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Phospholipase A_2-Mediated preparation of phosphatidylcholine containing ricinoleic acid and its anti-inflammatory effect on murine macrophage-like RAW264.7 cells

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ABSTRACT

Ricinoleic acid (RA) is a type of fatty acid found in castor oil and has been known to have anti-inflammatory effects. Phospholipids are useful functional compounds in food/medical fields due to their amphiphilic property and biocompatibility. The purpose of this study was to prepare phospholipid (PL) containing RA and to evaluate the anti-inflammatory activity of a prepared PL *in vitro*. Lyso-phosphatidylcholine (LPC) from egg yolk was subjected to phospholipase A_2-mediated esterification for the preparation of phosphatidylcholine containing RA at *sn*-2 position (2-RA-PC). The prepared 2-RA-PC was then evaluated for its anti-inflammatory activity against the murine macrophage-like cell line RAW264.7 stimulated by lipopolysaccharide. Using glycerol as solvent and formamide as water mimic, 2-RA-PC was successfully prepared. Upon optimizing the molar ratio (RA/LPC), the amount of glycerol, and the reaction time, a maximum yield of 57.5 mol% was obtained. Analysis of fatty acid compositions of substrate LPC, RA, and synthesized PC suggested almost all RA was incorporated into *sn*-2 position of LPC. Down regulation of mRNA expression of pro-inflammatory cytokines, interleukin 6 and 1β was higher for 2-RA-PC than for RA or Soy-PC, which suggests that the anti-inflammatory effects of RA were improved following phosphatidylation. Our data suggest that 2-RA-PC is a potential lipid for use as an anti-inflammatory compound.

1. Introduction

Ricinoleic acid (RA, 12-OH-18:1 n-9) is the main fatty acid found in castor oil. It typically has a hydroxyl group at the C12 position (Fig. 1). Although RA is not used in food materials, it has generated interest for its physiological functions as a laxative, labor inducing agent, and an anti-inflammatory agent (Boddu et al., 2015; Tunaru et al., 2012; Vieira et al., 2001). It is also used as one of the raw materials in RipStick creams and pomade for its moderate solubility in ethanol and acetic acid resulting in the appropriate viscosity. However, quality improvement is necessary owing to occasional cases of contact dermatitis (Inoue et al., 1998).

Phospholipids (PLs), especially glycero-PLs, are desirable substrates for use as functional compounds in various fields, such as the food industry or the medical industry. This is because of the amphiphilic properties and biocompatibility of PLs. These properties allow for the use of phospholipids as food emulsifiers or as raw materials for liposomes, which are used in drug delivery systems (Shah et al., 2017; Li et al., 2015). Phosphatidylated RA can be an appealing bio-active compound that has functions of both RA and phospholipids.

Chemical or enzymatic methods for the preparation of phosphatidylated RA are available. Borsotti et al. reported on the synthesis of phosphatidylcholine (PC) containing RA at *sn*-2 position (2-RA-PC) using a chemical method. However, the method requires several toxic compounds and involves a complex reaction pathway, which results in only a 10.3% yield (Borsotti et al., 2001). Alternatively, the enzymatic method proceeds with the reaction in simple and mild conditions with no harmful compounds. Vijeeta et al. reported on the synthesis of RA-PC from soya and egg-PC through phospholipase A_1-mediated esterification with a yield of only 10% (Vijeeta et al., 2004). This method utilized the *sn*-1 position for the binding of RA.

On the other hand, by using phospholipase A_2 (PLA_2), it is possible to combine the desired fatty acid into the glycero-PLs at the *sn*-2 position. Although PLA_2 catalyze hydrolysis reaction at *sn*-2 position of glycerophospholipids (Dennis et al., 2011), it could also catalyze esterification reaction in organic solvent or at low water environments

