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Oxygenation of Arachidonoyl Lysophospholipids by Lipoxygenases from Soybean, Porcine Leukocyte, or Rabbit Reticulocyte

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Oxygenation of arachidonoyl lysophosphatidylcholine (lysoPC) or arachidonoyl lysophosphatidic acid (lysoPA) by lipoxygenase (LOX) was examined. The oxidized products were identified by HPLC/UV spectrophotometry/mass spectrometry analyses. Straight-phase and chiral-phase HPLC analyses indicated that soybean LOX-1 and rabbit reticulocyte LOX oxygenated arachidonoyl lysophospholipids mainly at C-15 with the *S* form as major enantiomer, whereas porcine leukocyte LOX oxygenated at C-12 with the *S* form. Next, the sequential exposure of arachidonoyl-lysoPC to soybean LOX-1 and porcine leukocyte LOX afforded two major isomers of dihydroxy derivatives with conjugated triene structure, suggesting that 15(*S*)-hydroperoxyeicosatetraenoyl derivatives were converted to 8,15(*S*)-dihydroxyeicosatetraenoyl derivatives. Separately, arachidonoyl-lysoPA, but not arachidonoyl-lysoPC, was found to be susceptible to double oxygenation by soybean LOX-1 to generate a dihydroperoxyeicosatetraenoyl derivative. Overall, arachidonoyl lysophospholipids were more efficient than arachidonic acid as LOX substrate. Moreover, the catalytic efficiency of arachidonoyl-lysoPC as substrate of three lipoxygenases was much greater than that of arachidonoyl-lysoPA or arachidonic acid. Taken together, it is proposed that arachidonoyl-lysoPC or arachidonoyl-lysoPA is efficiently oxygenated by plant or animal lipoxygenases, C12- or C15-specific, to generate oxidized products with conjugated diene or triene structure.

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12), belonging to a diverse family of nonheme ferroproteins, catalyzes the regio- and stereospecific oxygenation of free polyunsaturated fatty acids, containing the (*Z*,*Z*)-1,4-pentadiene moiety (*1–3*). On the basis of their ability to oxygenate arachidonic acid at carbons 5, 12, and 15, the lipoxygenases (LOX) are designated 5-, 12-, and 15-LOX, respectively (*2–5*). Previously, 15-hydroperoxyeicosatetraenoic acid (15-HPETE), derived from oxygenation of arachidonic acid by 15-LOX, was found to be further converted to ketols in coral (*6*) and dihydroxyeicosatetraenoic acids in leukocyte (*4, 7*). Additionally, 14,15-hepoxilins of the A_3 and B_3 series were generated from arachidonic acid through 15-LOX and hydroperoxide isomerase activities present in garlic roots (*8*). Thus, arachidonic acid has been demonstrated to be converted to various oxidized compounds via 15-HPETE in plant and animal sources. Although free polyunsaturated fatty acids have been used as preferential substrates for LOXs (*3–5, 9*), phospholipids or fat can be also oxygenated by certain plant LOX isoenzymes (*10–12*). Some mammalian LOXs such as reticulocyte LOX or leukocyte-type LOX also oxygenate complex substrates such as phospholipids or biomembranes (*13−22*). Additionally, endothelial cell LOX can also oxidize phospholipids in LDL, where phospholipids exist as solubilized state (*23*). Separately, some oxidation products of unsaturated fatty acids, such as oxylipins, were observed to exist predominantly in esterified forms in plants (*24*). Besides, the lipoxygenase-catalyzed conversion of *N*-linoleoylethanolamine to 13-hydroxyoctadecanoylethanolamine was observed in cottonseed (*25*). Furthermore, linoleoyl-lysophosphatidylcholine (lysoPC) and linoleoyl-lysophosphatidic acid (lysoPA) have been found to be readily transformed to 13-hydroperoxy derivatives in the presence of C15-specific soybean LOX-1 (*26, 27*). This might be relevant to the previous observation that hydroxylinoleoyl-lysoPC and hydroxylinoleoyl-lysoPA were present in soy protein isolate (*28*).

From these, it is supposed that 15-hydroperoxyeicosatetraenoyl lysophospholipids, as possible precursors of multiply oxidized products, can be generated from C15-specific lipoxygenase-catalyzed oxygenation of arachidonoyl-lysophospholipids such as achidonoyl-lysoPC and arachidonoyl-lysoPA, which had been reported to exist in egg white (*29, 30*). As a precursor for further metabolism, 15- hydroperoxyeicosatetraenoyl lysophos-

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pholipids might be preferred to 13-hydroperoxyoctadecanoyl lysophospholipids, because 15-hydroperoxyeicosatetraenoyl group, but not 13-hydroperoxyoctadecanoyl group, could be subjected to further conversions as had been observed in the conversion of 15-hydroperoxyeicosatetraenoic acid (HPETE) to physiologically active products such as ketols (*6*), hepoxilin isomers (*8*), or dihydroxyeicosatetraenic acids (*4, 7*). In this regard, it is intriguing to see whether arachidonoyl lysophospholipids can be oxygenated by LOXs to generate multiply oxidized products in addition to hydroperoxyeicosatetraenoyl lysophospholipids. For this purpose, C15-specific LOX may be more favorable than C5-specifc LOX, because the former activity is known to be implicated in the oxygenation of phospholipids or fat (*10–13*).

In this study, the oxygenation of arachidonoyl-lysoPC or 1-arachidonoyl-lysoPA by soybean LOX-1, reticulocyte LOX, or leukocyte-type LOX was investigated with respect to substrate efficiency and selectivity. Additionally, the possible formation of multiply oxidized products of lysophospholipids was examined.

MATERIALS AND METHODS

Materials. Diarachidonoyl phosphatidylcholine (99%) and 1-arachidonoyl lysophosphatidic acid (lysoPA) (99%) were from Avanti Polar Lipid (Alabaster, AL). Soybean lipoxygenase (Tgype I-B), phospholipase A_2 (honey bee venom), 15-hydroxyeicosatetraenoic acid (HETE), 12-HETE, and Tween 20 were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Rabbit reticulocyte 15-lipoxygenase (668.4 units/mg of protein) was obtained from Biomol Inc. (Plymouth Meeting, PA). Leukocyte 12-lipoxygenase (porcine leukocyte, 51.8 units/mg of protein), potato 5-lipoxygenase (21.9 units/mg of protein), and other HETE standards were from Cayman Chemical Co. (Ann Arbor, MI). HPLC solvents were of HPLC grade, and other chemicals were of analytical grade. Arachidonoyl lysophosphatidylcholine (lysoPC) was prepared from PLA_2-catalyzed hydrolysis of diarachidonoyl phosphatidylcholine as described previously with a slight modification (*11, 26*). 15-Hydroperoxyeicosatetraenoic acid (15-HPETE) was prepared from the incubation of arachidonic acid with soybean LOX-1 as described before (*31*). Separately, 8(*S*),15(*S*)-dihydroxyeicosatetra-5*Z*,9*E*,11*Z*,13*E*-enoic acid was prepared from the incubation of 15(*S*)-HPETE with soybean LOX-1 as described previously (*32*).

