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Regulation of Lipoxygenase Activity by Polyunsaturated Lysophosphatidylcholines or Their Oxygenation Derivatives

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Lysophosphatidylcholines (lysoPCs) have been known to play a role as lipid mediators in various cellular responses. In this study, we examined whether lysoPC containing linoleoyl, arachidonoyl, or docosahexaenoyl groups or their peroxy derivatives affect lipoxygenase (LOX)-catalyzed oxygenation of native substrates. First, arachidonoyl lysoPC and docosahexaenoyl lysoPC were found to inhibit potato 5-LOX-catalyzed oxygenation of linoleic acid (LA) in a noncompetitive type with K_i values of 0.38 and 1.90 μM, respectively. Likewise, arachidonoyl lysoPC and docosahexaenoyl lysoPC also inhibited 5-LOX activity from rat basophilic leukemia cells-2H3 (RBL-2H3) with IC_{50} values (50% inhibitory concentration) of 18.5 $\pm$ 3.06 and 30.6 $\pm$ 1.06 μM, respectively. Additionally, arachidonoyl lysoPC and docosahexaenoyl lysoPC also inhibited 15-LOX activity from RBL-2H3 with IC_{50} values of 16.6 $\pm$ 1.3 and 24.1 $\pm$ 2.4 μM, respectively. In a separate experiment, where lysoPC peroxides were tested for the effect on soybean LOX-1, 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoyl lysoPC and 17(*S*)-hydroperoxy-4,7,10,13,15,19-docosahexaenoyl lysoPC potently inhibited soybean LOX-1 activity with K_i values of 6.8 and of 1.54 μM, respectively. In contrast, 13(*S*)-hydroperoxy-9,11-octadecadienoyl lysoPC was observed to stimulate soybean LOX-1-catalyzed oxygenation of LA markedly with AC_{50} value (50% activatory concentration) of 1.5 μM. Taken together, it is proposed that lysoPCs containing polyunsaturated acyl groups or their peroxy derivatives may participate in the regulation of LOX activity in biological systems.

KEYWORDS: Lipoxygenase; potato; soybean; RBL; arachidonoyl lysoPC; docosahexaenoyl lysoPC; hydroperoxy lysoPC

INTRODUCTION

Lipoxygenase (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) belongs to a diverse family of nonheme ferroproteins catalyzing the regio- and stereospecific oxygenation of free polyunsaturated fatty acids (*1, 2*). LOXs are designated as 5-, 12-, and 15-LOX on the basis of their ability to oxygenate arachidonic acid (AA) at carbons 5, 12, and 15, respectively, and they contribute to the formation of biologically active substances from polyunsaturated fatty acids (*2, 3*). It is well-known that in plant system, LOXs catalyze oxygenation of linoleic acid (LA) and linolenic acid to generate cellular regulators, such as jasmonic acid, traumatic acid, and alkenals, responsible for the growth regulation and wound healing (*4, 5*). In animals, LOXs are known to convert AA to lipid mediators, such as leukotrienes or lipoxins (*6, 7*), which are implicated in various inflammatory diseases such as asthma, rheumatoid arthritis, or inflammatory bowel disease (*8, 9*). Because LOX metabolites of polyunsaturated fatty acids are involved in cellular response under normal or disease conditions (*8*−*11*), the regulation of LOX-catalyzed oxygenation of polyunsaturated fatty acids may result in the alteration of a pathophysiological response in plants and animals.

Recently, lysophosphatidylcholine (lysoPC) has been proposed to play a role as a lipid mediator in some cellular responses (*12*). However, it is not clear whether the polyunsaturated acyl group in the structure of lysoPC is required for the cellular function. Nonetheless, lysoPCs with polyunsaturated acyl group are known to be abundant in plant and animal sources. For example, linoleoyl lysoPC was observed to be a major lysoPC in soybean extract (*13*), and arachidonoyl lysoPC was found to exist in egg white to a substantial level (*14, 15*). In addition, docosahexaenoyl lysoPC was one of major lipid components in shark liver extract (*16*). Furthermore, there has been evidence for the existence of oxidized derivatives of linoleoy lysoPC in the lipid fraction of heart extract, plasma, or soybean, suggesting that polyunsaturated lysoPCs may be oxygenated by cellular LOXs (*13, 17, 18*). Presumably in support of this, our recent studies demonstrated that polyunsaturated lysoPCs containing linoleoyl, arachidonoyl, or docosa-

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hexaenoyl groups were efficiently oxygenated by reticulocyte LOX, leukocyte LOX, or soybean LOX-1 (*19*−*21*). In this regard, it is supposed that lysoPCs with a polyunsaturated acyl group can affect the oxygenation of endogenous substrates by some LOXs in cells. Earlier studies demonstrated that phosphatidylcholines with a monodocosahexaenoyl group at C2 inhibited 5-LOX activity in rat basophilic leukemia (RBL) cells (*22*). Separately, 13-hydroperxyoctadecadienoic acid (13-HPODE) or phosphatidylcholine with monohydroperxyoctadecadienoyl group was found to stimulate soybean LOX-1 activity (*23, 24*). However, there has been no report on the effect of polyunsaturated lysoPCs on LOX activities.

