeded at a density of 2 × 10⁶ cells/well on 6-well plates (Corning Inc.) with DMEM. Two hours later, the dishes were washed three times with PBS to remove non-adherent cells, and the adherent cells were subsequently cultured in DMEM with FBS. Thereafter, the cells were cultured and treated in the same manner as the RAW 264.7 cells. All animal experiments were conducted in accordance with the guidelines for Animal Care and approved by the animal committee at the University of Tokyo.

**Preparation of Oxidized LDL and Lipoprotein Uptake Assay**

Low-density lipoprotein (LDL) was purified from serum using the standard sequential flotation ultracentrifugation method. After dialysis against PBS (pH7.4) at 4°C, the LDL (1 mg protein/mL) was exposed to 5 μM of CuSO₄ at 37°C for 20 hours. Then, the oxLDL was dialyzed against PBS (pH7.4) containing 1 mM EDTA at 4°C for 24 hours	extsuperscript{18}.

For the lipoprotein uptake assays, the cells were preincubated for 24 hours with various concentrations of LysoPS. Then, the medium was exchanged for FBS-free DMEM containing oxLDL at a concentration of 100 μg/mL with vehicle or LysoPS for another 24 hours. The cells were subsequently washed twice with PBS and the lipid contents were extracted from the pelleted cells with chloroform/methanol. The total cellular cholesterol level was then measured using standard enzymatic methods (Wako Pure Chemical Industries, Ltd.) and adjusted according to the cellular protein level	extsuperscript{19}.

**Analysis of the Gene Expression using Real-Time RT-PCR**

In order to assess the expression of scavenger receptors (Fig. 3), cells were preincubated for 24 hours with vehicle or 1 μM of LysoPS and the medium was exchanged for FBS-free DMEM containing oxLDL at a concentration of 100 μg/mL with vehicle or LysoPS. Meanwhile, for the assay of LysoPS receptors (Fig. 6), cells were incubated in FBS-free DMEM with or without LPS. After 24 hours, the total RNA of the cells was purified using the GenElute mammalian total RNA Miniprep kit (Sigma-Aldrich Co.) and
Fig. 1. LysoPS increased the uptake of oxLDL in the RAW 264.7 cells

RAW 264.7 cells were preincubated for 24 hours with vehicle or LysoPS and the medium was exchanged for FBS-free DMEM (containing vehicle, oxLDL or native LDL [100 μg/mL]) with LysoPS. After 24 hours, the cellular lipid contents were extracted with chloroform and methanol, and the total cellular cholesterol level was measured using enzymatic methods and adjusted to the cellular protein level (n = 4-6/group). (A, B) Effects of LysoPS on the cellular cholesterol contents in the LPS-untreated RAW 264.7 cells incubated with oxLDL. "None" represents cells treated without oxLDL. *P < 0.01 vs. oxLDL, †P < 0.05 vs. oxLDL + vehicle. (C) Effects of LysoPS on the cellular cholesterol levels in the LPS-treated RAW 264.7 cells incubated with oxLDL. †P < 0.05 vs. oxLDL + vehicle. (D) Effects of LysoPS on the cellular cholesterol contents in the LPS-untreated RAW 264.7 cells incubated without oxLDL. (E) Effects of LysoPS on the cellular cholesterol levels in the LPS-untreated RAW 264.7 cells incubated with native LDL. "None" represents cells incubated without native LDL. (F) Effects of 18:0 and 18:1 LysoPS on the cellular cholesterol levels in the LPS-untreated RAW 264.7 cells incubated with oxLDL. *P < 0.01 vs. oxLDL + vehicle.

hybridization probes and primers obtained from TaqMan (Applied Biosystems): CD36 (Mm00432403_m1), macrophage scavenger receptor 1 (MSR1, Mm00446214_m1), oxidized low-density lipoprotein receptor 1 (LOX1, Mm00454586_m1), Toll-like
Results

LysoPS Increased the Uptake of oxLDL in the RAW 264.7 Cells

We investigated the modulation of the uptake of oxLDL by LysoPS in order to assess the effects of LysoPS on foam cell formation. As shown in Fig. 1A, treatment with 18:1 LysoPS together with oxLDL increased the cellular cholesterol content by 1.94-fold compared with treatment with oxLDL alone. The extent of the increase in the cellular cholesterol levels depended on the dose of 18:1 LysoPS (Fig. 1B). When the RAW 264.7 cells were activated with LPS, the cellular cholesterol levels also increased (Fig. 1C). In contrast, when the RAW 264.7 cells were treated with 18:1 LysoPS only, without oxLDL, no changes in the cellular cholesterol levels were observed (Fig. 1D). These results indicate that the increase in the cellular cholesterol levels observed in the oxLDL-treated RAW 264.7 cells incubated with 18:1 LysoPS may have resulted from the enhanced uptake of oxLDL, rather than an increase in de novo cholesterol synthesis.