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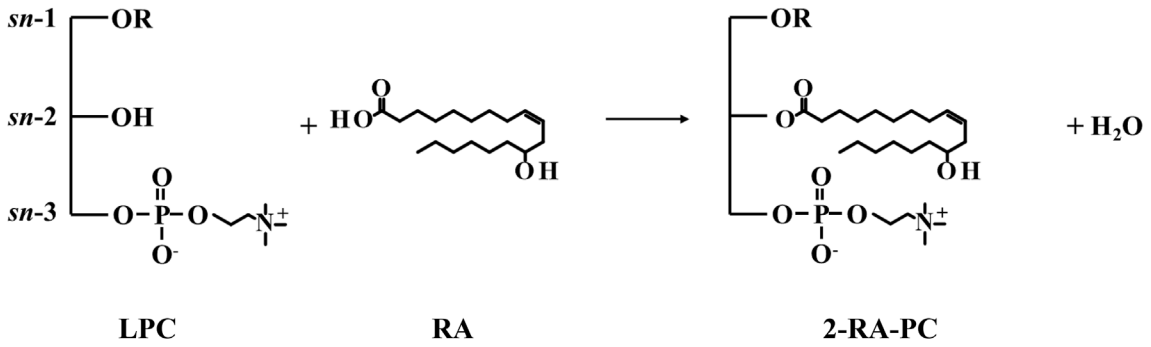

Fig. 1. Reaction scheme of PLA_2-mediated esterification of lysophosphatidylcholine (LPC) and ricinoleic acid (RA).

(Adlercreutz and Wehtje, 2004; Tanaka et al., 2010). The synthesis of PC containing docosahexaenoic acid (DHA) at the *sn*-2 position through esterification of soybean lyso-PC (LPC) and DHA mediated by PLA_2 (Hosokawa et al., 1995). Furthermore, we also reported the synthesis of PC containing conjugated linoleic acid at the *sn*-2 position (Yamamoto et al., 2006). However, the enzymatic synthesis and characterization of PL containing RA at the *sn*-2 position has not yet been reported.

In this study, we aimed to prepare 2-RA-PC mediated by PLA_2 (Fig. 1) and to evaluate its anti-inflammatory effect *in vitro*.

2. Materials and methods

2.1. Materials

LPC (L-α-lysophosphatidylcholine) from egg yolk, RA (> 80%) and 3-hydroxyltetradecanoic acid (> 98%, 3-OH-14:0) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Oleic acid (> 99%, 18:1 n-9) and 12-hydroxystearic acid (> 80%, 12-OH-18:0) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Industrial PLA_2 (Lecitase® 10L) from porcine pancreas was obtained from Novozymes A/S (Bagsværd, Denmark). PLA_2 was used after dialysis of Lecitase 10L using a dialysis membrane in distilled water, followed by freeze-drying. All solvents and other chemicals used in this study were analytical grade.

2.2. Enzymatic reaction

The reaction mixture used for 2-RA-PC preparation was: 0.02 mmol of LPC (11 mg), 0.3–3.0 mmol of RA (90–900 mg), 225–2200 mg of glycerol, 3.3×10^4 U of PLA_2 (6 mg), and 50 μL formamide containing 0.3 μmol of $CaCl_2$. Reaction was allowed to proceed at 40 °C, 900 rpm, in the dark. The reaction was terminated by the addition of methanol. Afterwards, chloroform and water were added to the reaction mixture to a final ratio of chloroform/methanol/water = 10:5:3 (v/v/v). The lipid fraction, which includes the synthesized 2-RA-PC, the remaining substrates LPC and RA, was obtained from the chloroform layer. The reaction mixture was subjected to thin layer chromatography and revealed a novel spot that had the same Rf value as the PC standard. Detection was done using both I_2 vapor and Dittmer reagent. Because PLA_2 (Lecitase 10L) is well known enzyme to recognize *sn*-2 position of glycerophospholipids rigidly, RA-PC synthesized in this reaction condition will be all 2-RA-PC.

2.3. Fatty acid compositions of LPC, RA, and 2-RA-PC

Fatty acid compositions of LPC, RA, and 2-RA-PC was measured by gas chromatography (GC). For LPC, sample was methyl-esterified using hydrochloric acid/methanol, as described by Jham et al. (1982). For RA and 2-RA-PC, samples were first methyl-esterified as same as LPC, followed by trifluoroacetylated. Briefly, adequate amount of sample was dissolved with 0.5 mL benzene and 0.1 mL of pyridine. Then, 10 μL of trifluoroacetic acid anhydrides was added and kept at 50 °C for 10 min. After cooling, 1 mL of 5% NH_3 aq. was added and shook for 5 min. Resulting benzene layer was analyzed by GC (GC-17A, Shimadzu, Kyoto, Japan). equipped with a flame ionization detector and a fused silica capillary column, DB-WAX (0.25 mm × 60 m, 0.25 μm; Agilent Technologies, CA, USA). The temperature of both the injector and detector were 250 °C. Helium at 80 kPa was used as the carrier gas. The column temperature was 190 °C.