In this regard, we examined the effect of lysoPCs containing polyunsaturated acyl groups and their oxygenated products on the oxygenation of native substrates by LOXs from plant or animal sources. Here, it is proposed that the role of polyunsaturated lysoPCs or their oxidized products differs according to the type of lysoPC or LOX. Arachidonoyl lysoPC and docosahexaenoyl lysoPC are potent inhibitors of potato 5-LOX, and their hydroperoxy derivatives are potent inhibitor of soybean LOX-1. In contrast, the hydroperoxy form of linoleoyl lysoPC is a strong stimulator of soybean LOX-1 activity.

MATERIALS AND METHODS

Materials. Dilinoleoyl phosphatidylcholine (DLPC), diarachidonoyl phosphatidylcholine, and didocosahexaenoyl phosphatidylcholine (purity, 99%) were from Avanti Polar Lipid (Alabaster, AL). Soybean LOX-1 (type I-B), phospholipase A_2 (PLA_2) (honey bee venom), and Tween 20 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). LA, AA, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 15-HETE (purity, 99%), and potato 5-LOX (14772 units/mg protein, >98%) were obtained from Cayman Chemical Co. (Ann Arbor, MI). High-performance liquid chromatography (HPLC) solvents were of HPLC grade, and other chemicals were of analytical grade. 1-Linoleoyl lysoPC, 1-arachidonoyl lysoPC, and 1-docosahexaenoyl lysoPC were prepared from PLA_2-catalyzed hydrolysis of corresponding diacyl phosphatidylcholines as described previously (*19*−*21, 25*). 13(*S*)-Hydroperoxy-9,11-octadecadienoic acid (13-HPODE, >98%) and 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE, >98%) were prepared as described before (*26*). Separately, the 13(*S*)-hydroperoxy-9,11-octadecadienoyl lysoPC (13-HPODE lysoPC), 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoyl lysoPC (15-HPETE lysoPC), or 17(*S*)-hydroperoxy-4,7,10,13,15,19-docosahexaenoyl lysoPC (17-HPDHA lysoPC) was prepared as described before (*27*); briefly, soybean LOX-1 (200 units/mL) was incubated with each lysoPC (20 μM) in borax buffer (50 mM, pH 9.0) at 25 °C for 30 min. Subsequently, the mixture was passed through a C_{18} column (2 cm × 1 cm), and the products were eluted with methanol. Finally, the eluate was concentrated under N_2 gas.

Determination of LOX Activities by UV Method and Lineweaver−Burk Plot Analysis of Inhibition of LOX Activities by Polyunsaturated LysoPCs or Oxidized LysoPCs. The LOX activity was determined by measuring the increase in absorbance at 234 nm due to the formation of conjugated dienes ($\varepsilon_{234} = 25000\ M^{-1}\ cm^{-1}$) at 25 °C as described before (*28, 29*). One unit was defined as the amount of LOX to generate 1 nmol of conjugated diene per min. Potato 5-LOX (4.5 units/mL) was incubated with LA (20−100 μM) in the presence of archidonoyl lysoPC or docosahexaenoyl lysoPC in Tris-HCl buffer (50 mM, pH 6.3) containing 0.02% Tween 20 at 25 °C for 1 min. Separately, soybean LOX-1 (5 units/mL) was incubated with LA (0−40 μM) in the presence of 15-HPETE lysoPC or 17-HPDHA lysoPC (0−5 μM) in borax buffer (50 mM, pH 9.0) at 25 °C for 1 min. The remaining LOX activity was determined by measuring the absorbance at 234 nm, and Lineweaver−Burk plot analyses were done as described before (*27*).

Determination of LOX Activity by FOX Assay. The FOX assay was carried out according to a slight modification of the procedure reported previously (*30*). Briefly, the oxygenation reaction was terminated by the addition of FOX reagent mixture (5 volumes), sulfuric acid (25 mM), xylenol orange (100 μM), and iron(II) sulfate (100 μM) in methanol:water (9:1). After 4 min of reaction, the absorbance at 575 nm, due to the formation of Fe^{3+} complex, was measured using a microplate reader (BIO-RAD model 550). For the blank, each substrate was incubated with lysoPC or oxidized lysoPC for the designated time, and then, to the mixture was added FOX reagent, followed by LOX. A standard curve of peroxide was prepared using H_2O_2 as standard peroxide.

