Arachidonic acid mobilization among phospholipids in murine mastocytoma P-815 cells: role of ether-linked phospholipids

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Abstract The ethanolamine-containing glycerophospholipids, choline-containing glycerophospholipids, and phosphatidylinositol fractions are major sources of arachidonic acid in murine mastocytes P-815 cloned cells. The choline-linked fraction contained high arachidonic acid content in 1-O-alkyl-2-acyl (18%) and 1,2-diacyl-sn-glycero-3-phosphocholine (11%), with smaller amounts in 1-O-alkyl-1'-enyl-2-acyl species, whereas the arachidonic acid content of the ethanolamine-linked fraction was high in 1-O-alkyl-1'-enyl-2-acyl (26%) and 1,2-diacyl species (15%) and low in 1-O-alkyl-2-acyl species. The uptake and transfer of [3H]arachidonic acid into the 1,2-diacyl and ether classes of choline-containing glycerophospholipids and ethanolamine-containing glycerophospholipids in mastocytoma cells were examined. There was very rapid incorporation of radioactive arachidonic acid into mastocytoma cells that leveled off after 30 min. By labeling cells with [3H]arachidonic acid for 7.5 min, the radioactivity was recovered in the choline-containing glycerophospholipids (43%), phosphatidylinositol (32%), and ethanolamine-containing glycerophospholipids (20%) with little in other phospholipids, neutral lipid, or free fatty acid fractions. Upon reincubation of the mastocytoma cells in the radiolabel-free medium, the radioactivity was gradually lost from the choline-containing glycerophospholipid fractions and, concomitantly, increased in ethanolamine-containing glycerophospholipids. At the zero time of reincubation, most of the radioactivity was recovered in the 1,2-diacyl species of both choline-containing glycerophospholipids and ethanolamine-containing glycerophospholipids. With increasing time of reincubation, the radioactivity rapidly decreased from 1,2-diacyl-sn-glycero-3-phosphocholine, with a concurrent increase in 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine and 1-O-alkyl-1'-enyl-2-acyl-sn-glycero-3-phosphoethanolamine. Such gradual transfer of arachidonic acid may result in its preferential enrichment in these ether phospholipids, especially in ethanolamine plasmalogens. —Yoshioka, S., S. Nakashima, Y. Okano, and Y. Nozawa. Arachidonic acid mobilization among phospholipids in murine mastocytoma P-815 cells: role of ether-linked phospholipids. J. Lipid Res. 1986. 27: 939-944.

Supplementary key words choline-containing glycerophospholipids • ethanolamine-containing glycerophospholipids • phosphatidylinositol fractions

Arachidonic acid is converted to the slow-reacting substance of anaphylaxis (SRS-A) by 5-lipoxygenase. The mastocytoma cells are known to produce large amounts of SRS-A when they are stimulated by Ca2+ ionophore (1). However, mastocytoma cells contained lower levels of secretary granules than those found in normal mast cells, and were observed to release much less histamine than mast cells, upon stimulation with either antigen, A23187, or compound 48/80 (2). There has been substantial evidence to account for the concept that the provocation of cell functions is coupled with a rapid increase in phosphatidylinositol (PI) metabolism (3). Actually, the little changes observed in the level of radiolabeled PI, diacylglycerol (DG), and phosphatidic acid (PA) indicate that PI turnover may not be operating in the transformed cells (S. Nakashima, S. Yoshioka, and Y. Nozawa, unpublished data). In a previous paper (2), we analyzed the compositional distribution of phospholipids (1,2-diacyl, 1-O-alkyl-2-acyl, 1-O-alkyl-1'-enyl-2-acyl species) and fatty acyl chains in murine mastocytoma cells. It was demonstrated that mastocytoma cells contained high levels of 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine (1-O-alkyl-2-acyl-GPC) and 1-O-alkyl-1'-enyl-2-acyl-sn-glycero-3-phosphoethanolamine (1-O-alkyl-1'-enyl-2-acyl-GPE), and also that ether lipids were rich in arachidonic acid as seen in the human platelets (4) and rabbit alveolar macrophages (5). Biological role(s) and significance(s) of these ether lipids are still obscure. However, considerable interest has been focused on 1-O-alkyl-2-acyl-GPC, since 1-O-alkyl-2-acetyl-GPC synthesized from 1-O-alkyl-2-acyl-GPC by phospholipase A2 hydrolysis acts as platelet-activating factor (PAF) (6). It is conceivable that since 1-O-alkyl-2-acyl-GPC and 1-O-

Abbreviations: PC, choline-containing glycerophospholipids; PE, ethanolamine-containing glycerophospholipids; PI, phosphatidylinositol; GPC, sn-glycero-3-phosphocholine; GPE, sn-glycero-3-phosphoethanolamine; SRS-A, slow-reacting substance of anaphylaxis; PA, phosphatidic acid; TLC, thin-layer chromatography; BSA, bovine serum albumin; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; GLC, gas-liquid chromatography; DG, diacylglycerol.