2.4. Calculation of reaction yield

The yield of 2-RA-PC prepared from the reaction was measured through high performance liquid chromatography (HPLC). The chloroform layer from the reaction mixture containing LPC, RA, and synthesized 2-RA-PC, was injected into the HPLC system which consists of a Waters 2695 Separations module and a reflective detector model 133 (GILSON). An Inert SIL 10 (4.6 × 250 mm, 5 μm, GL Science) column was used. Samples were then eluted using an isocratic elution of the mobile phase with a ratio of acetonitrile/methanol/sulfuric acid = 100:10:0.05 (v/v/v). The flow rate was maintained at 1.0 mL/min, while the column temperature was maintained at 40 °C. Calibration curve was prepared with 2-RA-PC, and reaction yield of 2-RA-PC was then calculated following the equation below.

$$\text{Yield (mol\%)} = \text{synthesized 2-RA-PC (mol)/substrate LPC (mol) x 100} \quad (1)$$

2.5. Cell culture

Murine macrophage-like cell line RAW264.7 was purchased from DS Pharma Biomedical (Osaka, Japan). RAW264.7 cells (5×10^4 cells/well) were pre-incubated in 24-well plates with 1 mL RPMI 1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. 2-RA-PC which was purified from reaction mixture using PLC plate (Silica gel 60, 2 mm, Merck), Soy-PC (H. Holstein Co., Ltd., Tokyo Japan), and RA were then added into the culture media, respectively, and the cells were incubated for an additional 24 h. Each sample was added into the culture medium as an ethanolic solution. Final concentration of ethanol was adjusted to 0.1% in the culture medium without cytotoxicity. Afterwards, cell inflammation was induced using lipopolysaccharide (LPS, final concentration of 0.1 μg/mL) for 6 h in the presence of 2-RA-PC, Soy-PC or RA.

2.6. Quantitative real-time RT-PCR

After stimulation with LPS, the RAW264.7 cells were washed with PBS three times. Total RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. Afterwards, cDNA was synthesized from total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems Japan

Ltd, Tokyo, Japan). Quantitative real time RT-PCR was then performed using the ABI Prism 7500 (Applied Biosystems Japan Ltd, Tokyo, Japan). Cycling conditions for PCR were as follows; 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min. PCR primers and TaqMan® probes were obtained from TaqMan® Gene Expression Assays (Applied Biosystems Japan Ltd, Tokyo, Japan); IL-6: Mm00446190_ml, IL-1β: Mm00434228_ml, 18S: Mm02601777_g1.

2.7. Statistical analysis

For 2-RA-PC preparation: All values are expressed as mean ± SD (n = 3). Statistical differences were determined by the Scheffe's *F* test at $P < 0.05$. For evaluation of anti-inflammatory effect: All values are expressed as mean ± SE (n = 3). Statistical differences were determined by the Tukey's *F* test at $P < 0.05$ or $P < 0.01$.

3. Results and discussion

3.1. Effect of the molar ratio of RA/LPC on the RA-PC preparation

The effect of the molar ratio of RA/LPC on the 2-RA-PC preparation was first investigated with reaction mixture of 0.02 mmol of LPC, 0.02–0.40 mmol of RA, 550 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 24 h. 2-RA-PC synthesis proceeded with the increase in molar ratio of RA/LPC. The yield of 2-RA-PC reached a peak of 40.5 mol% at a molar ratio of 10. This may be due to the increased contact frequency in this range of molar ratios (Fig. 2). However, at a molar ratio of 20, the yield of 2-RA-PC decreased (20.7 mol%).