LC/MS Determination of Inhibitory Effect of Polyunsaturated LysoPC on RBL-2H3 LOX. Rat basophilic leukemia cells (RBL-2H3, American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% ($v\ v^{-1}$) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY) at 37 °C in 5% CO_2 (*31, 32*). The RBL-2H3 cells, suspended in Tris-HCl buffer (50 mM, pH 7.4), were sonicated at 0 °C for 5 min and then subjected to centrifugation (10000*g*, 30 min) at 4 °C to obtain cell extract (9.1 ± 1.2 mg protein/mL). Finally, the extract (50 μL) was incubated with AA in the presence of arachidonoyl lysoPC or docosahexaenoyl lysoPC (0−50 μM) in Tris-HCl buffer (50 mM, pH 7.4) containing 2.5 mM $CaCl_2$ and 2.5 mM ATP at 37 °C for 15 min. The reaction was terminated by including 100 μL of solvent mixture (acetonitrile: methanol:acetic acid; 145:55:0.1) in the above incubation mixture (*32*), and the final mixture, after centrifugation (10000*g*, 30 min), was subjected to LC/ESI-MS analysis, which was performed using a MSD spectrometer (HP 1100 series LC/MSD, Hewlett-Packard, United States) with a ZORBAX Eclipse XDB C_{18} column (5 μm, 50 mm × 4.6 mm, Agilent Technologies, United States) and precolumn (20 mm × 3 mm, 3.5 μm, Waters, Ireland). The mobile phase (methanol: H_2O:formic acid = 250:50:0.3) was delivered at 0.3 mL/min. 5-HETE or 15-HETE was identified in comparison with each standard HETE and quantified by measuring the intensity at *m/z* 319 with ESI-MS system employing negative selected ion monitoring mode.

RP-HPLC Analysis of Regulation of Soybean LOX-1 Activity by 13-HPODE LysoPC or 15-HPETE LysoPC. Soybean LOX-1 (4 units/mL) was incubated with LA (100 μM) in the presence of 13-HPODE lysoPC or 15-HPETE lysoPC (2 μM) in borax buffer (50 mM, pH 9.0) at 25 °C, and the change of LOX activity was monitored at 234 nm for 30 min. Separately, after 30 min of oxygenation, an aliquot (100 μL) of the reaction mixture was taken and mixed with cold methanol (100 μL) containing 0.5 μg of 15-HETE as a standard. Then, the final mixture was injected into RP-HPLC system (Hitachi L-7100 pump, Japan), equipped with ZORBAX Eclipse XDB C_{18} column (5 μm, 50 mm × 4.6 mm, Agilent Technologies) containing a C_{18} precolumn (3.5 μm, 20 mm × 3 mm, Waters, Ireland), which was eluted with mobile phase (methanol/H_2O/formic acid; 70:30:0.1). The flow rate was 0.5 mL/min, and the elution was monitored at 234 nm.

Activation of Soybean LOX-1 Activity by 13(*S*)-Hydroperoxy-9,11-octadecadienoic Acid or 13-HPODE LysoPC. Soybean LOX-1 (4 units/mL) was incubated with LA (100 μM) in the presence of 13-HPODE lysoPC or 13(*S*)-hydroperoxy-9,11-octadecadienoic acid in borax buffer (50 mM, pH 9.0) at 25 °C for 10 min, and the remaining LOX activity was determined by measuring the increase of absorbance at 234 nm. Separately, the concentration-dependent effect of 13(*S*)-hydroperoxy-9,11-octadecadienoic acid or 13-HPODE lysoPC was examined by varying the concentration of each lipid peroxide (0−10 μM). The activation fold was calculated by comparing the LOX activity (up to 1 min) in the presence of activator with that in the absence of lipid activator.

Statistical Analysis. Results were expressed as means ± SD. Statistical significance was evaluated using Student's *t* test, and $p < 0.05$ was considered statistically significant.

RESULTS

Recent studies have shown that polyunsaturated lysoPCs are alternative substrates of LOXs such as reticulocyte 15-LOX, soybean LOX-1, and leukocyte 12-LOX (*19*−*21*). However, the rate for the oxygenation of polyunsaturated lysoPCs by LOX seemed to differ greatly according to the oxygenation time. From

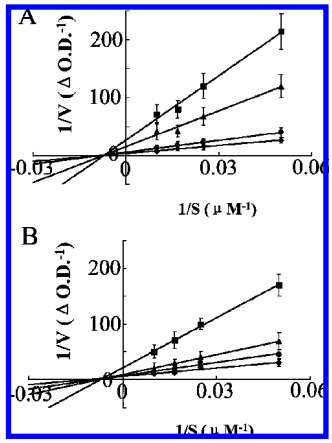

Figure 1. Inhibitory effect of arachidonoyl lysoPC or docosahexaenoyl lysoPC on potato 5-LOX activity. Potato 5-LOX (4.5 units/mL) was incubated with LA (20−100 μM) in the presence of inhibitors in Tris-HCl (50 mM, pH 6.3) containing 0.02% Tween 20 at 25 °C. (**A**) Arachidonoyl lysoPC (◆, 0 μM; ●, 0.3 μM; ▲, 0.9 μM; and ■, 2.7 μM). (**B**) Docosahexaenoyl lysoPC (◆, 0 μM; ●, 1.5 μM; ▲, 4.5 μM; and ■, 9.2 μM). The remaining LOX activity was determined by measuring the absorbance at 234 nm. Results are expressed as a mean ± SD value of triplicate experiments.