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alk-1'-enyl-2-GPE are rich in arachidonic acid, these two phospholipids may serve as the efficient arachidonate pool. However, the mechanism(s) for abundance of the fatty acid in these lipids has not yet been clarified. Experiments with platelets and macrophages have shown the discrepancy between endogenous levels of arachidonic acid and extent of radiolabeling, suggesting possible differences in arachidonic acid turnover and metabolism between ether and diacyl phospholipids (7-10). It was, therefore, of interest to investigate the arachidonic acid mobilization in 1-O-alk-1'-enyl-2-acyl, 1,2-diacyl, as well as in 1-O-alkyl-2-acyl phospholipids in murine mastocytoma P815 cloned cells.

MATERIALS AND METHODS

Materials

Phospholipase C (Bacillus cereus) was obtained from Boehringer-Mannheim, GmbH. Silica gel H and primulin dye were purchased from Sigma Chemical Company, St. Louis MO. Phosphatidylethanolamine plasmalogen (bovine brain) and D-1,2-diestearoyl glycerol ether were the products of Serdary Research Laboratories, London, Ontario, Canada. [5,6,8,9,11,12,14,15-3H]Arachidonic acid (86 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Fatty acid methyl ester standards were obtained from Nihon Chromato Industrial Co., Tokyo. All chemicals were of reagent grade.

Cell line

As described previously (2), cloned cell line of transformed murine mast cell, mastocytoma P815, derived from the Dunn-Potter's mastocytoma was transplanted in the abdominal cavities of DBA/2 mice. The tumor-bearing mice were kindly provided from the Department of Biochemistry (Drs. A. Ichiyama and H. Hasegawa), Hamamatsu Medical College.

Isolation and purification of mastocytoma cells

The mastocytoma cells were harvested from the abdominal cavity of the tumor-bearing mice on the 7th day after transplantation (1-3 × 10⁶ cells/mouse) and were purified using a BSA density gradient method (II). Purity of cell preparations was over 95% and the viability was over 90% as assessed by 0.05% toluidine blue staining. Finally cells were suspended in the mast cell medium (150 mM NaCl, 3.7 mM KCl, 3 mM Na₂HPO₄, 3.5 mM KH₂PO₄, 5.6 mM dextrose, 1 mg/ml BSA, 0.1% gelatin, and 10 units/ml heparin, pH 6.8). Polyethylene or siliconized glassware was used during cell preparation and incubation.

Thin-layer chromatography (TLC) solvent system

Several solvent systems were used for separation of the cellular lipids and derivatives by TLC: system I, chloroform-methanol-13.5 N ammonia water 65:35:6 (v/v); system II, chloroform-acetone-methanol-glacial acetic acid-water 3:4:1:0.5 (v/v); system III, petroleum ether-dietfyl ether-glacial acetic acid 90:10:1 (v/v); system IV, toluene.

Phospholipid metabolism

Isolated cells were incubated with [³H]arachidonic acid (1 μCi/10⁶ mastocytoma cells) at 37°C for pulse chase and continuous labeling. For pulse chase, the cells radiolabeled for 7.5 min were washed twice with the mast cell medium containing 1 mM EGTA and finally suspended in mast cell medium with 1 mM CaCl₂ and 0.5 mM MgCl₂ to give a final concentration of 2.1 × 10⁷ mastocytoma cells/ml.

Extraction and fractionation of lipids

Washed cells were added with 4 ml of chloroform-methanol 1:2 (v/v) and lipids were extracted by the method of Bligh and Dyer (12). Individual phospholipids were separated by two-dimensional chromatography on silica gel H plates containing 2.5% magnesium acetate, using solvent system I for the first dimension, and solvent system II for the second dimension in the presence of butylated hydroxytoluene (5 mg/ml) in the developing solvents. After visualization with a primulin spray reagent, the areas corresponding to individual phospholipids were extracted from the scraped gel powder by the method of Bligh and Dyer (12).