Assay of LOX Activities in Oxygenation of Arachidonic Acid or Arachidonoyl- Lysophospholipids. Activities of various LOXs were monitored by measuring the increase in absorbance at 234 nm due to the formation of hydroperoxide ($\epsilon_{234} = 25000\ M^{-1}\ cm^{-1}$) at 25 °C as described before (*1, 32, 33*). One unit of leukocyte LOX or reticulocyte LOX is defined as the amount of LOX capable of producing 1 nmol of conjugated diene per minute, and 1 unit of soybean LOX-1 or potato LOX is defined as the amount of LOX that can produce 1 μmol of conjugated diene per minute. The reaction mixture (500 μL) includes reticulocyte LOX (2 units/mL) in 50 mM phosphate buffer (pH 7.4), porcine leukocyte LOX (1 unit/mL) in 100 mM phosphate buffer (pH 7.5) containing 5 mM EDTA and 0.03% Tween 20, or soybean LOX-1 (0.0025 unit/mL) in 50 mM borax buffer (pH 9.0) at 25 °C. The reactions were started by including each substrate (100 μM) into the reaction mixture.

Determination of Kinetic Values in LOX-Catalyzed Oxygenation of Arachidonic Acid or Arachidonoyl Lysophospholipids. Reticulocyte LOX (2 units/mL), leukocyte LOX (1 unit/mL), or soybean LOX-1 (0.0025 unit/mL) was incubated with each arachidonoyl derivative substrate of various concentrations as described above. The values of kinetic parameters were obtained according to Lineweaver–Burk plot analyses as described before (*26, 27*).

RP-HPLC Separation of Oxygenation Products of Arachidonoyl Lysophospholipids. Oxygenation of arachidonoyl-lysoPC was started by including soybean LOX-1 (0.01 unit/mL) in 150 μL of 50 mM borax buffer (pH 9.0) containing arachidonoyl-lysoPC (100 μM) or including reticulocyte LOX (40 units/mL) or leukocyte LOX (30 units/mL) in 150 μL of 50 mM phosphate buffer (pH 7.4) containing arachidonoyl-lysoPC (100 μM). After 10 min, the reaction products were injected into a RP-HPLC system (Hitachi L-7100 pump), equipped with a ZORBAX Eclipse XDB C_{18} column (5 μm, 50 × 4.6 mm, Agilent Technologies), which was eluted (flow rate = 1 mL/min) with a gradient system of solvent B (acetic acid/acetonitrile/H_2O; 0.05:80:20) in solvent A (methanol/H_2O; 10:90), 30–75% from 0 to 50 min. Separately, arachidonoyl-lysoPA (100 μM) was oxygenated by soybean LOX-1, reticulocyte LOX, or leukocyte LOX as described above, and the oxygenation products were analyzed by HPLC with a gradient solvent system of solvent B (acetic acid/acetonitrile/H_2O; 0.05:80:20) in solvent A (methanol/H_2O; 10:90), 50–75% from 0 to 50 min, and the effluent was monitored at 234 nm using the diode array type UV detector (Hitachi L-7400 detector).

Determination of Position Specificity in LOX-Catalyzed Oxygenation of Arachidonoyl Lysophospholipids. Arachidonoyl-lysoPA (400 μM) or arachidonoyl-lysoPC (400 μM) was incubated with soybean LOX-1 (0.01 unit/mL), reticulocyte LOX (40 units/mL), or leukocyte LOX (30 units/mL) in 2 mL of the respective incubation buffer described above. After 30 min of incubation, the reaction products were reduced with $NaBH_4$ and then subjected to alkaline hydrolysis in 1 N NaOH (*26*). After 30 min of incubation at room temperature under N_2, the mixture was subjected to lipid extraction as described previously (*23*). Finally, the lipid extract was analyzed by a SP-HPLC system equipped with a silica gel column (4 μm, 150 × 3.9 mm, Waters), which was eluted (1 mL/min) with *n*-hexane/isopropyl alcohol/acetic acid (100:1:0.05), and the effluent was monitored at 234 nm. The identification of hydrolysis products was carried out in comparison with each standard, 15-HETE or 12-HETE.

Determination of Stereoselectivity in LOX-Catalyzed Oxygenation of Lysophospholipids. 15-HETE, prepared by SP-HPLC as described above, was subjected to chiral-phase HPLC equipped with a Chiralcel OD-H column (250 × 4.6 mm, 5 μm, Daicel Chemical Industries, Ltd.) employing the solvent system of *n*-hexane/isopropyl alcohol/trifluoroacetic acid (100:3:0.05) for separation of the *R* and *S* enantiomers. Separately, the stereochemistry of 12-HETE was analyzed using the solvent system of *n*-hexane/isopropyl alcohol/trifluoroacetic acid (100:2:0.05) as described above. The flow rate of the solvent system was 0.5 mL/min, and the effluent was monitored at 234 nm.

LC-ESI/MS Analysis. LC-ESI/MS analysis was performed using a MSD spectrometer (HP 1100 series LC/MSD, Hewlett-Packard) equipped with a ZORBAX Eclipse XDB C_{18} column (5 μm, 50 × 4.6 mm, Agilent Technologies), which was eluted (0.8 mL/min) with an isocratic system of solvent B (acetate/acetonitrile/H_2O; 0.05:80:20) in solvent A (methanol/H_2O; 10:90): 45% from 0 to 15 min for arachidonoyl-lysoPC and 60% from 0 to 30 min for arachidonoyl-LPA. The products were monitored by UV detection at 234 or 268 nm or by an ESI/MS system employing positive-ion scan mode or selected ion monitoring (SIM) mode.