Table 1. K_i Values of LysoPC Derivatives in Inhibition of Potato 5-LOX or Soybean LOX-1 Activity[a]

	inhibitors	K_i (μM)
potato 5-LOX	linoleoyl lysoPC	>100
	arachidonoyl lysoPC	0.38 ± 0.10
	docosahexaenoyl lysoPC	1.90 ± 0.5
soybean LOX-1	15-HPETE	26.57 ± 5.57
	15-HPETE lysoPC	6.80 ± 0.76
	17-HPDHA lysoPC	1.54 ± 0.24

[a] The inhibitory effect of each lysoPC derivative was determined as described in **Figures 1** and **4**, and the K_i values were obtained by Lineweaver−Burk plot analysis. Results are expressed as a mean ± SD value of triplicate experiments.

this, it was supposed that oxygenation products of lysoPCs might affect the oxygenation of substrate by LOXs. In an attempt to explain this phenomenon, we examined the effect of lysoPCs or peroxy lysoPC derivatives on LOX activity.

Inhibition of Potato 5-LOX by Arachidonoyl LysoPC or Docosahexaenoyl LysoPC. First, potato 5-LOX was incubated with LA in the presence of each lysoPC in Tris-HCl buffer (50 mM, pH 6.3) containing 0.02% Tween 20, and the remaining activity of LOX was determined by UV method. When arachidonoyl lysoPC was tested for the inhibition of potato 5-LOX-catalyzed oxygenation of LA, it was found to inhibit potato 5-LOX activity strongly. The double-reciprocal plot analysis indicated that arachidonoyl lysoPC and docosahexaenoyl lysoPC inhibited potato 5-LOX, displaying a quasi-noncompetitive type of inhibition (**Figure 1**). In contrast, linoleoyl lysoPC had no remarkable inhibition up to 100 μM. In comparison (**Table 1**), the most potent in inhibiting potato 5-LOX activity was arachidonoyl lysoPC (K_i, 0.38 μM), followed by docosahexaenoyl lysoPC (K_i, 1.90 μM) and linoleoyl lysoPC (K_i, >100 μM), suggesting that the number of double bonds in acyl chain may determine the inhibitory action of lysoPC.

Inhibitory Effect of Arachidonoyl LysoPC or Docosahexaenoyl LysoPC on LOXs from RBL-2H3 Cells. Subsequently, we examined the effect of these polyunsaturated lysoPCs on the activity of 5-LOX or 15-LOX from RBL-2H3 cells. Because the LOX activity in RBL-2H3 cell extract was too low to be detected by UV method, liquid chromatography/mass spectrometry (LC/MS) analysis was used to determine the amount of hydroxyeicosatetraenoic acid (HETE) generated from oxygenation of AA by LOXs from RBL-2H3 cells. First, when the effect of arachidonoyl lysoPC on the formation of 5-HETE, 5-lipoxygenation product, was assessed, arachidonoyl lysoPC was found to cause a dose-dependent inhibition of 5-HETE formation with an IC_{50} value of approximately 18.5 μM (**Figure 2A**, open bar); a significant decline of 5-HETE was observed when 5-LOX was exposed to arachidonoyl lysoPC at ≥10 μM ($p < 0.05$). In addition, docosahexaenoyl lysoPC was also observed to inhibit 5-LOX activity from RBL-2H3 cell in a dose-dependent mode with an IC_{50} value of 30.6 μM (**Figure**

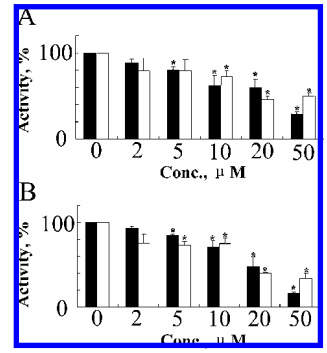

Figure 2. Inhibitory effect of arachidonoyl lysoPC or docosahexaenoyl lysoPC on LOX activities from RBL-2H3 cells. RBL-2H3 cell extract (0.5 mg of protein) was incubated with AA (50 μM) in the presence of arachidonoyl lysoPC (2−50 μM, open bar) or docosahexaenoyl lysoPC (2−50 μM, filled bar). The formation of 5-HETE (**A**) or 15-HETE (**B**) was determined by LC/MS analyses as described in the Materials and Methods. *$P < 0.05$, significantly different from control (without lysoPC). Results are expressed as a mean ± SD value of triplicate experiments.