In the PLA_2-mediated esterification of LPC and oleic acid, excess amount of oleic acid caused high viscosity and high polarity in the reaction mixture, and resulted in lower reaction rate, but did not affect the reaction yield (Egger et al., 1997). In our previous study on the preparation of PC containing conjugated fatty acids, it was also reported that the yield increased with the increase in the molar ratio of conjugated fatty acid/LPC up to a molar ratio of 40 (Yamamoto et al., 2006). Therefore, the present result was typical for the reaction using RA as acyl donor. In the current study, the high polarity of RA, brought about by its hydroxyl group, might cause the deactivation of PLA_2. Therefore, the optimal molar ratio of RA/LPC was determined to be 10.

3.2. Effect of the amount of glycerol on the 2-RA-PC preparation

The effect of the amount of glycerol on the 2-RA-PC preparation was then investigated with the reaction mixture of 0.02 mmol of LPC, 0.20 mmol of RA, 225–2200 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 24 h. The yield increased with the addition of glycerol up to 1100 mg (Fig. 3). A maximum yield of 46.1 mol% was obtained; however, a decrease in yield was observed with the addition of glycerol beyond 1100 mg. The increase in yield in the range of 225–1100 mg of glycerol is attributed to the increase in the dispersibility of reaction components such as RA, LPC, and PLA_2. However, in the range of 1100–2200 mg of glycerol, the dilution effect will be high, resulting in a low yield. Therefore, optimal amount of glycerol was determined to be 1100 mg.

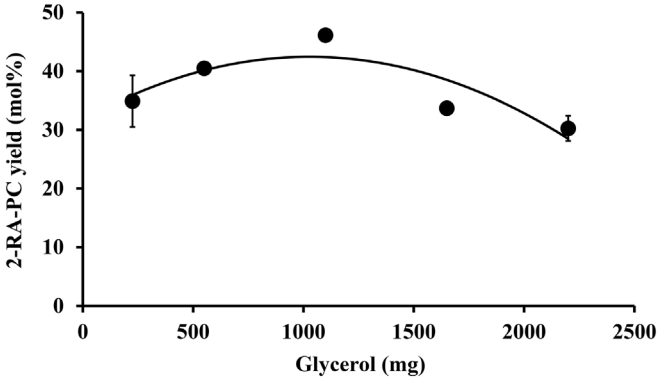

Fig. 3. Effect of the amount of glycerol on 2-ricinoleic acid-phosphatidylcholine (2-RA-PC) preparation. Reaction conditions: 0.02 mmol of lysophosphatidylcholine, 0.20 mmol of RA, 225–2200 mg of glycerol, 3.3×10^4 U of phospholipase A_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 24 h.

3.3. Effect of the reaction time on the 2-RA-PC preparation

The effect of the reaction time on the 2-RA-PC preparation was examined with reaction mixture of 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$, with a reaction time varying from 6 ~ 72 h. The yield increased with the reaction time, but reached a plateau at 48 h, where a maximum yield of 57.5 mol% was obtained (Fig. 4). Therefore, the optimum reaction condition for 2-RA-PC synthesis was determined to be 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 48 h.

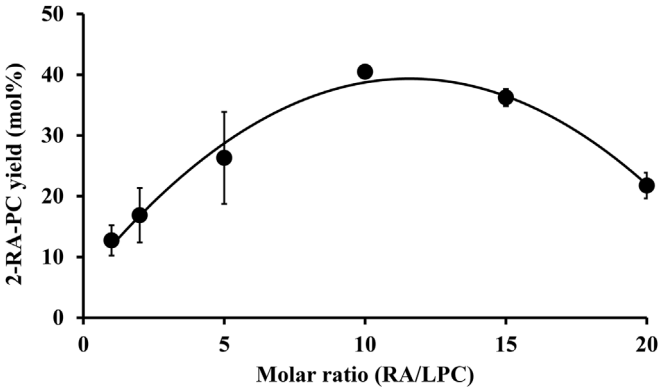

Fig. 2. Effect of molar ratio (ricinoleic acid (RA)/lysophosphatidylcholine (LPC) on 2-RA-phsophatidylcholine (PC) preparation. Reaction conditions: 0.02 mmol of LPC, 0.02–0.40 mmol of RA, 550 mg of glycerol, 3.3×10^4 U of phospholipase A_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 24 h.