In order to investigate whether the effects of 18:1 LysoPS were specific to oxLDL, we challenged RAW 264.7 cells with native LDL. As shown in Fig. 1E, we observed no modulation of the increase in the cellular cholesterol levels in the RAW 264.7 cells by LysoPS when challenged with native LDL, ruling out the possibility that LysoPS increased the capacity for phagocytosis against lipoproteins in general. Regarding the molecular species of LysoPS, both 18:0 and 18:1 LysoPS exhibited similar effects (Fig. 1F).

LysoPS Increased the Uptake of oxLDL in the MPMs

We next examined the effects of LysoPS on foam cell formation in the MPMs. As shown in Fig. 2A, treatment with 18:1 LysoPS increased oxLDL incorporation by 3.36-fold compared with treatment with oxLDL alone. The extent of the increase in the cellular cholesterol levels depended on the dose of 18:1 LysoPS (Fig. 2B). When the MPMs were activated with LPS, the cellular cholesterol levels also increased (Fig. 2C). In contrast, when the MPMs were treated with 18:1 LysoPS only, without oxLDL, no changes in the cellular cholesterol levels were observed (Fig. 2D). These results indicate that the increase in the cellular cholesterol levels observed in the oxLDL-treated RAW 264.7 cells incubated with 18:1 LysoPS may have resulted from the enhanced uptake of oxLDL, rather than an increase in de novo cholesterol synthesis.

Analysis of X-box Binding Protein-1 (XBP-1) Splicing

Cells were preincubated for 24 hours with vehicle or 1 μM of LysoPS and the medium was exchanged for FBS-free DMEM with vehicle or 1 μM of LysoPS. After 24 hours, the total RNA of the cells was purified and subjected to reverse transcription, as described above. Utilizing the obtained cDNAs, PCR amplification of XBP-1 cDNA, which included the region at which endoplasmic reticulum stress (ER stress) induces the processing of XBP-1 mRNA, was performed, followed by the digestion of the PCR products with BstI and electrophoresis on a 1% agarose gel. The processing of XBP-1 mRNA with ER stress resulted in the elimination of the BstI cutting site. PstI (-) represents DNA bands derived from spliced XBP-1 mRNA with ER stress, while PstI (+) represents DNA bands derived from un-processed XBP-1 mRNA.

Statistical Analysis

The results are expressed as the mean ± SD. Differences between two groups were evaluated using Student’s t-test, and differences between more than two groups were assessed using a one-way ANOVA followed by multiple comparison tests. *P-values less than 0.05 were deemed to be statistically significant (**P<0.05, ***P<0.01).
Fig. 2. LysoPS increased the uptake of oxLDL in the MPMs

MPMs were preincubated for 24 hours with vehicle or LysoPS and the medium was exchanged for FBS-free DMEM containing oxLDL (100 μg/mL) with vehicle or LysoPS. After 24 hours, the cellular lipid contents were extracted with chloroform and methanol and the total cellular cholesterol level was measured using enzymatic methods and adjusted to the cellular protein level (n=6-9/each group). (A) Effects of LysoPS on the cellular cholesterol contents in the LPS-untreated MPMs incubated with oxLDL. “None” represents cells incubated without oxLDL. *P<0.01 vs. oxLDL+vehicle. (B) Effects of LysoPS on the cellular cholesterol contents in the LPS-treated MPMs incubated in the presence of oxLDL. *P<0.01 vs. oxLDL+vehicle.

Fig. 3. LysoPS increased the expression of scavenger receptors

RAW 264.7 cells were preincubated for 24 hours with vehicle or 1 μM of LysoPS and the medium was exchanged for FBS-free DMEM with vehicle or 1 μM of LysoPS. After 24 hours, mRNA was extracted and subjected to a real-time PCR analysis for CD36, MSR1, LOX-1 and TLR4. GAPDH was utilized as an internal control.