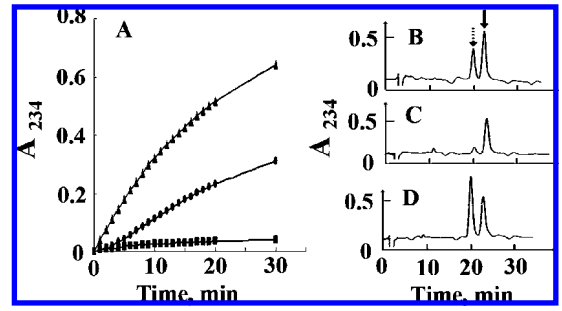

Figure 3. Effect of 13-HPODE lysoPC or 15-HPETE lysoPC on soybean LOX-1-induced oxygenation of LA. Soybean LOX-1 (4 units/mL) was incubated with LA (100 μM) in the presence or absence of 13-HPODE lysoPC (2 μM) or 15-HPETE lysoPC (2 μM) in borax buffer (50 mM, pH 9.0) at 25°C. (**A**) Time course of soybean LOX-1-catalyzed oxygenation of LA in the presence of 13-HPODE lysoPC or 15-HPETE lysoPC. ●, Control (without lysoPC); ▲, 13-HPODE lysoPC (2 μM);and ■, 15-HPETE lysoPC (2 μM). (**B**−**D**) RP-HPLC chromatogram of products from soybean LOX-1-catalyzed oxygenation of LA in the presence of 13-HPODE lysoPC or 15-HPETE lysoPC. After 30 min of incubation, an aliquot (100 μL) of the reaction mixture was taken and mixed with cold methanol (100 μL) containing 0.5 μg of 15-HETE as a standard, and the mixture was subjected to RP-HPLC analysis (flow rate, 0.5 mL/min; UV detection, 234 nm). (**B**) Products from incubation of LOX with LA alone; (**C**) products from incubation of LOX with LA in the presence of 15-HPETE lysoPC (2 μM); and (**D**) products from incubation of LOX with LA in the presence of 13-HPODE lysoPC (2 μM). The compound from each peak was identified by coinjection with 15-HETE standard (solid arrow) or 13-HPODE standard (dotted arrow).

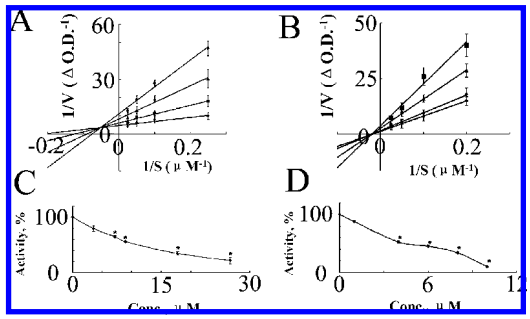

Figure 4. Inhibitory effect of 15-HPETE lysoPC and 17-HPDHA lysoPC on soybean LOX-1 activity. Soybean LOX-1 (5 units/mL) was incubated with LA (4−40 μM) in the presence of inhibitors in borax buffer (50 mM, pH 9.0) at 25 °C. (**A**) 15-HPETE lysoPC (◆, 0 μM; ●, 0.3 μM; ▲, 1 μM; and ■, 3 μM). (**B**)17-HPDHA lysoPC (◆, 0 μM; ●, 0.4 μM; ▲, 1.3 μM; and ■, 4 μM). Separately, soybean LOX-1 (10 units/mL) was incubated with 100 μM LA in the presence of 15-HPETE lysoPC (**C**) at 0−27 μM or 17-HPDHA lysoPC (**D**) at 0−10 μM in borax buffer (50 mM, pH 9.0) at 25 °C. The formation of 13-HPODE was determined by FOX assay as described in the Materials and Methods. *P < 0.05, significantly different from control (no inhibitor). Results are expressed as a mean ± SD value of triplicate experiments.

2A, filled bar); a remarkable reduction of 5-HETE was expressed by docosahexaenoyl lysoPC at ≥ 5 μM (p < 0.05).

Next, the effect of lysoPC on the formation of 15-HETE, a product from 15-LOX-catalyzed oxygenation of AA, was assessed. As demonstrated in **Figure 2B**, arachidonoyl lysoPC and docosahexaenoyl lysoPC inhibited 15-LOX activity in RBL-2H3 cell extract in a dose-dependent mode. When the IC_{50} value was determined, it was found to be 16.6 μM for arachidonoyl lysoPC and 24.1 μM for docosahexaenoyl lysoPC. In contrast, linoleoyl lysoPC at 100 μM showed no remarkable inhibitory effect on either 5-LOX activity or 15-LOX activity in RBL cells. Thus, it was found that arachidonoyl lysoPC and docosahexaenoyl lysoPC inhibited 5- LOX as well as 15-LOX in RBL cells.