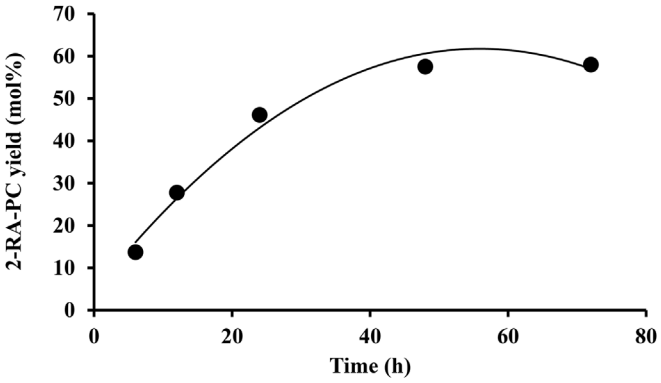

Fig. 4. Effect of reaction time on 2-ricinoleic acid-phosphatidylcholine (2-RA-PC) preparation. Reaction conditions: 0.02 mmol of lysophosphatidylcholine, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of phospholipase A_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 6–72 h.

Table 1
Fatty acid compositions of LPA, RA, and 2-RA-PC (mol%).

	LPC	RA	2-RA-PC*
C16:0	71.7 ± 0.2	1.5 ± 0.1	37.0 ± 0.1
C18:0	24.5 ± 0.3	1.3 ± 0.1	20.2 ± 0.2
C18:1	3.8 ± 0.3	4.2 ± 0.2	5.8 ± 0.1
C18:2	0	5.6 ± 0.2	3.4 ± 0.1
RA	0	83.6 ± 1.6	32.6 ± 0.9
others	0	3.8 ± 0.2	1.0 ± 0.1

*: Reaction conditions: 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 48 h.

Table 2
PC yield from several fatty acids.

Substrate fatty acid	PC yield (mol%)
RA (12-OH-18:1 n-9)	57.5 ± 1.3[a]
18:1 n-9 (Oleic acid)	72.8 ± 3.8[b]
12-OH-18:0	21.7 ± 2.6[c]
3-OH-14:0	13.4 ± 1.0[d]

Reaction conditions: 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 48 h.
[ab]: Different letters are significance with each other ($P < 0.05$).

3.4. Fatty acid compositions of LPC, RA, and 2-RA-PC

Fatty acid compositions of LPC, RA, and 2-RA-PC prepared under optimum condition were shown in Table 1. Theoretically, concentration of RA in 2-RA-PC is to be half value of that of substrate RA because fatty acids should be incorporated into *sn*-2 position of substrate LPC *via* PLA_2 catalyzed esterification. In fact, our previous report revealed that conjugated linoleic acids used as substrate fatty acids are incorporated into *sn*-2 position under the similar reaction condition with PLA_2 (Yamamoto et al., 2006). In the current study, the concentration of RA detected in 2-RA-PC (32.6 mol%) was slightly lower than that of substrate RA (83.6/2 = 41.8 mol%). This result indicated that (I): almost all RA was incorporated into *sn*-2 position of LPC, and (II): RA was not so suitable substrate for PLA_2-catalyzed esterification than the other fatty acids such as stearic acid (C18:0), C18:1n-9 and C18:2n-6. In the view of substrate specificity for hydroxyl fatty acids of PLA_2 was further described in section 3.5.

3.5. Effect of structure of substrate hydroxyl fatty acids

In the optimum reaction conditions, several hydroxyl fatty acids such as 12-OH-18:0 and 3-OH-14:0 as well as RA, and 18:1 n-9 (oleic acid) were used as substrate for PC preparation. Highest yield was obtained with 18:1 n-9 (72.8 ± 3.8 mol%) followed by RA (57.5 ± 1.3 mol%), 12-OH-18:0 (21.7 ± 2.6 mol%), and 3-OH-14:0 (13.4 ± 1.0 mol%), respectively (Table 2). These results suggest that hydroxyl group in fatty acid chain inhibits PC synthesis by PLA_2 by comparing with substrate RA and C18:1 n-9. In addition, unsaturated hydroxyl fatty acid will be better substrate than that of saturated hydroxyl fatty acid by comparing with substrate RA and 12-OH-18:0. Further, from low yields of PC synthesis with substrate 12-OH-18:0 and 3-OH-14:0, position of hydroxyl group might be also important for substrate specificity of ester synthesis by PLA_2.