HPLC Analysis of DiHETE Derivatives Generated from Oxidation of Arachidonoyl Lysophospholipids. Arachidonoyl-lysoPC (200 μM) or arachidonoyl-lysoPA (200 μM) was incubated with soybean LOX-1 (0.02 unit/mL) in 40 mL of 5 mM borax buffer (pH 9.0) at 4 °C, and 1 h later, the reaction mixture, after pH adjustment to pH 7.0, was further incubated with leukocyte LOX (30 units/mL) for 2 h at room temperature. Separately, each arachidonoyl lysophospholipid was initially exposed to soybean LOX-1 (0.04 unit/mL) at pH 9.0 and then additional soybean LOX-1 (0.2 unit/mL) at pH 7.0 as described above. The final reaction mixture was partially purified using a C_{18} extraction column (1 × 3 cm), which was eluted with methanol. The products from the eluate, after $NaBH_4$ reduction and alkaline hydrolysis (*26, 27*), were partially purified using a C_{18} extraction column (1 × 3 cm). Finally, the sample, after dilution in methanol, was loaded onto a C_{18} HPLC column (5 μm, 50 × 3.9 mm, Agilent), which was eluted (1 mL/min) with a gradient system of solvent B (methanol/acetate; 100:0.1) in solvent A (H_2O/acetate; 100:0.1), 50−100% from 0 to 60 min. The effluent was monitored at 268 nm to detect diHETE isomers. Separately, 8(*S*,*R*),15(*S*)-dihydroxyeicosatetra-5*Z*,9*E*,11*E*,13*E*-enoic acid isomers were generated from the exposure of 15(*S*)-HPETE (30 μg/mL) to hematin (5 μg/mL) in phosphate buffer (50 mM, pH 7.0) at 37 °C for 2 h (*34*). The oxidized products, after $NaBH_4$ reduction and partial purification on C_{18} extraction, were analyzed by RP-HPLC, and the structure of diHETE was confirmed by LC-ESI/MS.

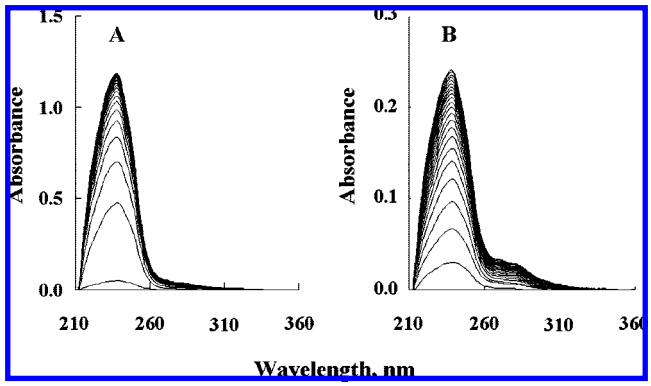

Figure 1. Change of UV spectra during oxygenation of arachidonoyl-lysoPC by soybean LOX-1 or leukocyte LOX. (**A**) Soybean LOX-1 (0.01 unit/mL) was incubated with arachidonoyl-lysoPC (100 μM) in 0.5 mL of borax buffer (50 mM, pH 9.0) at 25 °C. (**B**) Separately, leukocyte LOX (1 unit/mL) was incubated with arachidonoyl-lysoPC (100 μM) in 0.5 mL of phosphate buffer (50 mM, pH 7.4) at 25 °C. The change of UV spectra was scanned every minute (2000 nm/min).

Statistical Analysis. Values were expressed as means ± SE. All statistical analyses were performed using an SPSS program for window. Statistical assessments were performed using ANOVA for the initial demonstration of significance at $P < 0.05$, followed by post hoc Duncan's multiple-range test (*35*).

RESULTS

LOX-Catalyzed Conversion of Arachidonoyl-LysoPC to Hydroperoxyeicosatetraenoyl-LysoPC. When soybean LOX-1 was incubated with arachidonoyl-lysoPC (100 μM) in 50 mM borax buffer (pH 9) at 25 °C, a time-dependent increase of absorption at 234 nm was observed (**Figure 1A**), consistent with the formation of conjugated dienes. Likewise, a similar change of UV spectrum was also observed when arachidonoyl-lysoPC was incubated with leukocyte LOX (**Figure 1B**) or reticulocyte LOX. Next, to identify the oxygenation products of arachidonoyl-lysoPC, the products from the exposure of arachidonoyl-lysoPC to soybean LOX-1 were partially purified using C_{18} extraction column and then subjected to RP-HPLC. **Figure 2A** shows that a peak with a retention time of 19 min appeared as a predominant product. In ESI/MS analysis (**Figure 2B**), the product was found to show the mass spectrum characteristic of a compound corresponding to hydroperoxy derivative of arachidonoyl-lysoPC: molecular ions at *m/z* 576 ($[M + H]^+$), *m/z* 598 ($[M + Na]^+$), and *m/z* 614 ($[M + K]^+$). Thus, hydroperoxyeicosatetraenoyl-lysoPC is obtained as a major oxygenation product during soybean LOX-1 catalyzed oxygenation of arachidonoyl-lysoPC. The same result was also obtained when the products from the incubation of arachidonoyl-lysoPC with reticulocyte LOX or leukocyte LOX were analyzed by LC-ESI/MS.

Determination of Position Specificity and Stereospecificity in Oxygenation of Arachidonoyl-LysoPC. To establish the position of oxygenation of arachidonoyl chain in arachidonoyl-lysoPC, hydroperoxyeicosatetraenoyl-lysoPC was subjected to $NaBH_4$ reduction, followed by alkaline hydrolysis, to afford hydroxyeicosatetraenoic acid (HETE), which was further purified by SP-HPLC. When HETE, a final product, was compared with standard HETE, most of the HETE (retention time, 12.5 min) obtained from the incubation of arachidonoyl-lysoPC with soybean LOX-1 was identified to be 15-HETE (**Figure 3A**, solid line arrow). A similar result was also observed when arachidonoyl-lysoPC was incubated with reticulocyte LOX (data not shown). Separately (**Figure 3B**), when the oxygenation position

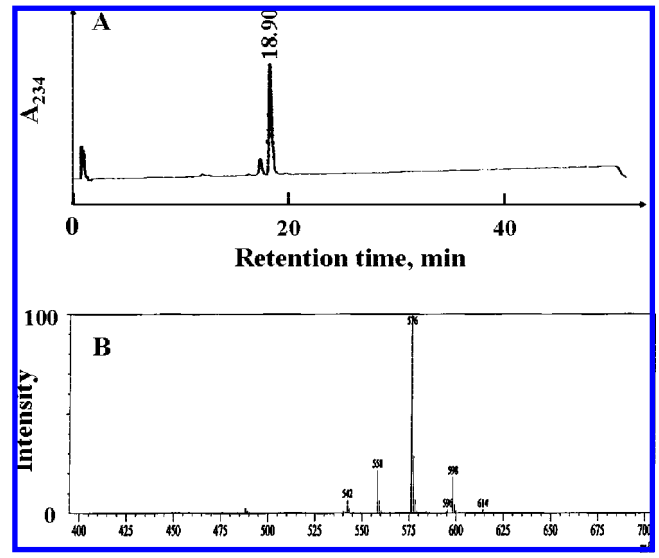

Figure 2. LC-ESI/MS analysis of products from oxygenation of arachidonoyl-lysoPC by soybean LOX-1. (**A**) The product, obtained from 10 min of incubation of arachidonoyl-lysoPC (400 μM) with soybean LOX-1 (0.01 unit/mL) in 150 μL of borax buffer (50 mM, pH 9.0), was injected into a ZORBAX Eclipse XDB C_{18} column (5 μm, 50 × 4.6 mm), which was eluted (0.8 mL/min) with a gradient solvent system of acetate/acetonitrile/H_2O (0.5:80:20) in methanol/H_2O (90:10) as described under Materials and Methods. (**B**) Representative mass spectrum of peroxy derivative of arachidonoyl-lysoPC. The mass spectrum of the major peak (retention time, 19 min) in **A** was obtained by ESI/MS system using positive-ion scan mode as described under Materials and Methods.