Inhibition of Soybean LOX-1 by 15-HPETE LysoPC or 17-HPDHA LysoPC. After establishing the regulation of LOXs activity by lysoPCs, we wanted to see whether oxidized lysoPCs can affect the oxygenation of native substrates by soybean LOX-1. For this purpose, soybean LOX-1 was incubated with LA in the presence of 15-HPETE lysoPC or 17-HPDHA lysoPC in borax buffer (50 mM, pH 9.0), and the remaining LOX activity was determined by UV spectrometry and RP-HPLC analyses. As shown in **Figure 3A**, the initial rate (up to 1 min) for oxygenation of LA by soybean LOX-1 decreased to <20% of control level in the presence of 15-HPETE lysoPC (2 μM), indicating that 15-HPETE lysoPC inhibited soybean LOX-1 activity potently. Separately, in an attempt to reaffirm the inhibitory effect of 15-HPETE lysoPC on soybean LOX-1, the inhibitory action of 15-HPETE lysoPC was evaluated by RP-HPLC analysis. As demonstrated in **Figure 3**, as compared to control (**Figure 3B**), the inclusion of 15-HPETE lysoPC (2 μM) inhibited the soybean LOX-1-catalyzed formation of 13-HPODE by approximately 82% (**Figure 3C**), close to the inhibition level observed with UV spectrometry assay (**Figure 3A**). In addition, 17-HPDHA lysoPC was also found to display a similar inhibition of soybean LOX-1, based on UV spectrometry and RP-HPLC analysis (data not shown). In a separate experiment, the inhibition of soybean LOX-1 by oxidized lysoPCs was determined by UV spectrometry and assessed kinetically using Lineweaver−Burk plot analysis.

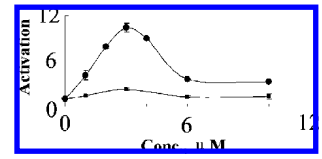

Figure 5. Activatory effect of 13-HPODE lysoPC or 13-HPODE on soybean LOX-1 activity. Soybean LOX-1 (4 units/mL) was incubated with 100 μM LA in the presence of 13-HPODE lysoPC (0−10 μM) or 13-HPODE (0−10 μM) in borax buffer (50 mM, pH 9.0) at 25 °C for 1 min. The activity of soybean LOX-1 was determined by measuring the absorbance at 234 nm. Results are expressed as a mean ± SD value of triplicate experiments (●, 13-HPODE lysoPC; ■, 3-HPODE).

Figure 4A indicated that 15-HPETE lysoPC showed a mixed type inhibition of soybean LOX-1 activity. A similar result was also observed with 17-HPDHA lysoPC (**Figure 4B**). In comparison (**Table 1**), the most potent inhibitor was 17-HPDHA lysoPC (K_i, 1.54 μM), followed by 15-HPETE lysoPC (K_i, 6.80 μM) and 15-HPETE (K_i, 26.57 μM). In an independent experiment, where the inhibitory action of 17-HPDHA lysoPC or 15-HPETE lysoPC was confirmed by FOX assay, 15-HPETE lysoPC and 17-HPDHA lysoPC were found to inhibit soybean LOX-1-catalyzed oxygenation of LA with an IC_{50} value (50% inhibitory concentration) of 14.3 and 5.1 μM, respectively (**Figure 4C**,**D**). In contrast, 15-HETE lysoPC up to 20 μM did not inhibit soybean LOX-1 activity (data not shown).

