Macrophage Uptake Behavior and Anti-inflammatory Response of Bovine Brain- or Soybean-derived Phosphatidylserine Liposomes

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Abstract: Phosphatidylserine (PtdSer) is mainly derived from the bovine brain cortex or soybean lecithin. We investigated macrophage uptake behavior and the anti-inflammatory response induced by liposomes containing bovine brain- (B-PSL) or soybean-derived PtdSer (S-PSL). The size of B-PSL and S-PSL was very similar. There were no significant differences in the uptake of B-PSL and S-PSL by Raw 264.7 macrophage cells. Addition of B-PSL or S-PSL decreased the production of the inflammatory cytokines, IL-1α, IL-6 and TNF-α, in lipopolysaccharide-treated Raw 264.7 cells, but there were no differences between them. These results suggest that S-PSL may be used as an anti-inflammatory agent.

Key words: cytokine, inflammation, liposome; phosphatidylserine, soybean lipid

1 Introduction

Phosphatidylserine (PtdSer) comprises serine and two fatty acids (Fig. 1A), and is the most abundant negatively charged phospholipid in eukaryotic membranes, mainly in the inner leaflet of the plasma membrane and in endocytic membranes¹,². PtdSer supplementation shows several health benefits, such as improvement of cognitive (memory) functions in middle-aged and elderly people³⁴⁵, reduction in stress-induced activation of the hypothalamo-pituitary-adrenal axis in healthy men⁶, suppression of attention-deficit hyperactivity disorder symptoms and short-term auditory memory in children⁷, reduction in cortisol responses to physical exercise⁸⁹, and improvement of exercise capacity in healthy young men⁹.

In apoptotic cells, PtdSer acts as the ‘eat me’ signal and stimulates their uptake by phagocytes (e.g., macrophages) via PtdSer receptors. PtdSer-dependent phagocytosis of apoptotic cells by macrophages decreases inflammatory cytokine production such as interleukin (IL)-6 and tumor necrosis factor (TNF) -α⁴¹⁰¹¹. Artificial PtdSer-containing liposome (PSL) that can mimic the effects of apoptotic cells

Fig. 1 (A) Structure of PtdSer. (B) The size of B-PSL and S-PSL.

Bovine brain-derived PtdSer

Soybean-derived PtdSer

|   | B-PSL | S-PSL |
|---|-------|-------|
| Size (nm) | 302 ± 11 | 293 ± 13 |

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Accepted June 11, 2018 (received for review May 21, 2018)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
are useful for the treatment of inflammation-related diseases, such as myocardial infarction\textsuperscript{12}, rheumatoid arthritis\textsuperscript{35}, retinal ischemia-reperfusion injury\textsuperscript{34}, and obesity\textsuperscript{13}.

PtdSer can be derived from either animal (mainly bovine brain cortex) or plant sources (mainly soybean lecithin), but there is a difference in fatty acid composition between them. The fatty acids in bovine brain-derived PtdSer are primarily stearic acid (C18:0) in the R1 position and oleic acid (C18:1n-9) in the R2 position, whereas soybean-based PtdSer contains linoleic acid (C18:2n-6) in both positions (Fig. 1A)\textsuperscript{15}. However, there is little data to assess whether the difference in fatty acid composition affects macrophage uptake behavior and the anti-inflammatory response.

In the present study, bovine brain-derived PtdSer-containing liposomes (B-PSL) or soybean-derived PtdSer-containing liposomes (S-PSL) were synthesized and their uptake by macrophages and the anti-inflammatory response were investigated.

2 Materials and Methods

2.1 PtdSer synthesis

PtdSer derived from bovine brain or soybean (Glycine max; purity ≥ 98%) and phosphatidylcholine (PtdCho) (purity ≥ 98%; both from Sigma-Aldrich, St. Louis, MO, USA) were dissolved in chloroform/methanol (90:10, v/v). PSL was prepared from a lipid mixture of PtdSer (14 mM) and PtdCho (33 mM) at a molar ratio of 3:7, with or without the fluorescent dye, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (ammonium salt; Avanti Polar Lipids, Inc., Alabaster, Alabama, USA). The solvent was removed under nitrogen with stirring and dried in a desiccator overnight. The liposomal pellet was then resuspended in PBS (10 mg/mL).

2.2 PSL diameter measurement

Milli-Q water (900 µL; pH 7.3) was added to the PSL solution (100 µL). The diameter of the samples was determined using a Zetasizer (Malvern Instruments, Malvern, UK) with a helium/neon laser at a detection angle of 173° at 25°C.