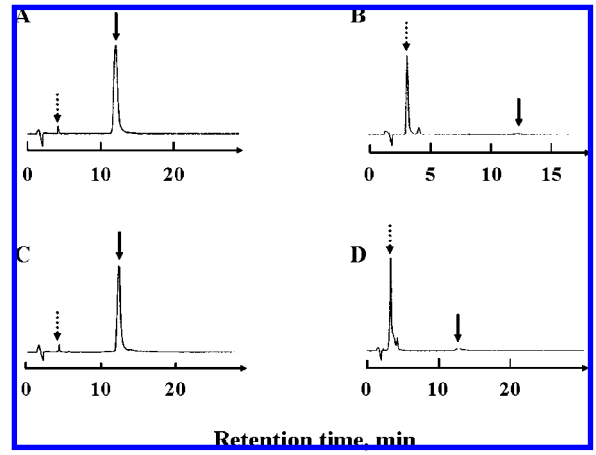

Figure 3. Determination of oxygenation position of oxidized arachidonoyl-lysoPC or arachidonoyl-lysoPA by SP-HPLC: (**A**) incubation of arachidonoyl-lysoPC with soybean LOX-1 at pH 9.0; (**B**) incubation of arachidonoyl-lysoPC with leukocyte LOX at pH 7.4; (**C**) incubation of arachidonoyl-lysoPA with soybean LOX-1 at pH 9.0; (**D**) incubation of arachidonoyl-lysoPA with leukocyte LOX at pH 7.4. Arachidonoyl-lysoPC or arachidonoyl-lysoPA was incubated with each LOX for 30 min, and after $NaBH_4$ reduction and alkaline hydrolysis, the final products were loaded onto an SP-HPLC column, which was eluted with *n*-hexane/isopropyl alcohol/acetic acid (100:1:0.05). The flow rate was 1.0 mL/min, and the effluent was monitored at 234 nm. Finally, each peak was identified by co-injection with corresponding standard HETE (15-HETE, solid line arrow; 12-HETE, dotted line arrow).

in leukocyte LOX-catalyzed oxygenation of arachidonoyl-lysoPC was determined as described above, the predominant HETE product (retention time, ca. 3.2 min) was identified as 12-HETE (dotted line arrow), and the minor one (≤5%) with elution time of about 12.5 min was 15-HETE. Taken together,

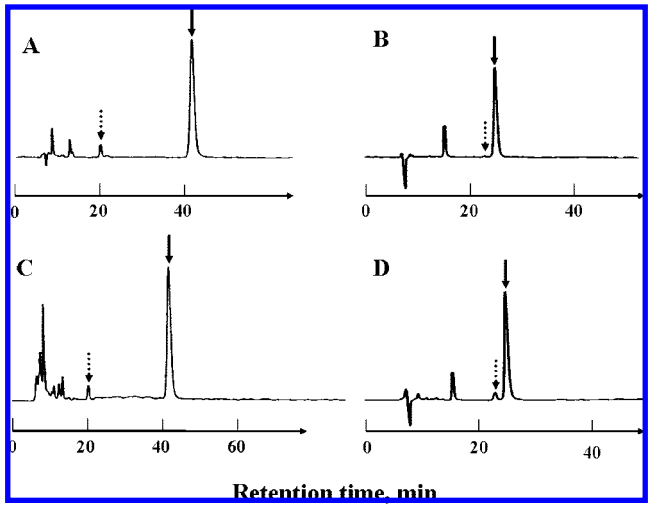

Figure 4. Chiral-phase HPLC of HETE derived from oxygenation of arachidonoyl-lysoPC and arachidonoyl-lysoPA by lipoxygenases: (**A**) 15-HETE derived from exposure of arachidonoyl-lysoPC to soybean LOX-1; (**B**) 12-HETE derived from exposure of arachidonoyl-lysoPC to leukocyte LOX; (**C**) 15-HETE derived from exposure of arachidonoyl-lysoPA to soybean LOX-1; (**D**) 12-HETE from exposure of arachidonoyl-lysoPA to leukocyte LOX. 15-HETE, prepared as described in **Figure 3**, was analyzed by chiral-phase HPLC, equipped with a Chiralcel OD-H column, which was eluted with the solvent system of *n*-hexane/isopropyl alcohol/trifluoroacetic acid (100:3:0.05). Separately, 12-HETE was analyzed using the solvent system of *n*-hexane/isopropyl alcohol/trifluoroacetic acid (100:2:0.05). The flow rate was 0.5 mL/min, and the effluent was monitored at 234 nm. Finally, each peak was identified by co-injection with the corresponding standard HETE stereoisomer, (*S*) isomer (solid line arrow) or (*R*) isomer (dotted line arrow) of 12-HETE or 15-HETE.

it is suggested that arachidonoyl-lysoPC is oxygenated mainly at C-15 by soybean LOX-1 or reticulocyte LOX and mainly at C-12 by leukocyte LOX, following the positional specificity for oxygenation of arachidonic acid (*14–17*). Next, the stereospecificity in the oxygenation of arachidonoyl-lysoPC was determined by chiral-phase HPLC. When 15-HETE, prepared as described in **Figure 3A**, was subjected to chiral-phase HPLC analysis in comparison with each standard 15-HETE stereoisomer, most of the 15-HETE (retention time, 42 min) was observed to correspond to 15(*S*)-HETE (**Figure 4A**). Also, the same stereospecificity for the (*S*) form was demonstrated by 15-HETE, produced from the incubation of arachidonoyl-lysoPC with reticulocyte LOX (data not shown). Meanwhile, most of the 12-HETE (retention time, 25 min), obtained from leukocyte LOX-catalyzed oxygenation of arachidonoyl-lysoPC as described in **Figure 3B**, was identified to be 12(*S*)-HETE (**Figure 4B**). Thus, the three LOXs used here expressed a common (*S*) stereospecificity in oxygenation of arachidonoyl-lysoPC.