Activation of Soybean LOX-1 Activity by 13-HPODE LysoPC. In contrast to the inhibitory effect of 15-HPETE lysoPC or 17-HPDHA lysoPC on soybean LOX-1 activity, 13-HPODE lysoPC did not demonstrate any inhibitory effect. Rather, 13-HPODE lysoPC stimulated soybean LOX-1-catalyzed oxygenation of LA remarkably. In related experiment (**Figure 3A**), where the oxygenation of LA by soybean LOX-1 was monitored for 30 min, the activation of soybean LOX-1 activity by 13-HPODE lysoPC (2 μM) differed according to the incubation time (0−30 min); the activation degree (>7-fold) in the early phase (up to 1 min) of oxygenation was greater than that (2.1-fold) in the late phase (20−30 min). In additional experiments to confirm the activatory action of 13-HPODE lysoPC, soybean LOX-1 was incubated with LA in the presence of 13-HPODE lysoPC (2 μM) for 30 min, and the final reaction products were subjected to RP-HPLC analysis. As compared to control (**Figure 3B**), the inclusion of 13-HPODE lysoPC (2 μM) enhanced soybean LOX-1-catalyzed formation of 13-HPODE by 2.3-fold (**Figure 3D**), similar to 2.1-fold activation (30 min of incubation) observed with UV assay (**Figure 3A**). Separately, a similar activation was also found when the LOX activity was determined by FOX assay (data not shown). In a related study, where the concentration-dependent activation of soybean LOX-1 by 13-HPODE lysoPC was further examined (**Figure 5**), the initial rate (up to 1 min) for oxygenation of LA by soybean LOX-1 was augmented by 13-HPODE lysoPC in a concentration-dependent mode up to 3 μM with an AC_{50} value (50% activatory concentration) of 1.5 μM. Moreover, the maximal activation fold was no less than 10-fold. However, the increase of 13-HPODE lysoPC concentration beyond 3 μM tended to decrease the activatory effect, and the activation degree at 6 or 10 μM was limited to approximately 4-fold. Although 13(*S*)-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) also enhanced LOX-1 activity, its maximal activatory effect was less than 2-fold (**Figure 5**). Meanwhile, 13(*S*)-hydroxy-9,11-octadecadienoyl lysoPC (up to 10 μM) did not show any remarkable

stimulation of soybean LOX-1 activity. Separately, 13-HPODE lysoPA was tested for the activation of soybean LOX-1 activity, but its maximal activatory effect was not higher than 1.4-fold (data not shown). Thus, it is supposed that the hydroperoxy group as well as the lysoPC form of 13-HPODE lysoPC may be important for the stimulation of soybean LOX-1 activity.

DISCUSSION

LOX had been known to be regulated, negatively or positively, by peroxy derivatives of unsaturated fatty acids (*33, 34*). Among complex lipids, phosphatidylcholine, containing a docosahexaenoyl acyl group at C2, inhibited 5-LOX activity in RBL cells (*22*), and sphingomyelin inhibited platelet 12-LOX activity (*35*). In contrast, monohydroperoxylinoleoyl phosphatidylcholine (25 μM) stimulated soybean LOX-1-cactalyzed oxygenation of DLPC by 2.4-fold (*23*). Thus, the LOX activity seemed to be regulated by lipids or their peroxides. Our present study provides a positive support for the regulation of LOX activity by polyunsaturated lysoPCs or corresponding oxidized products.

Noteworthy, the K_i value (K_i, 0.4−2 μM) of arachidonoyl lysoPC or docosahexaenoyl lysoPC in potato 5-LOX-catalyzed LA oxygenation was relatively small. Our previous studies indicated that polyunsaturated lysoPC could be utilized as substrates for LOXs, rabbit reticulocyte LOX, porcine leukocyte LOX, and soybean LOX-1 (*19*−*21*). However, arachidonoyl lysoPC and docosahexaenoyl lysoPC were poor substrates for potato 5-LOX (*20, 21*). Therefore, it is supposed that the inhibitory effect of polyunsaturated lysoPCs on potato 5-LOX may be due to the binding of polyunsaturated lysoPCs to a site other than the binding site of fatty acid substrates. A support for this may come from the noncompetitive inhibition of 5-LOX by arachidonoyl lysoPC or docosahexaenoyl lysoPC (**Figure 1**). Furthermore, the present finding, together with previous reports (*22, 35*), indicates that the carboxyl end of substrates may not be crucial for the inhibition of potato 5-LOX by lipids (*36, 37*). Although the inhibitory action of arachidonoyl lysoPC or docosahexaenoyl lysoPC was smaller for RBL 5-LOX than potato 5-LOX, this might be explained by the assumption that the inhibitory action of lysoPCs was suppressed in the presence of proteins in RBL cell extract. Separately, our current data revealed that soybean LOX-1-catalyzed oxygenation of LA was inhibited strongly by 15-HPETE lysoPC or 17-HPDHA lysoPC (**Figure 4**). Noteworthy, the K_i values of 15-HPETE lysoPC (K_i, 6.80 μM) and 17-HPDHA lysoPC (K_i, 1.54 μM) in the inhibition of soybean LOX-1 activity are much smaller than that of 15-HPETE (K_i, 26.6 μM), indicating that 15-HPETE lysoPC and 17-HPDHA lysoPC correspond to potent inhibitors of soybean LOX-1. The noncompetitive or mixed type inhibition suggests that the binding site of lysoPCs may not necessarily coincide with that of fatty acid substrate. This might be supported by the previous finding that a binding site for lipid hydroperoxide is distinct from that for substrates (*2, 4*). A further support may be from an additional report (*38*) that saturated monohydric alcohols inhibited soybean LOX-1 through hydrophobic bonding to a binding site. In this regard, the greater inhibitory action of 15-HPETE lysoPC, as compared to 15-HPETE, may be ascribed to the higher binding affinity of lysoPC form, as compared to acid form, toward soybean LOX-1. This might be in a good agreement with the previous finding that arachidonoyl lysoPC and docosahexaenoyl lysoPC showed smaller K_m values than the corresponding acid substrate (*20, 21*). Alternatively, it is conceivable that the strong inhibitory action of 15-HPETE lysoPC or 17-HPDHA lysoPC may be due to their role as a substrate for further oxygenation. However, this possibility is not supported by the finding that 15-HPETE lysoPC or 17-HPDHA lysoPC is resistant to further oxygenation by soybean LOX-1, in contrast to the further oxygenation of 15-HPETE by soybean LOX-1 (*20, 21*). In addition, it was questioned whether soybean LOX-1 could be inactivated during the incubation of soybean LOX-1 with 15-HPETE lysoPC, as had been suggested from the inactivation of soybean LOX-1 by 15-HPETE (*39*). However, 15-HPETE lysoPC up to 50 μM did not exhibit a significant inactivation of soybean LOX-1 activity during 20 min of preincubation (data not shown).

