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ASSESSMENT OF PHOSPHOLIPID DEACYLATION-REACYLATION CYCLES BY A STABLE ISOTOPE TECHNIQUE

Toyoyasu Kuwae, Patricia C. Schmid, and Harald H. O. Schmid

The Hormel Institute, University of Minnesota, Austin, MN 55912

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Incorporation of ^{18}O into glycerophospholipids was determined after incubating mouse peritoneal exudate cells for 1 or 2 h in media containing 40% $H_2{}^{18}O$. Gas chromatography-mass spectrometry of hydrogenated fatty acid methyl esters showed highest amounts of ^{18}O in choline phospholipids and phosphatidylinositol. Acyl groups generally present at the sn-1 position contained at least as much carbonyl ^{18}O as those at the sn-2 position. Considering the route of ^{18}O incorporation via free fatty acid derived through ester hydrolysis in $H_2{}^{18}O$, acyl turnover in certain peritoneal exudate cell phospholipids may equal or exceed 20% per h.

It is generally accepted that the glycerophospholipids of biological membranes undergo deacylation-reacylation through phospholipase and acyltransferase activities (1,2). These reactions are involved not only in the establishment and maintenance of phospholipid molecular species, but also, together with the synthesis and hydrolysis of acyl-CoA, in the regulation of the levels of lysophospholipids and free fatty acids (3). It is therefore important to quantify acyl turnover in intact cells or organelles. When deacylation occurs in a medium containing $H_2{}^{18}O$, the resulting fatty acid will contain ^{18}O in its carboxyl group. Subsequent reacylation will incorporate one-half of its ^{18}O into the carbonyl group of the newly formed ester. We have

Abbreviations: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TLC, thin-layer chromatography; GC, gas chromatography; MEM, minimum essential medium; PBS(-), Dulbecco's phosphate buffer minus Ca^{2+} and Mg^{2+}; PEC, peritoneal exudate cells; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

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used this approach to assess the turnover rates of phospholipid acyl groups in mouse peritoneal exudate cells (PEC), consisting primarily of macrophages, and report the results in the present communication.

Materials and Methods

Male mice (ICR strain, Charles River Laboratories, Inc. Wilmington, MA), 7-12 weeks old, were injected i.p. with 2 ml of sterile 3% aqueous thioglycolate (Brewer thioglycolate, Difco Laboratories, Detroit, MI) and sacrificed by bleeding 4 days later. Peritoneal exudate cells (PEC) were harvested by lavage of the peritoneal cavity twice with Dulbecco's phosphate buffer, PBS(-), containing 10 U heparin/ml, pH 7.2, excluding Ca^{2+} and Mg^{2+}. PEC preparations containing red blood cells were discarded. The cells were passed through stainless steel mesh (200 mesh) and pooled from 13-15 mice (2-2.5 x 10^7 cells/mouse). The pooled PEC were washed twice with Eagles minimum essential medium (MEM; Sigma Chemical Company, St. Louis, MO), pH 7.2, spun at 150 x g for 5 min and suspended (5 X 10^7 cells/ml) in complete MEM (containing 5% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 mM Hepes buffer, pH 7.2). More than 90% of the cells were viable by trypan blue exclusion and more than 80% of PEC were macrophages by Wright-Giemsa staining of smears.

In the ^{18}O incorporation experiments, PEC (10^8 cells/ml) were suspended in doubly-concentrated complete MEM. To 0.5 ml of cell suspension was added 0.4 ml of $H_2{}^{18}O$ (97.3 atom % ^{18}O; MSD Isotopes, Montreal, Canada) and 0.1 ml water in a plastic tube (2025 Falcon's tubes). The final suspension, containing 5 X 10^7 cells and 40% $H_2{}^{18}O$, was incubated for up to 2 h at 37°C under 95% oxygen/5% CO_2 in a shaking incubator. Thereafter, 6 ml of ice-cold PBS(-) was added and the cells were washed twice with the same medium. Lipids were extracted (4), assayed (5) for phosphorus (approximately 30 nmol lipid P/10^6 cells), redissolved in chloroform, and stored at -20°C until used.

Phospholipids were separated by two-dimensional thin-layer chromatography (TLC) on layers of silica gel H containing 7.5% magnesium acetate (6) using chloroform/methanol/conc. ammonia/water (65:35:5:1, by volume) in the first direction, and chloroform/acetone/methanol/acetic acid/water (30:40:10:7:5, by volume) in the second direction. Plates were exposed to iodine vapor to locate the lipid fractions and they were scraped off for phosphorus assay (5).