LOX-Catalyzed Conversion of 15-Hydroperoxyeicosatetraenoyl-LysoPC to Dihydroxyeicosatetraenoyl-LysoPC. Previously, it had been reported that 15(*S*)-HPETE was converted to conjugated triene derivatives, corresponding to analogues of C15-series leukotrienes in leukocytes (*7*). In an attempt to prove that leukocyte LOX can generate the leukotriene-type derivatives of arachidonoyl-lysoPC, arachidonoyl-lysoPC was first exposed to soybean LOX-1 at pH 9.0 to produce 15-hydroperoxyeicosatetraenoyl-lysoPC, and then the mixture, after pH adjustment to pH 7.0, was further incubated with leukocyte LOX (*7*). As exhibited in **Figure 5A**, the sequential exposure of arachidonoyl-lysoPC to soybean LOX-1 and leukocyte LOX led to a time-dependent increase in the absorbance at 268 nm with shoulders at 259 and 279 nm, indicative of conjugated triene structure. Subsequently, the oxidized products from sequential oxygenation of arachidonoyl-lysoPC were subjected to LC-ESI/MS analyses. As shown in **Figure 5B**, peak I (6.4 min) and peak II (7.6 min) appeared as major compounds when the chromatogram was analyzed by SIM monitoring at *m/z* 576, corresponding to $[M + H]^+$ of dihydroxyeicosatetraenoyl-lysoPC or 15-hydroperoxyeicosatetraenoyl-lysoPC (**Figure 5B**, inset). Additionally, peaks I and II exhibited the same mass spectrum as peak II (**Figure 5C**): 576.2 ($[M + H]^+$), *m/z* 598.2 ($[M + Na]^+$), and *m/z* 614.2 ($[M + K]^+$). When the peaks were analyzed by UV absorption monitoring, it was found that peaks I and II were detected by UV absorption at 268 and 234 nm, respectively (**Figure 5B**). Thus, it is supposed that peak I corresponds to dihydroxyeicosatetraenoyl-lysoPC, whereas peak II is 15-hydroperoxyeicosatetraenoyl-lysoPC as disclosed in **Figure 2B**. However, the molecular ion at *m/z* 608.2, corresponding to $[M + H]^+$ of dihydroperoxyeicosatetraenoyl-lysoPC, was not detected under the conditions used above, excluding the possibility that leukocyte LOX caused another oxygenation of 15-hydroperoxyeicosatetraenoyl-lysoPC. In a separate experiment to elucidate the structure of dihydroxylated derivatives of conjugated triene, the oxidized products were subjected to $NaBH_4$ reduction and alkaline hydrolysis to afford dihydroxyeicosatetraenoic acid (diHETE). When the final products were analyzed by RP-HPLC employing UV monitoring at 268 nm (**Figure 6A**), it turned out that three peaks, peak I (20.9 min), peak II (21.6 min), and peak III (22.1 min), appeared as major diHETE products. In repeated experiments, peaks I and III appeared to be isomers of a similar quantity in HPLC profile, suggesting that two peaks may be diHETE isomers of the same double-bond geometry (*7*). In support of this, the HPLC analysis (**Figure 6B**) indicated that peaks I and III coincided with two peaks of 8,15(*S*)-dihydroxyeicosatetra-5*Z*,9*E*,11*E*,13*E*-enoic acid isomers, which were generated from the exposure of 15(*S*)-hydroperoxyeicosatetraenoic acid (HPETE) to hematin (*34*). Meanwhile, peak II, which appeared as a minor component, was found to comigrate with 8(*S*),15(*S*)-dihydroxyeicosatetra-5*Z*,9*E*,11*Z*,13*E*-enoic acid (**Figure 6C**), derived from further oxygenation of 15(*S*)-HPETE by soybean LOX-1 (*32*), implying that arachidonoyl-lysoPC might be susceptible to LOX-catalyzed double oxygenation. However, the double oxygenation was not observed during the incubation of arachidonoyl-lysoPC with excess soybean LOX-1 alone.

LOX-Catalyzed Conversion of Arachidonoyl-LysoPA to Oxidized Derivatives. Subsequently, the exposure of arachidonoyl-lysoPA to soybean LOX-1 led to the same UV spectral change as observed with arachidonoyl-lysoPC (**Figure 7A**). Also, a similar result was obtained when arachidonoyl-lysoPA was incubated with reticulocyte LOX or leukocyte LOX. When the major product from oxygenation of arachidonoyl-lysoPA by soybean LOX-1 were analyzed by LC-ESI/MS, it was found to possess a mass spectrum characteristic of hydroperoxyeicosatetraenoyl-lysoPA: molecular ions at *m/z* 490.3 ($[M + H]^+$), *m/z* 512.3 ($[M + Na]^+$), and *m/z* 528.3 ($[M + K]^+$). When the position of oxygenation of the arachidonoyl chain in arachidonoyl-lysoPA was determined by SP-HPLC, it was demonstrated that HETE (12.5 min), derived from oxygenation of arachidonoyl-lysoPA by soybean LOX-1, corresponded to 15-HETE (**Figure 3C**) and that HETE (3.2 min), derived from oxygenation of arachidonoyl-lysoPA by leukocyte LOX, corresponded to 12-HETE (**Figure 3D**). Next, the stereospecificity in the oxygenation of arachidonoyl-lysoPA was determined by chiral-phase HPLC (**Figure 4**); 15-HETE, obtained as described in **Figure 3C**, behaved like 15(*S*)-HETE (**Figure 4C**), whereas 12-HETE, obtained as described in **Figure 3D**, behaved like