Fig. 4. Modulation of the expression of inflammatory mediators by LysoPS in the LPS-untreated and LPS-treated RAW 264.7 cells and MPMs

RAW 264.7 cells treated without LPS (A) or with LPS (B) or MPMs treated without LPS (C) or with LPS (D) were incubated in FBS-free DMEM with vehicle or 10 μM of LysoPS. After four hours, mRNA was extracted and subjected to a real-time PCR analysis for IL-6, TNFα, MMP-2, MMP-9 and MCP-1. 18S was utilized as an internal control.
the expression of scavenger receptors.

**Lysophosphatidylserine (LysoPS) Affected the mRNA Levels of Inflammatory Mediators**

In addition to cholesterol uptake via scavenger receptors, macrophages have important functions in inflammation as related to the pathogenesis of atherosclerosis. Therefore, we next analyzed the modulation of the expression of IL-6, TNFα, MMP-2, MMP-9 and MCP-1 in the RAW 264.7 cells and MPMs by LysoPS. Consequently, we found that the expression of TNFα and MMP-9 was suppressed in the LPS-treated RAW 264.7 cells and LPS-treated MPMs (Fig. 4B, D), while the expression of IL-6 and MCP-1 was suppressed in the LPS-treated MPMs (Fig. 4D).

**Lysophosphatidylserine (LysoPS) Suppressed Endoplasmic Reticulum Stress in the RAW 264.7 Cells**

Recently, endoplasmic reticulum (ER) stress has been demonstrated to be involved in the macrophage-related pathogenesis of atherosclerosis. Therefore, we next analyzed the effects of LysoPS on ER stress in the RAW 264.7 cells and MPMs. The results for XBP-1 splicing induced by ER stress are shown in Fig. 5. The ratio of the unspliced form to the spliced form of XBP-1 mRNA was higher in the RAW 264.7 cells challenged with LysoPS, whereas no notable findings were observed for the effect of LysoPS on ER stress in the MPMs (data not shown).

**The Lysophosphatidylserine (LysoPS) Receptor Expression Differed among the LPS-Untreated and LPS-Treated RAW 264.7 Cells and MPMs**

As described above, an increment in oxLDL uptake in response to LysoPS was observed in all three models (LPS-untreated and LPS-treated RAW 264.7 cells and MPMs), while the effects on inflammatory cytokines and ER stress differed somewhat among the cell models.

In order to elucidate the different responses to LysoPS between the cell models, we investigated the possibility that the pattern of LysoPS receptor expression differed in each model. We therefore analyzed the expression of LysoPS receptors in the LPS-untreated and LPS-treated RAW 264.7 cells and MPMs. First, we confirmed that all the previously reported LysoPS receptors (P2Y10, GPR34, A630033H20, GPR174 and G2A) were expressed in both the RAW 264.7 cells and MPMs (Fig. 6A, B). Next, we investigated the LysoPS receptor expression using real-time PCR and found that the expression levels of the LysoPS receptors differed largely among the cell models. Compared with that observed in the LPS-untreated RAW 264.7 cells, the mRNA levels of P2Y10 and GPR174 were significantly higher in the LPS-treated RAW 264.7 cells, whereas the mRNA levels of GPR34 and G2A were significantly lower (Fig. 6C, D). In the MPMs, the mRNA levels of P2Y10 and GPR174 were significantly higher, while the mRNA levels of GPR34 and G2A were significantly lower (Fig. 6C, D). The results for the expression of LysoPS receptors indicate the possibility that differences in various biological effects of LysoPS among the cell models may result from differences in the expression patterns of LysoPS receptors.

**Discussion**

In this study, we investigated the effects of LysoPS on several factors involved in the pathogenesis of atherosclerosis and found that (1) LysoPS enhanced the uptake of oxLDL, (2) LysoPS decreased the expression of inflammatory mediators and (3) LysoPS attenuated ER stress in RAW 264.7 cells. These results suggest that LysoPS possesses proatherosclerotic properties with regard to foam cell formation and antiatherosclerotic properties with respect to inflammation.

Regarding foam cell formation, the results of the present study suggest that LysoPS accelerates plaque formation. No previous studies have investigated the effects of LysoPS on foam cell formation, although LysoPA, a more well-known LPL, increases the expression of CD36 by activating PPAR. Indeed, LysoPS increased the CD36 expression together with that of other scavenger receptors in this study (Fig. 3), although the extent of the increase in CD36 was not as great as that observed in cases in which LysoPS works as a potent PPAR agonist. Other possible mechanisms for the LysoPS-mediated increase in oxLDL uptake include the augmentation of efferocytosis by LysoPS, and LysoPS reportedly enhances apoptotic cell engulfment by MPMs via G2A signaling. However, since LysoPS did not increase the uptake of native LDL in this study, LysoPS may not simply increase the capacity of macrophages for phagocytosis, but rather increases the receptor-mediated oxLDL uptake.