It is known that PLA_2 from porcine pancreas has preference of short/middle chain fatty acid to long/unsaturated fatty acid for its substrate of esterification reaction (Mingarro et al., 1994). In addition, 10*t*, 12*c* configuration of conjugated linoleic acid is the best substrate among the other isomers of conjugated linoleic acid in similar reaction condition of the current study (Yamamoto et al., 2006). Preference of PLA_2 on the position of hydroxyl group in fatty acid structure was first revealed in this study.

3.6. Anti-inflammatory effect of 2-RA-PC

Anti-inflammatory effects of 2-RA-PC were investigated by using macrophage-like RAW264.7 cells stimulated by LPS. Excessive mRNA expression of pro-inflammatory cytokines such as IL-6 and IL-1β induced by LPS was down-regulated by the treatment with 2-RA-PC and RA (Fig. 5A and B). Comparing the expression of IL-1β mRNA expression, the significant down-regulation was observed only by 2-RA-PC ($P < 0.01$), but not RA and Soy-PC (Fig. 5B). This result shows that anti-inflammatory effect of RA is enhanced by phosphatidylation.

Physiological functions of several bioactive compounds are often improved through phosphatidylation (Shuto et al., 1988; Takami and Suzuki, 1994; Yamamoto et al., 2008). For example, cytotoxicity of phosphatidylated genipin was found to be higher against several cancer cell lines than that of genipin (Takami and Suzuki, 1994). Phosphatidylated terpenes, such as geraniol and farnesol, showed stronger antiproliferative effects than their non-phosphatidylated counterparts (Yamamoto et al., 2008). These reports suggest that enhancement of physiological functions of phosphatidylated compounds are depend on the increase in cellular intake by the amphiphilic property of phospholipids. The same mechanism may explain the improved anti-inflammatory effect of 2-RA-PC after phosphatidylation of RA.

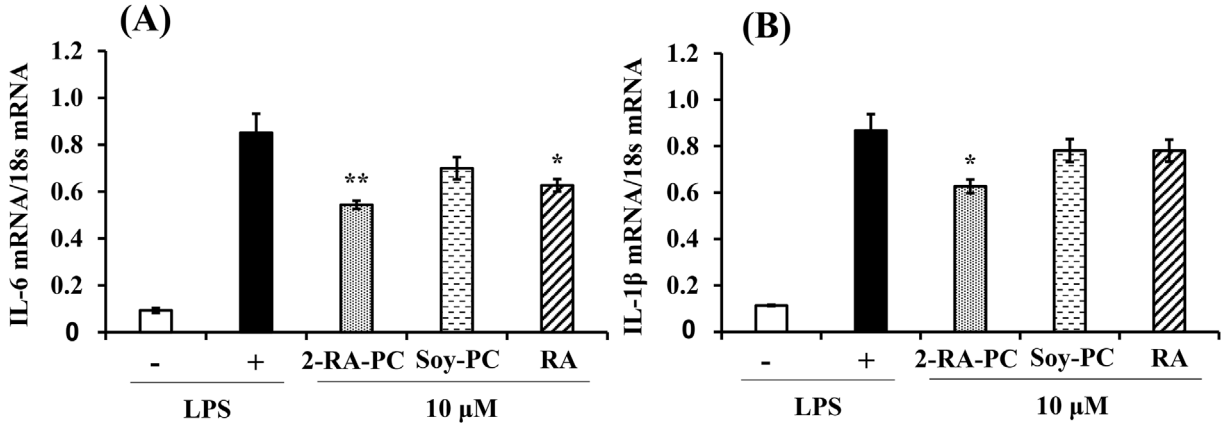

Fig. 5. Down-regulation of interleukin 6 and 1β mRNA expression in lypopolysccharide-stimulated RAW264.7 cells by 2-ricinoleic acid-phosphatidylcholine (2-RA-PC).

4. Conclusion

2-RA-PC was successfully prepared with optimal reaction mixture: 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$, for a reaction time of 48 h. This resulted in a yield of 57.5 mol%. The anti-inflammatory effect of 2-RA-PC was found to be higher than that of free RA and Soy-PC, suggesting that 2-RA-PC is a potential anti-inflammatory lipid.

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