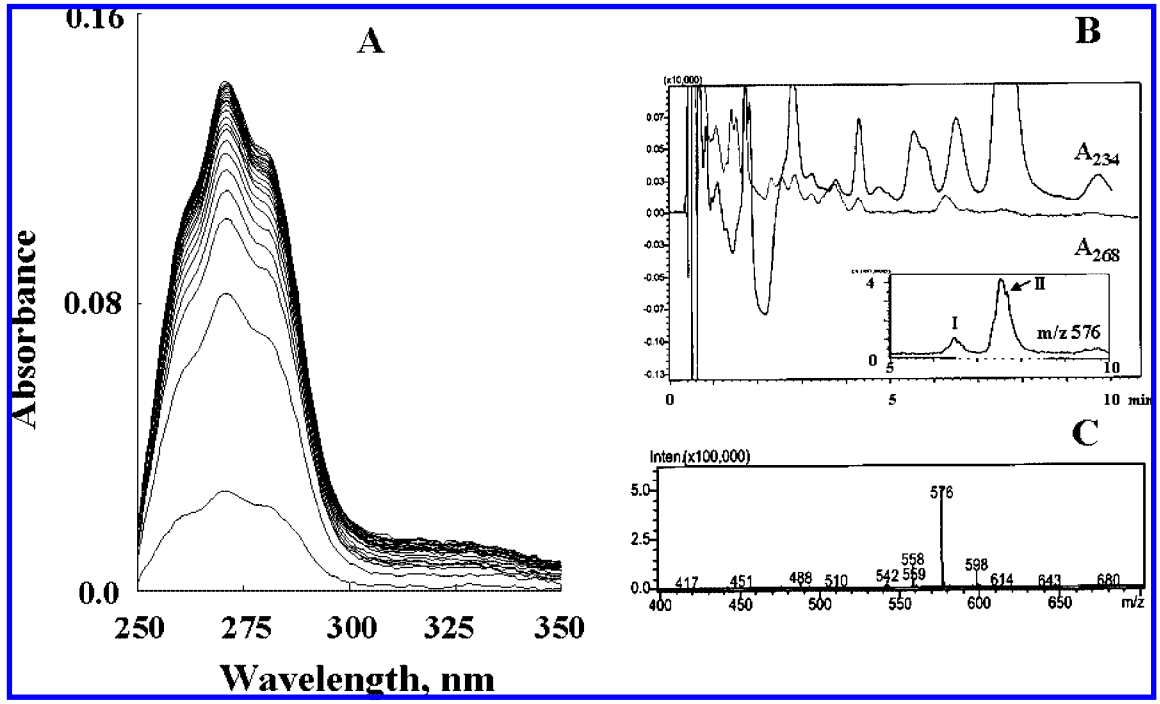

Figure 5. Analysis of products from the sequential oxygenation of arachidonoyl-lysoPC to soybean LOX-1 and leukocyte LOX: (**A**) Arachidonoyl-lysoPC (200 μM) was incubated with soybean LOX-1 (0.004 unit/mL) in 5 mM borax buffer (pH 9) for 1 h at 4 °C, and then the mixture, after pH had been adjusted to pH 7.0, was further incubated with leukocyte LOX (30 units/mL) at room temperature for 2 h. The UV spectral change in differential spectrometry was monitored with the cycle time of 5 min. (**B**) The final oxygenation products were analyzed by LC-EMI/MS. The products were monitored by UV detection at 234 nm (upper line) and 268 nm (lower line) and monitored by SIM at *m/z* 576 (inset). (**C**) Representative mass spectrum of conjugated triene derivate of arachidonoyl-lysoPC (retention time = 6.3 min in **B**, lower line).

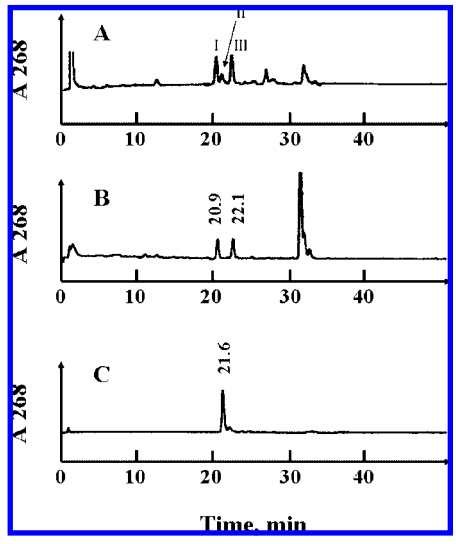

Figure 6. RP-HPLC chromatogram of diHETEs. The oxidized products (diHETEs), prepared as described under Materials and Methods, was loaded into a C_{18} column, which was eluted with a gradient system of solvent B (methanol/acetic acid; 100:0.1) in solvent A (H_2O/acetic acid; 100:0.1): (**A**) Sequential exposure of arachidonoyl-lysoPC to soybean LOX-1 and leukocyte LOX; (**B**) exposure of 15(*S*)-HPETE to hematin; (**C**) incubation of 15(*S*)-HPETE with soybean LOX-1. The effluent was monitored at 268 nm (flow rate = 1 mL/min).

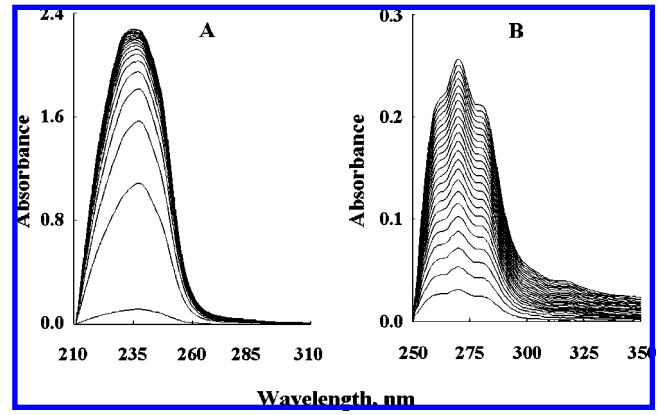

Figure 7. Change of UV spectra during oxygenation of arachidonoyl-lysoPA by soybean LOX-1. (**A**) Soybean LOX-1 (0.04 unit/mL) was incubated with arachidonoyl-lysoPA (200 μM) in 0.5 mL of borax buffer (5 mM, pH 9.0) at 4 °C for 1 h. The UV spectral change (scan speed = 2000 nm/min) in differential spectrometry was monitored with the cycle time of 1 min. (**B**) The above incubation mixture (**A**), after pH adjustment to pH 7.0, was further incubated with excess soybean LOX-1 (0.2 unit/mL) at 25 °C for 2 h. The UV spectral change (scan speed = 2000 nm/min) in differential spectrometry was monitored with the cycle time of 5 min.

12(*S*)-HETE (**Figure 4D**). Subsequently, the further conversion of 15-hydroperoxyeicosatetraenoyl-lysoPA by LOX was examined. As demonstrated in **Figure 7B**, the primary exposure of arachidonoyl-lysoPA to soybean LOX-1 at pH 9.0, followed by the extended exposure to excess soybean LOX-1 at pH 7.0, gradually caused the appearance of UV spectrum displaying maximal absorbance at 271 nm, as had been demonstrated in double oxygenation of arachidonic acid by soybean LOX-1 (*32*). Furthermore, the formation of dihydroperoxy derivative was confirmed by a mass spectrum characteristic of dihydroperoxy-eicosatetraenoyl-lysoPA: molecular ions at *m/z* 522.3 ($[M + H]^+$), *m/z* 544.2 ($[M + Na]^+$), and *m/z* 560.3 ($[M + K]^+$). Separately, the sequential exposure of arachidonoyl-lysoPA to soybean LOX-1 and leukocyte LOX also caused the same UV

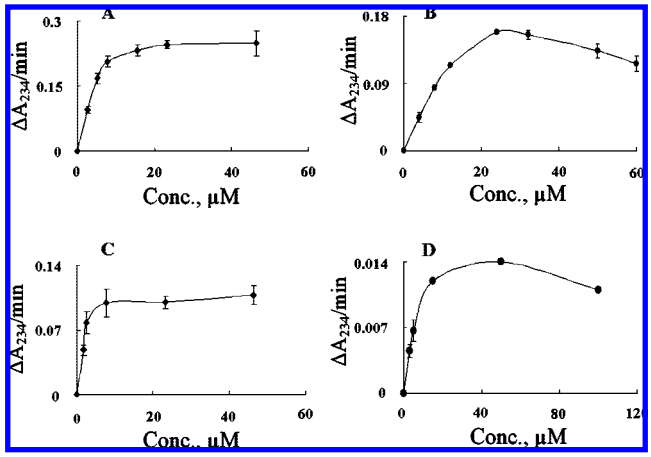

Figure 8. Effect of substrate concentration on LOX-catalyzed oxygenation of arachidonoyl-lysoPC or arachidonoyl-lysoPA. Soybean LOX-1 (0.0025 unit/mL) was incubated with arachidonoyl-lysoPC (**A**) or arachidonoyl-lysoPA (**B**) of various concentrations (3–60 μM) in borax buffer (50 mM, pH 9.0). Separately, leukocyte LOX (1 unit/mL) was incubated with arachidonoyl-lysoPC (**C**) or arachidonoyl-lysoPA (**D**) of various concentrations (3–100 μM) in 100 mM phosphate buffer (pH 7.5) containing 5 mM EDTA and 0.03% Tween 20. Data are expressed as a means ± SD value of triplicate assays.

spectral change as observed with arachidonoyl-lysoPC (data not shown). Thus, double oxygenation by soybean LOX-1 seems to be determined by the type of lysophospholipid.