Individual phospholipid classes were isolated by preparative TLC on the same adsorbent, using chloroform/methanol/conc. ammonia/water (65:35:5:1, by volume) to obtain a fraction containing diphosphatidylglycerol, PE and PC, and fractions of pure PI and PS. Pure PE and PC were obtained by refractionation on regular silica gel H using chloroform/methanol/water (65:25:4, by volume). The fractions were made visible with 0.1% ethanolic 2',7'-dichlorofluorescein, scraped off, eluted from the adsorbent with chloroform/methanol/water (30:50:20, by volume), chloroform and water were added to the eluate, and the lower chloroform

layer was taken to dryness under N_2. Lipids were transesterified with 0.2 M methanolic NaOH (45°C, 1 h) for analysis of fatty acid methyl esters by gas chromatography (GC) using a Packard 428 gas chromatograph equipped with a flame ionization detector and a Spectra-Physics 4270 computing integrator. An aluminum column packed with 10% Alltech CS-10 on 100/120 Chrom W-AW (Alltech Associates) was programmed from 180 to 220°C at 3°C/min.

We have ascertained that this alkaline methanolysis procedure does not lead to an exchange of ester carbonyl oxygen and that catalytic hydrogenation of the methyl esters (PtO_2 in ethyl acetate, 40 psi H_2 for 3 h) simplifies the GC-mass spectrometric analysis (manuscript in preparation). GC-mass spectrometry was carried out with a DuPont DP-102 instrument equipped with a glass jet separator. A fused silica capillary column, 30 m X 0.25 mm, coated with SE-30 (Supelco) was used isothermally at 220°C to separate the hydrogenated fatty acid methyl esters. Helium at 1 ml/min was used as carrier gas and the outlet of the column was positioned into the jet separator. Spectra were recorded at 70 eV and areas of m/e 74 and 76 (methyl ester groups including both oxygens) were calculated and their ratios used to determine the natural abundance of ^{18}O and its incorporation under experimental conditions.

Results

The phospholipid composition of PEC (>80% intraperitoneal macrophages) is given in Table I. After 2 h of incubation, it remained essentially the same. Choline and ethanolamine phospholipids amount to about 65% of all glycerophospholipids. For the purpose of the present study, they were not subfractionated into alkylacyl, alkenylacyl, and diacyl species.

The fatty acid compositions of the four major classes of glycerophospholipids are listed in Table II. Palmitic (16:0) and

TABLE I
Phospholipid composition (%) of mouse peritoneal exudate cells before (A) and after (B) incubation for 2 h

	A	B
Phosphatidylcholine[a]	38.6 ± 1.2	39.4 ± 1.6
Phosphatidylethanolamine[a]	26.9 ± 0.7	25.8 ± 0.7
Phosphatidylserine	9.0 ± 0.3	8.8 ± 0.4
Phosphatidylinositol	5.0 ± 0.5	5.2 ± 0.3
Phosphatidic acid	0.4 ± 0.1	0.3 ± 0.1
Diphosphatidylglycerol + bis(monoacyl)glycerophosphate	4.5 ± 0.4	4.4 ± 0.6
Sphingomyelin	15.6 ± 1.3	16.2 ± 0.5

Cells were incubated and lipids extracted and analyzed as described in Experimental; values are mean ± S.D. of 4 independent analyses.

[a] Including diacyl, alkylacyl, and alkenylacyl species.

TABLE II

Constituent fatty acids (wt %) of major phospholipids before (A) and after (B) incubation

	PC[a]		PE[a]		PS		PI	
	A	B	A	B	A	B	A	B
16:0	34.9	36.5	7.8	8.2	9.1	9.4	7.7	7.1
16:1	2.2	2.3	0.5	0.5	0.7	0.7	0.5	0.6
17:0	0.7	0.7	0.7	0.7	0.9	0.9	0.7	0.8
18:0	8.6	9.5	21.9	21.9	41.8	41.8	40.2	39.2
18:1	17.9	17.7	10.0	9.9	13.8	14.1	11.2	9.5
18:2(n-6)	16.0	15.3	8.1	8.1	12.5	12.5	5.4	5.7
20:3(n-6)	1.4	1.6	1.9	2.0	2.7	2.7	1.6	2.1
20:4(n-6)	10.0	10.0	27.2	27.2	6.7	6.5	26.2	26.7
22:4(n-6)	2.2	1.7	6.9	7.0	4.1	4.2	2.3	2.6
22:5(n-6)	1.4	0.2	0.8	1.2	0.3	0.3	--	0.2
22:5(n-3)	2.0	1.6	5.8	5.5	3.7	3.5	2.3	2.8
22:6(n-3)	2.8	2.8	8.7	8.0	4.2	3.7	1.9	2.9

Phospholipids were isolated and their fatty acids analyzed as described in Experimental; values are averages of 4 independent analyses.