Separation of 1,2-diacyl-, 1-O-alkyl-2-acyl-, and 1-O-alk-1'-enyl-2-acyl classes of choline-containing glycerophospholipids (PC) and ethanolamine-containing glycerophospholipids (PE)

The choline- and ethanolamine-containing fractions, which had been extracted from mastocytoma cells, and phosphatidylethanolamine plasmalogen (Serdary) were treated with phospholipase C to produce their 1,2-diradyl glycerols. The formed 1,2-diradyl glycerols and D-1,2-diesterglyceroether (Serdary) were acetylated by the method of Soodsm, Mims, and Harlow (13) to yield 1,2-diradyl-3-acetylglycerols. The three species were separated by TLC on silica gel H by the method of Sugiuura, Masuzawa, and Waku (14) using solvent system III for 1,2-diacyl and 1-O-alkyl-2-acyl and solvent system IV for 1-O-alk-1'-enyl-2-acyl. Spots were identified by comigration with authentic standards.

Analysis of percent distribution of arachidonic acid in each phospholipid class

For determination of the relative percentage of arachidonic acid distribution, phospholipids were separated by TLC as mentioned above, scraped off the plate, extracted with chloroform-methanol 9:1 (v/v), transmethylated with 10% BF₃ in CH₃OH, and then subjected to gas-liquid chromatography (GLC) as described below. The arachi-
Arachidonic acid contents were determined using margaric acid methyl ester as an internal standard.

Analysis of labeled arachidonic acid in phospholipid methyl ester

In order to investigate the distribution of $^3$H radioactivity, the 1,2-diradyl-3-acetylgllycerols of PC and PE and other phospholipids were separated by TLC as mentioned above, and the areas corresponding to 1,2-diradyl-3-acetyl-glycerol and phospholipids were scraped off the TLC plate, to measure the radioactivity in a scintillation counter (Beckman, LS-9000) with scintillation liquid [toluene-Triton X-100-water-2,5-diphenyloxazole-2,2'-p-phenylenedioxybis(5-phenyloxazole) 800 ml:200 ml:50 ml:3.3 g:0.24 g] (15). Recovery of radioactivity from the plate was not less than 95%. Counting efficiency was determined with an external standard.

GLC analysis of fatty acids

The fatty acid methyl esters were determined by GLC (16). Peaks of each chromatogram were identified by comparison of their relative retention times with those of authentic standards. Analysis was carried out on a Shimazu GC-6A gas chromatograph with a glass column packed with 10% diethylenglycol succinate on Chromosorb W 80-100 mesh (Nihon Chromato Industrial Co., Tokyo). The flow rate of carrier nitrogen gas was approximately 50 ml/min. The column temperature was maintained at 200°C, and the injector and ion detector temperatures were 250°C.

RESULTS AND DISCUSSION

In a previous study (2), we reported the detailed analyses of phospholipid class composition and fatty acyl chain distribution in murine mastocytoma cells. Analyses of mastocytoma 1-O-alkyl-2-acyl-GPC and 1-O-alk-1'-enyl-2-acyl-GPE revealed a significant enrichment of arachidonic acid at the sn-2 position when compared among three subclasses of PC and PE. These previous results indicated that the heterogeneous distribution of arachidonic acid among glycerophospholipids may be due to the different metabolic activities of the molecular species of glycerophospholipids.

In the present study, for an understanding of the mechanism of preferential enrichment of arachidonic acid in 1-O-alkyl-2-acyl-GPC and 1-O-alk-1'-enyl-2-acyl-GPE, we investigated arachidonic acid mobilization among diacyl and ether phospholipids in $^3$H]arachidonic acid-labeled mastocytoma cells.

Incorporation of $^3$H]arachidonic acid into mastocytoma cells

The incorporation of $^3$H]arachidonic acid into intact mastocytoma cells was examined over a 1-hr time course. Results are shown in Fig. 1. Uptake of $^3$H]arachidonic acid with mastocytoma was very rapid and saturated at 30 min. In the present study, we chose 7.5 min for labeling cells to prevent rearrangement of $^3$H]arachidonic acid among phospholipids during a longer incubation time.