Determination of Kinetic Values in LOX-Catalyzed Oxygenation of Arachidonoyl Lysophospholipids. Subsequently, the effect of substrate concentration on LOX-catalyzed oxygenation of arachidonoyl-lysoPC or arachidonoyl-lysoPA was examined. As demonstrated in **Figure 8**, the enzyme activity followed classical Michaelis–Menten kinetics when each enzyme was incubated with arachidonoyl-lysoPC or arachidonoyl-lysoPA. Overall, the maximal oxygenation rate was reached at lower concentrations of arachidonoyl-lysoPC (**Figure 8A**,**C**) rather than arachidonoyl-lysoPA (**Figure 8B**,**D**). In Lineweaver–Burk plot analysis, the K_m and V_m values in oxygenation by soybean LOX-1 were estimated to be 5.4 μM and 97.0 units/μg of protein, respectively, for arachidonoyl-lysoPC, and 15.4 μM and 147.8 units/μg of protein, respectively, for arachidonoyl-lysoPA, in contrast to 12.4 μM and 92.2 units/μg of protein, respectively, for arachidonic acid (**Table 1**). Thus, the catalytic efficacy (V_m/K_m) as substrate of soybean LOX-1 was greatest for arachidonoyl-lysoPC, followed by arachidonoyl-lysoPA and arachidonic acid. Next, when the V_m/K_m values in the oxygenation by rabbit reticulocyte LOX were compared (**Table 1**), arachidonoyl-lysoPC was found to be much more efficient than arachidonic acid or arachidonoyl-lysoPA as substrates for reticulocyte LOX. The catalytic efficacy of arachidonoyl-lysoPC for reticulocyte LOX was approximately 10-fold greater than that of arachidonic acid. In an additional study, the oxygenation of arachidonoyl-lysoPC or arachidonoyl-lysoPA by leukocyte LOX was examined. **Table 1** indicates that the V_m/K_m value of arachidonoyl-lysoPC or arachidonoyl-lysoPA in leukocyte LOX-catalyzed oxygenation is somewhat greater than that of arachidonic acid, suggesting that arachidonoyl-lysophospholipids are also favorable substrates of leukocyte LOX. In contrast, the oxygenation of arachidonoyl-lysoPC or arachidonoyl-lysoPA by potato LOX (*27*) was negligible.

DISCUSSION

It is well established (*1–5*) that free polyunsaturated fatty acids are endogenous substrates for LOXs. Additionally, phospholipids containing polyunsaturated fatty acyl chains are also known to be oxygenated by some LOXs (*13–20*). Our previous studies demonstrated that linoleoyl-lysoPC was no less efficient than linoleic acid as substrate for soybean LOX-1 as well as mammalian LOXs, such as leukocyte LOX or reticulocyte LOX (*26, 27*). The present study demonstrates that arachidonoyl-lysoPC and arachidonoyl-lysoPA are also oxygenated efficiently by soybean LOX-1, reticulocyte LOX, or leukocyte LOX. Moreover, arachidonoyl-lysoPC possesses a greater catalytic efficiency than arachidonic acid or arachionoyl-lysoPA as substrate for the three LOXs (*36*), indicating that of the lipids tested, arachidonoyl-lysoPC is the most efficient substrate for leukocyte LOX. The lower efficacy of arachidonoyl-lysoPA, compared to arachidonoyl-lysoPC, as a substrate of soybean LOX-1 or reticulocyte LOX may be the result of a negative charge property adopted by arachidonoyl-lysoPA, as suggested from its higher K_m value. Previously, it had been reported that LOXs had a substantially higher affinity for monomeric substrate than for substrate incorporated into micelles (*9, 26, 37*). Alternatively, the lower catalytic efficacy of arachidonoyl-lysoPA, compared to arachidonoyl-lysoPC, may be due to its greater cmc value (*38, 39*). However, the latter idea is not supported by present observations that arachidonoyl-lysoPC and arachidonoyl-lysoPA are utilized as substrates of soybean LOX-1 or reticulocyte LOX at concentrations much below their cmc values (>50 μM).

It is well-known (*40, 41*) that when acting on free arachidonic acid, soybean LOX-1 and leukocyte LOX produce 15(*S*)-HPETE and 12(*S*)-HPETE, respectively. Such regio- and stereospecificities were also announced in the oxygenation of arachidonoyl-lysophospholipids by soybean LOX-1, reticulocyte LOX, or leukocyte LOX. Thus, esterification of the arachidonoyl group at the glycerol backbone did not alter the regio- or stereospecificity of LOX in the oxygenation of arachidonoyl-lysophospholipids (*42, 43*). Furthermore, the esterification of the carboxyl group in lysophospholipids had no negative effect on the catalytic rate of LOX. All of these indicate that the carboxyl group may not be crucial for the structural requirement as substrates of soybean LOX-1, rabbit reticulocyte LOX, or porcine leukocyte LOX.