[a] Including diacyl, alkylacyl, and alkenylacyl species.

arachidonic acids (20:4) are the major representatives of the C_{16} and C_{20} fatty acids, whereas the C_{18} acids include 18:0, 18:1, and 18:2, and C_{22} acids consist of 22:4, 22:5, and 22:6.

Figure 1 shows the atom percent excess of ^{18}O in the acyl groups of the major phospholipids after 1 and 2 h of incubation

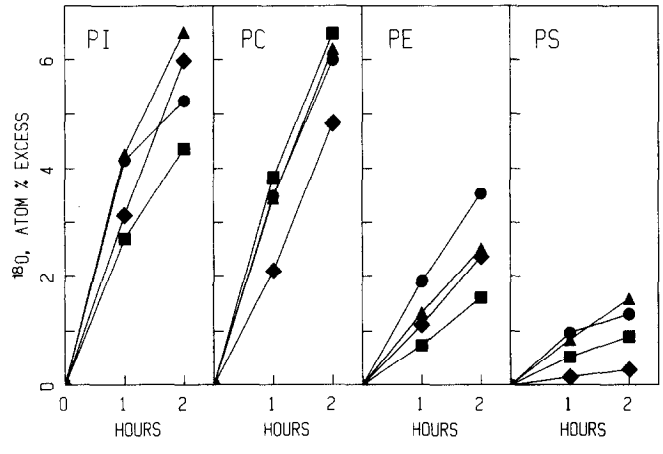

FIG. 1. Atom % excess of ^{18}O in ester carbonyl groups of phospholipids after 1 and 2 h of incubation in 40% $H_2{}^{18}O$. Fatty acid methyl esters were hydrogenated and analyzed by GC-mass spectrometry as described in Experimental. ●—● 16:0, ▲—▲ 18:0, ■—■ 20:0, ◆—◆ 22:0. Values are averages of 3 analyses from the same experiment; standard deviations were less than 5% of the mean.

in 40% $H_2{}^{18}O$. Phosphatidylinositol (PI) and phosphatidylcholine (PC) contain the highest amount of ^{18}O, distributed fairly evenly among fatty acids of different chain lengths. A 4% excess of ^{18}O after 1 h is equivalent to a 10% excess in 100% $H_2{}^{18}O$. We assume that ^{18}O can enter ester carbonyl only via free fatty acid and that free fatty acid can acquire carboxyl ^{18}O only through hydrolysis. Thus, each hydrolysis and reacylation should lead to the incorporation of only one-half of the newly acquired carboxyl ^{18}O. On that basis we can assume that "turnover" of acyl groups in PC and PI can be as high as 20% per h. After 2 h of incubation at 0°C, ^{18}O excess in acyl groups was only 0.01-0.1%. Our data indicate that ^{18}O uptake is high in palmitic acid which is expected to be esterified at the sn-1 position of glycerol. It is interesting to note that the percentage of ^{18}O in the C_{20} fatty acids, representing primarily arachidonic acid, is lowest in PE and PI and also relatively low in PS. This supports the concept of selective and direct transfer of the arachidonoyl group from PC to other phospholipids without hydrolysis to the free acid (7-11). Such transacylations would not introduce ^{18}O into these phospholipids until sufficient ^{18}O-containing 20:4 appears in PC.

Discussion

Incubations in $H_2{}^{18}O$ have previously been used for studies on the mechanism of action of lysophospholipase-transacylase from rat lung (12) and of snake venom phospholipases (13,14). Our present data represent the first attempt to use incubations in $H_2{}^{18}O$ for the assessment of phospholipid turnover, through deacylation-reacylation, in living cells. Quantitative interpretation is difficult since acyl hydrolysis can involve both glycerolipids and acyl-CoA, as well as other molecules

containing long-chain acyl groups. Obviously, de novo phospholipid synthesis and degradation beyond deacylation will also have to be taken into account. Our data also represent average values for the principal glycerophospholipids derived from all subcellular compartments and membranes. It can be expected that metabolically active compartments such as the endoplasmic reticulum exhibit higher than average acylation rates. The stable isotope technique reported here can be used to assess such differences without the problems encountered by the introduction of exogenous substrates or tracers.

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