Meanwhile, 13-HPODE lysoPC (AC_{50}, 1.5 μM), but not 13-HODE lysoPC, was found to be a potent activator of soybean LOX-1. Noteworthy, the activatory action of 13-HPODE lysoPC was greater than that of 13-HPODE in soybean LOX-1-catalyzed LA oxidation. Moreover, such an activatory action of 13-HPODE lysoPC was much greater than that of monohydroperoxylinoleoyl phosphatidylcholine in soybean LOX-1-catalyzed oxidation of DLPC (*23*). Furthermore, the activatory effect seemed to be specific to 13-HPODE lysoPC, since 13-HPODE lysoPA was less activatory than 13-HPODE lysoPC. From these, it is proposed that 13-HPODE lysoPC may be a selective activator of soybean LOX-1. Although the mechanism for the activatory action of 13-HPODE lysoPC was not elucidated, it might be related primarily to the direct effect of the peroxide group. Earlier, it was reported that free fatty acid peroxides regulated soybean LOX-1 activity via removal of the kinetic lag period (*2, 4*). Likewise, it is conceivable that one mechanism for activation by 13-HPODE lysoPC may be related to removal of the kinetic lag period (*24*). Taken together, it appeared that the role of lysoPCs, inhibitory or activatory, might be determined by the type of lysoPC or LOX.

Concerning the presence of lysoPC in vivo, it had been reported that polyunsaturated lysoPCs such as linoleoyl lysoPC or arachidonoyl lysoPC existed in plasma and egg white to a substantial level (*25, 26, 40*). In addition, docosahexaenoyl lysoPC was one of the major lipid components in shark liver extract (*16*). From this, it is possible to surmise that arachidonoyl lysoPC or docosahexaenoyl lysoPC may participate in the negative regulation of LOX activity in vivo, at least in animal systems. Although the formation of lysoPC peroxides in vivo system was not examined here, linoleoyl lysoPC, arachidonoyl lysoPC, and docosahexaenoyl lysoPC were observed to be readily oxygenated by 12-LOX or 15-LOX (*19*−*21*). Moreover, the existence of 13-HODE lysoPC in soybean protein extract or human alveolar fluid (*13, 17*) may imply that 13-HODE lysoPC is generated from LOX-catalyzed oxygenation of linoleoyl lysoPC via 13-HPODE lysoPC, a peroxide precursor of 13-HODE lysoPC (*19*). In this respect, it is possible that LOX-catalyzed oxygenation of LA in plant or animal sources may be regulated by lysoPC peroxides.

The present availability of oxygenated derivatives of polyunsaturated lysophopsholipids may contribute to further elucidation of in vivo regulation of LOX activity by lysophospholipids or their oxidized products, which need be clarified in future studies using plant or animal cells.

ABBREVIATIONS USED

LysoPC, lysophosphatidylcholine; LOX, lipoxygenase; AC_{50}, 50% activatory concentration; IC_{50}, 50% inhibitory concentration; PLA_2, phospholipase A_2; LA, linoleic acid; AA, arachidonic acid; DLPC, dilinoleoyl phosphatidylcholine; 13-H(P)ODE, 13(*S*)-hydro(pero)xy-9,11-octadecadienoic acid; 15-H(P)ETE, 15(*S*)-hydro(pero)xy-5,8,11,13-eicosatetraenoic acid; 13-HPODE lysoPC, 13(*S*)-hydroperoxy-9,11-octadecadienoyl lysoPC; 15-HPETE lysoPC, 15(*S*)-hydroperoxy-5,8,11,13-eicosatetraenoyl lysoPC; 17-HPDHA lysoPC, 17(*S*)-hydroperoxy-4,7,10,13,15,19- docosahexaenoyl lysoPC.

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