While LysoPS exhibits proatherosclerotic properties, as demonstrated above, we also observed various antiatherosclerotic properties of LysoPS. For example, LysoPS attenuated the expression of inflammatory mediators as well as ER stress, both of which are considered to be atherogenic. Although no previous studies have investigated the effects of LysoPS on the expression of inflammatory mediators or ER stress in macrophages, several reports have documented the
**Fig. 5.** LysoPS suppressed ER stress in the RAW 264.7 cells.

RAW 264.7 cells were preincubated for 24 hours with vehicle or 1 µM of LysoPS and the medium was exchanged for FBS-free DMEM with vehicle or 1 µM of LysoPS. After 24 hours, mRNA was extracted. The modulation of ER stress by LysoPS was investigated using the cells described above. Pst(-) describes bands derived from spliced XBP-1 mRNA with ER stress, while Pst(+) describes bands derived from un-processed XBP-1 mRNA. LPS-untreated (left) and LPS-treated (right) RAW 264.7 cells.

**Fig. 6.** The LysoPS receptor mRNA levels in macrophages depended on the status of inflammation.

RAW 264.7 cells or MPMs were incubated in FBS-free DMEM with vehicle or LPS. After 24 hours, mRNA was extracted and subjected to a real-time PCR analysis. The expression levels of known LysoPS receptors (P2Y10, GPR34, A630033H20, GPR174 and G2A) in the LPS-untreated and LPS-treated RAW264.7 cells and MPMs were analyzed using real-time PCR. (A, B) RT-PCR for P2Y10, GPR34, A630033H20, GPR174 and G2A using a cDNA library prepared from RAW 264.7 cells (A) and MPMs (B). (C, D) Expression levels of P2Y10 (C) and GPR34, A630033H20, GPR174 and G2A (D) in the LPS-untreated and LPS-treated RAW264.7 cells and MPMs. GAPDH was utilized as an internal control. *P < 0.01 vs. LPS-untreated RAW 264.7 cells, †P < 0.05 vs. LPS-untreated RAW 264.7 cells.
effects of LysoPA and LysoPC on inflammatory cytokines and/or ER stress in other cells involved in atherosclerotic diseases. In particular, LysoPA promotes the release of the chemokine CXCL1 and the accumulation of macrophages in the endothelium of atherosclerotic lesions, while LysoPC promotes the expression of IL-8, a proinflammatory and proadhesive chemokine. Regarding ER stress, LysoPA reportedly attenuates ER stress and ER stress-associated apoptosis in murine mesenchymal stem cells.

Another interesting finding is that the effects of LysoPS on inflammatory mediators and ER stress were somehow dependent on the cell status. Although we did not elucidate the underlying mechanisms, we found that the expression patterns of LysoPS receptors differed largely between the LPS-untreated RAW 264.7 cells, LPS-treated RAW 264.7 cells and MPMs. In order to investigate the possible clinical usefulness of antagonists or agonists against specific LysoPS receptors, the physiological properties of each receptor must be clarified in future studies.

At present, no studies have elucidated the involvement of LysoPS in atherosclerotic diseases in human subjects, and data regarding the concentrations of LysoPS in human or murine plasma and serum are hardly available. Our preliminary findings suggested that the LysoPS level in mouse plasma is 30-50 nM, while the LysoPS level in mouse serum, in which platelets are fully activated, is 200-300 nM, similar to our observations for S1P in a previous paper. These results suggest that the LysoPS concentration may be much higher in atherosclerotic lesions, where platelets are activated.

In summary, LysoPS accelerates foam cell formation, while LysoPS suppresses the expression of inflammatory mediators and attenuates ER stress in macrophages. These results suggest that LysoPS possesses bilateral properties in the pathogenesis of atherosclerosis.

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Conflicts of Interest

None.

References

17) Kurano M, Iso ON, Hara M, Noiri E, Koike K, Kadowaki T, Tsukamoto K: Plant sterols increased IL-6 and TNF-alpha secretion from macrophages, but to a lesser