Distribution of arachidonic acid among phospholipid classes

The sn-1 positions of PC and PE fractions are composed principally of two saturated fatty acids, palmitic and stearic acids. In contrast, the sn-2 position is abundant in polyunsaturated fatty acids, especially oleic acid, linoleic acid, and arachidonic acid (2). Arachidonic acid distribution in individual phospholipids is shown in Table 1. PC and PE contained 30% and 47% of all arachidonic acids associated with phospholipids, respectively; its content in PI was 15%. As for the PC fraction, arachidonic acid contents were found to be high in 1-O-alkyl-2-acyl species (18%), and 1,2-diacyl species (11%) with a small amount in 1-O-alk-1'-enyl-2-acyl species (1%). In the PE fraction, on the other hand, the order of arachidonic acid content was as follows: 1-O-alk-1'-enyl-2-acyl (26%) > 1,2-diacyl (15%) > 1-O-alkyl-2-acyl (6%). A high content of arachidonic acid in 1-O-alkyl-2-acyl-GPC and 1-O-alk-1'-enyl-2-acyl-GPE has also been observed in rabbit neutrophils (17) and rabbit alveolar macrophages (5).

Changes in distribution of $^3$H]arachidonic acid among phospholipid classes

To observe the changes in distribution of arachidonic acid in phospholipids, mastocytoma cells were incubated with $^3$H]arachidonic acid for 7.5 min and then transferred into the radiolabel-free medium (0 time) for further incubation up to 300 min. Incubation of intact mastocytoma cells with $^3$H]arachidonic acid led to incorporation of this radiolabel...
TABLE 1. Distribution of arachidonic acid in phospholipid classes of mastocytoma P-815

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>Mol Percent</th>
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<tbody>
<tr>
<td>Phosphatidylethanolamine 1-2-Diacyl</td>
<td>15.0 ± 0.9</td>
</tr>
<tr>
<td>Phosphatidylethanolamine 1-0-Alkyl-2-acyl</td>
<td>5.7 ± 3.3</td>
</tr>
<tr>
<td>Phosphatidylethanolamine 1-O-Alkyl-2-acyl</td>
<td>26.2 ± 1.1</td>
</tr>
<tr>
<td>Phosphatidylylcholine     1-2-Diacyl</td>
<td>10.5 ± 2.7</td>
</tr>
<tr>
<td>Phosphatidylylcholine     1-0-Alkyl-2-acyl</td>
<td>18.3 ± 3.4</td>
</tr>
<tr>
<td>Phosphatidylylcholine     1-0'-eny1-2-acyl</td>
<td>1.3 ± 1.9</td>
</tr>
<tr>
<td>Phosphatidylserine        3.4 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Phosphatidylinositol      15.6 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin             4.1 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

Phospholipids and classes were separated as described under Materials and Methods. Arachidonic acid content was determined by GLC using margaric acid methyl ester as an internal standard. The results are presented as the mean ± standard deviation of three different animals.

into various phospholipids. Table 2 shows that almost all of the radioactivity of [3H]arachidonic acid incorporated into cells was recovered in the phospholipid fraction. The radioactivity in the neutral lipid plus the free fatty acid fraction was less than 3% of the total activity incorporated into cells. In mastocytoma cells, the extremely low level of free [3H]arachidonic acid is thought to be maintained by the combined action of arachidonoyl-CoA synthetase and acyltransferase activities promoting efficient esterification of free arachidonic acid into phospholipids. After 7.5 min of prelabeling, the majority of the radioactivity was recovered in PC (43%) followed, in decreasing order, by PI (32%), PE (20%), and PS (2%). No significant 3H radioactivity was observed in the medium during reincubation. The total radioactivity in the phospholipid and neutral lipid fractions was not significantly changed during incubation up to 300 min. However, the radioactivity in the PE fraction gradually increased with time, whereas that in PC concomitantly decreased. Little or no significant change was seen in PI. These findings strongly suggest the redistribution of [3H]arachidonic acid between the PC and PE fractions, i.e., the transfer from the former to the latter. However, from the data showing a decrease in PC, an increase in PE, and no change in PI we cannot rule out a transfer of [3H] radioactivity from PC to PI to PE. Actually, Irvine and Dawson (18), using liver microsomes and exogenous substrate, have reported a substantial transfer of radiolabeled arachidonic acid from PI to PC.