2.3 Uptake of PSLs by macrophages

PSL labeled with the fluorescent dye was used to examine the uptake of PSL by macrophages. The amount of fluorescent dye was 1 mol% of total lipid. Raw 264.7 cells were maintained in Dulbecco’s modified Eagle medium (Wako, Osaka, Japan) and incubated at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}. Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL) (all from Gibco, Invitrogen Co., Grand Island, NY, USA). Raw 264.7 cells (5 × 10\textsuperscript{4}) were grown in 24-well plates at 37°C for 24 h and were further incubated at 37°C for 1 or 24 h after adding PSL. After 1 and 24 h, the cells were washed three times with PBS and lysed in 200 µL of lysis buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5 mM MgCl\textsubscript{2} and 0.05% NP-40 substitute). Fluorescence intensity was detected using a microplate reader (Synergy HT, BIORAD Instruments Inc., Winoski, VT, USA). Total protein concentration was determined by the Bradford method (Coomassie Brilliant Blue G-250 reagent; BIO-RAD Lab., Hercules, CA, USA) and detected by absorbance at 595 nm. The results are presented as the fluorescence intensity per mg of total protein.

2.4 PSL-induced anti-inflammatory response

Media were supplemented with 10% heat-inactivated or non heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL) (all from Gibco). Raw 264.7 cells (2.5 × 10\textsuperscript{4}) were grown in 24-well plates at 37°C for 24 h. After 1 h of lipopolysaccharide (LPS) treatment (100 ng/mL), PSLs (300 µg/mL) were added to the cells. At 6 and 24 h the media were collected and the level of inflammatory cytokines (IL-1α, IL-6 and TNF-α) was examined using Ready-SET-Go ELISA kit (Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer’s instructions.

2.5 Statistical analysis

Results are expressed as mean ± SD of three or four samples. All determinations were carried out in duplicate for each sample. Statistical analysis of the differences between groups was performed using two-tailed Student’s t test or one-way ANOVA followed by Tukey’s multiple comparison test. P < 0.05 was considered statistically significant.

3 Results

We examined the size of B-PSL and S-PSL because the size of PSL can affect their uptake by macrophages\textsuperscript{17, 18}. The size of B-PSL and S-PSL is 302 ± 13 and 293 ± 13 nm, respectively, meaning that there is no difference in size between the two groups (Fig. 1B).

Next, we investigated the uptake of PSL by macrophages using Raw 264.7 macrophage cells. Fluorescence intensity was examined 1 and 24 h after addition of fluorescein-labeled PSL to Raw 264.7 cells. As shown in Fig. 2, there was no significant difference in the uptake of B-PSL and S-PSL by Raw 264.7 cells.

Non-heat-inactivated FBS contains activated complement components that may affect the inflammatory response of macrophages. Thus, we examined whether the production of inflammatory cytokines (IL-1α, IL-6, and TNF-α) in macrophages can be influenced by heat-inactivated or non-
heat-inactivated FBS. After LPS treatment, the level of TNF-α was higher in the medium containing 10% non-heat-inactivated than heat-inactivated FBS, but in the case of IL-1α and IL-6, opposite results were obtained.

Addition of B-PSL or S-PSL to LPS-treated Raw 264.7 cells significantly decreased the production of IL-1α and IL-6 at 6 h, but the TNF-α level was significantly reduced in Raw 264.7 cells treated with S-PSL than B-PSL. On the other hand, there were no significant differences at 24 h between the two treatments (Fig. 3). These results indicate that in spite of the difference in fatty acid composition between B-PSL and S-PSL, there are no differences in macrophage uptake behavior and anti-inflammatory response between them.

**4 Discussion**

Bovine brain-derived PtdSer is not suitable for medical or dietary supplement uses because of infectious diseases such as bovine spongiform encephalopathy. Hence, PtdSer made from soybean lecithin has been used as a safe alternative to bovine brain-derived PtdSer. Intake of soybean-derived PtdSer showed similar results to bovine-derived PtdSer, for example, in the reduction in cortisol responses to physical exercise and in the improvement of cognitive functions.

Another benefit of PtdSer intake is its anti-inflammatory effects. PtdSer binds to PtdSer receptors on the surface of phagocytes, such as T-cell immunoglobulin- and mucin domain-containing molecule (Tim)-4, Tim-1, and brain-specific angiogenesis inhibitor 1. The binding between PtdSer and PtdSer receptors stimulates anti-inflammatory signaling pathways, such as c-Src/PI3K/STAT3 or c-Src/FAK/Rac pathways, and reduces the production of inflammatory mediators such as IL-1, IL-6, and TNF-α.

In our study, B-PSL or S-PSL treatment significantly de...
increased the production of inflammatory cytokines in Raw 264.7 cells exposed to LPS (Fig. 3).

No differences were found in macrophage uptake behavior and anti-inflammatory response between B-PSL and S-PSL (Figs. 2 and 3), whereas there is a clear difference in fatty acid composition between them (Fig. 1A). PtdSer receptors specifically bind to the polar head group of PtdSer in a stereospecific fashion, but not to the fatty acid group of PtdSer \(^{21-23}\). As shown in Fig. 1A, bovine brain-derived PtdSer and soybean-based PtdSer have the same polar head group, and these structural properties may result in similar macrophage uptake behavior and anti-inflammatory response between B-PSL and S-PSL. On the other hand, several groups have reported that liposomes prepared with PtdCho alone have no or very little effect on macrophages of weight training-induced over-training. Biol. Sport 15, 135-144 (1998).

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