It is well-known that arachidonic acid is converted to leukotrienes via an intermediate, 5- or 15-HPETE (*4, 7, 44*). The present study provides supportive evidence for porcine leukocyte LOX-catalyzed conversion of 15(*S*)-hydroperoxyeicosatetraenoyl lysophospholipids to conjugated triene derivatives, corresponding to analogues of C15-series leukotrienes (*7*). This is well supported by gradual UV spectral change during sequential exposure of arachidonoyl lysophospholipids to soybean LOX-1 and porcine leukocyte LOX. In further support, the reduction of oxidized lysophospholipids, followed by alkaline hydrolysis, produced diHETE isomers comigrating with isomers of 8,15(*S*)-diHETE (*34*). Concerning the mechanism for the formation of dihydroxyeicosatetraenoyl derivatives, there may be two possibilities: one is the epoxide intermediate pathway and the other the dioxygenation pathway. According to the former, 15(*S*)-hydroperoxyeicosatetraenoyl-lysoPC may be transformed by leukocyte LOX to dihydroxyeicosatetraenoyl-lysoPC isomers via 14,15-epoxide intermediate, as established for the conversion of arachidonic acid to two isomers of 8,15(*S*)-diHETE in leukocytes (*7*). A support for this may come from the finding

Table 1. Kinetic Values in Oxygenation of Arachidonic Acid or Arachidonoyl-Lysophospholipids by Soybean LOX-1, Leukocyte LOX, and Reticulocyte LOX[a]

enzyme	substrate	K_m (μM)	V_m (units/mg)	V_m/K_m (units/mg/μM)
soybean LOX-1	arachidonic acid	12.4 ± 3.5a	92.2 ± 3.0a	7.4 ± 0.3a
	arachidonoyl-lysoPA	15.4 ± 1.7a	147.8 ± 2.3b	9.6 ± 1.0a
	arachidonoyl-lysoPC	5.37 ± 1.6b	97.0 ± 8.2a	20.2 ± 4.0b
reticulocyte LOX	arachidonic acid	7.7 ± 1.6a	63.2 ± 11.9a	8.2 ± 1.5a
	arachidonoyl-lysoPA	8.6 ± 2.3a	33.5 ± 2.9b	4.1 ± 1.2b
	arachidonoyl-lysoPC	2.6 ± 0.2b	199.8 ± 16.7c	81.3 ± 2.8c
leukocyte LOX	arachidonic acid	25.4 ± 9.8a	140.2 ± 2.4a	6.2 ± 0.1a
	arachidonoyl-lysoPA	3.6 ± 0.5b	201.5 ± 9.7b	54.5 ± 3.4b
	arachidonoyl-lysoPC	5.0 ± 0.3b	404.6 ± 21.2c	81.5 ± 4.8c

[a] Soybean LOX-1 (0.0025 unit/mL) was incubated with arachidonic acid, arachidonoyl-lysoPA, or arachidonoyl-lysoPC of various concentrations in 500 μL of 50 mM borax buffer (pH 9.0) at 25 °C. Porcine leukocyte LOX (2 units/mL) was incubated with each substrate of various concentrations (3–100 μM) in 500 μL of 100 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.03% Tween 20 at 25 °C. Reticulocyte LOX (2 units/mL) was incubated with each substrate of various concentrations (3–100 μM) in 500 μL of 50 mM phosphate buffer (pH 7.4) at 25 °C. Kinetic values were obtained from Lineweaver–Burk plot as described before (*24*). One unit of leukocyte or reticulocyte LOX is expressed as 1 nmol of oxygenation product per minute, and that of soybean LOX-1 as 1 μmol of product per minute. Values were expressed as means ± SE of at least three independent experiments. Means with the same letter are not significantly different ($P < 0.05$, $n = 3$).

that the chromatographic profile of two 8,15-diHETE isomers (**Figure 6B**) is the same as that of two 8(*S*,*R*),15(*S*)-dihydroxyeicosatetra-5*Z*,9*E*,11*E*,13*E*-enoic acid isomers, resulting from hematin-induced conversion of 15(*S*)-HPETE (*34*). Separately, the formation of dihydroperoxyeicosatetraenoyl-lysoPC was not significant during the sequential lipoxygenation of arachidonoyl-lysoPC by soybean LOX-1 and leukocyte LOX; moreover, soybean LOX-1 in excess did not convert the hydroperoxy derivative with conjugated diene structure to further oxygenation product, indicating that arachidonoyl-lysoPC was resistant to double oxygenation by soybean LOX-1. Meanwhile, arachidonoyl-lysoPA was oxygenated by soybean LOX-1 to produce dihydroperoxyeicosatetraenoyl-lysoPA with conjugated triene structure, consistent with double oxygenation of arachidonoyl-lysoPA by soybean LOX-1. Thus, the further conversion of 15(*S*)-hydroperoxyeicosatetraenoyl lysophospholipids differed greatly according to the base type of lysophospholipid. From these data, it is proposed that 15-hydroperoxyeicosatetraenoyl-lysoPA can be subjected to further metabolism in cells containing 12- or 15-LOXs (*7*). Previous studies demonstrated that 15-HPETE was further converted to ketols in coral (*6*), hepoxilin isomers in garlic roots (*8*), or diHETEs in leukocytes (*4, 7*). Likewise, it is likely that 15-hydroperoxyeicosatetraenoyl-lysophospholipids can be further metabolized to various oxidized metabolites in plant or animal sources. Lysophosphatidylcholine (lysoPC) is known to be generated from phosphatidylcholine by at least three enzymes, phospholipase A_2, lipoprotein-specific phospholipase A_2, and lecithin/cholesterol acyltransferase or endothelial lipase (*45–47*). In turn, lysoPC is further converted to lysoPA in the presence of lysophospholipase D (*48*). According to previous reports, arachidonoyl-lysoPC and arachidonoyl-lysoPA were found to exist in egg white to a substantial level (*29, 30*), and docosahexaenoyl-lysoPC was reported to be one major lipid component in shark liver extract (*49*). Meanwhile, lysophospholipids with a linoleoyl or linoleonoyl group were found to be present in plant sources (*28, 50*). Nonetheless, the formation of peroxy derivatives of lysophospholipids has not been extensively examined in plant or animal sources.

Concerning the physiological role of lysophospholipids in plants, one role of these lysophospolipid derivatives may be a carrier of oxidized acyl group, consistent with earlier proposals that lysoPC, membrane-permeable, may be a preferred physiological carrier of polyunsaturated fatty acids such as docosahexaenoic acid (*51, 52*). Also, it is likely that 15-hydroperoxyeicosatetraenoyl-lysophospholipids may interfere with the activities of LOXs by competing with native substrates. Additionally, it is conceivable that the initial oxygenation of lysophospholipids with polyunsaturated fatty acyl chains may promote the lipid peroxidation of cellular membanes of plant or animals. A recent report demonstrates that lysoPC can induce the mycorrhiza-specific phosphate transporter gene in potato roots and cause a rapid extracellular alkalinization in tomato cells (*53*). Separately, oxygenated fatty acids such as oxylipids have been known to have key roles as potent regulators in response to pathogenesis and herbivory in plants (*54*). Taken together, it would be curious to see whether lysophospholipids with oxidized acyl chains might be physiologically active in plants.

The present availability of hydroperoxydiene or conjugated triene derivatives of arachidonoyl-lysophopsholipids may contribute to further elucidation of physiological roles of lysophospholipids with oxidized unsaturated fatty acyl chains, which need to be clarified in future study using plant or animal cells.

ABBREVIATIONS USED

lysoPC, lysophosphatidylcholine; lysoPA, lysophosphatidic acid; LOX, lipoxygenase; HETE, hydroxyeicosatetraenoic acid; diHETE, diydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; SP, straight phase; RP, reverse phase.

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