To investigate the different contributions of 1,2-diacyl and ether phospholipids in the arachidonic acid transfer, we treated PE and PC with phospholipase C and acetylated them, in order to separate 1-O-alkyl-2-acyl, 1-O-alkyl-1'-eny1-2-acyl, and 1,2-diacyl compounds. Changes in [3H]arachidonic acid distribution of the ether and diacyl subclasses of choline- and ethanolamine-containing phospholipids are shown in Fig. 2. As indicated in Table 2 (the 7.5-min radiolabeling (0 time) studies), [3H]arachidonic acid is preferentially directed into PC (43%), while ethanolamine phospholipids are less readily labeled (20%). The ethanolamine- as well as choline-containing ether phospholipids were poorly labeled at the beginning of incubation and most of the radioactivity was recovered in 1,2-diacyl-GPC and -GPE. The relative rates of incorporation of [3H]arachidonic acid into the choline and ethanolamine phospholipids indicate that, for both phospholipids, the ether and 1,2-diacyl classes represent distinct pools, with the latter species of phospholipids being favored over the former species. As for the PC (Fig. 2A), the level of radioactivity of 1,2-diacyl species was greatly decreased with progress of incubation time, concomitantly with its appreciable increase in the 1-O-alkyl-2-acyl species. The CoA independent transfer of arachidonate from PC to 1-O-alkyl-2-lyso-GPC has been demonstrated (19, 20). The 1-O-alkyl-1'-eny1-2-acyl species did not show any change. On the other hand, the PE fraction (Fig. 2B) displayed a distinctly different profile. No changes in the level of [3H]arachidonic acid were seen in 1,2-diacyl and 1-O-alkyl-2-acyl species. However, the radioactivity in 1-O-alkyl-1'-eny1-2-acyl compound showed significant enhancement. The transfer of arachidonate from PC to 1-acyl-2-lyso-GPE was reported.

TABLE 2. Changes in distribution of [3H]arachidonic acid in mastocytoma cell lipids

<table>
<thead>
<tr>
<th>Component</th>
<th>Time(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>mol %</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>31.8 ± 2.7</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>43.2 ± 4.1</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>20.2 ± 2.7</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Phosphatic acid</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

Cells were labeled for 7.5 min with [3H]arachidonic acid (2.1 μCi/2.1 × 10⁷ cells) and then transferred into the radiolabel-free medium (0 time) for further incubation. Samples were taken at indicated times for determination of the radioactivity in individual lipid fractions separated by TLC. The results are presented as the mean ± standard deviation of three different experiments.
Fig. 2. Changes in distribution of [3H]arachidonic acid. Mastocytoma cells were incubated with [3H]arachidonic acid (2.1 μCi/2.1 x 10⁷ cells) for 7.5 min, then washed with [3H]arachidonic acid-free medium, and reincubated for the time intervals indicated. PC and PE were resolved into their 1,2-diacyl and ether components as described under Materials and Methods, and contents of [3H]arachidonic acid were determined. (A) PC; (B) PE; (C) 1,2-diacyl type; (D) 1-O-alkyl-2-acyl type; (E) 1-O-alk-1′-enyl-2-acyl type. The results are presented as the mean ± standard deviation of three different experiments.

(21, 22), but this pathway does not operate in the mastocytoma cells. These alterations in [3H]arachidonate distribution may reflect the transfer of the fatty acid from the 1,2-diacyl species of PC. The loss of [3H]arachidonate radioactivity from 1,2-diacyl-GPC could be largely accounted for by its increase in 1-O-alk-1′-enyl-2-acyl-GPE and 1-O-alkyl-2-acyl-GPC. However, a small portion of [3H]arachidonate may be transferred to PI, as observed in Table 2. This suggests that [3H]arachidonate incorporation via the Hill and Lands pathway (23) occurs principally in the 1,2-diacyl phospholipids and that the label in the ether-linked phospholipids is incorporated by exchange of acyl chains at the sn-2 position of 1,2-diacyl-GPC. These observations provide supporting evidence for the hypothesis that mobilization of [3H]arachidonate into plasmenylethanolamine may occur by shuttling arachidonate from PC to the plasmalogen phospholipid (24). Two mechanisms can be considered for achieving this selective enrichment of arachidonate in 1-O-alkyl-2-acyl-GPC and 1-O-alk-1′-enyl-2-acyl-GPE. One is that, as Hirata et al. (25) have reported in stimulated lymphocytes, an active conversion of PE to PC takes place via stepwise methylation of PE with S-adenosylmethionine. However, this theory seems inconsistent with our results indicating that 1-O-alk-1′-enyl-2-acyl-GPC showed no change. The second is transacylation via transacylase acting on intact phospholipid molecule as seen in human platelets (24) and mouse lymphocytes and macrophages (26) or reacylation via a phospholipase A₂-acyltransferase system. However, we are unable to differentiate these two possibilities from the observations obtained in the present study.

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