Plasmalogen status influences docosahexaenoic acid levels in a macrophage cell line: insights using ether lipid-deficient variants

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Abstract   Previously, this laboratory reported the isolation of variants, RAW.12 and RAW.108, from the macrophage-like cell line RAW 264.7 that are defective in plasmalogen biosynthesis [Zoeller, R.A. et al. **1992.** *J. Biol. Chem.* 267: 8299–8306]. **Fatty acid analysis showed significant changes in the mutants in the ethanolamine phospholipids (PE), the only phospholipid class in which the plasmalogen species, plasmenylethanolamine, contributes significantly. Within the PE fraction, docosapentaenoic (DPA; 22:5n–3) and docosahexaenoic (DHA; 22:6n–3) acids were reduced by approximately 50% in the variants while the levels of arachidonic acid (AA; 20:4n–6) remained unaffected. The decrease in DHA was accompanied by a 50% decrease in labeling PE with [3H]DHA over a 90-min period. Restoration of plasmenylethanolamine by supplementing the growth medium with *sn*-1-hexadecylglycerol (HG) completely reversed these changes in RAW.108. Pre-existing pools of plasmenylethanolamine were not required for restoration of normal [3H]DHA labeling; addition of HG only during the labeling period was sufficient. Due to the loss of Δ1′-desaturase in RAW.12, HG supplementation resulted in the accumulation of plasmenylethanolamine's immediate biosynthetic precursor, plasmanylethanolamine. Even though this latter phospholipid contained only the ether functionality (lacking the vinyl ether double bond) it was sufficient to restore wild type-like fatty acid composition and DHA labeling of the ethanolamine phospholipids, identifying the ether bond as a structural determinant for this specificity. In summary, we have used these mutants to establish that the plasmalogen status of a cell can influence the levels of certain polyunsaturated fatty acids. These results support the notion that certain polyunsaturated fatty acids, such as DHA, can be selectively targeted to plasmalogens and that this targeting occurs during de novo biosynthesis, or shortly thereafter, through modification of nascent plasmalogen pools.**—Gaposchkin, D. P., and R. A. Zoeller. **Plasmalogen status influences docosahexaenoic acid levels in a macrophage cell line: insights using ether lipid-deficient variants.** *J. Lipid Res.* **1999.** 40: **495–503.**

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Ether-linked species are found as contributors to both ethanolamine phospholipids (PE) and choline phospholipids (PC) in a variety of animal cell types, including macrophages (1–3). In most tissues, the predominant ether-linked PC species is plasmanylcholine (1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine) while plasmenylethanolamine (1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine) is the primary ether-linked PE species. An exception to this is myocardium in which up to 35% of the PC is in the plasmenyl form. Any phospholipid, like plasmenylethanolamine or plasmenylcholine, that bears a vinyl ether (a *cis* double bond adjacent to the ether linkage) at the *sn*-1 position of the glycerol backbone is classified as a "plasmalogen." Putative functions for plasmalogens include regulation of certain PKC isozymes (4, 5), their possible role in membrane–membrane fusion events (6), and the ability to serve as endogenous antioxidants (7). It has also been suggested that plasmalogens serve as a reservoir for polyunsaturated fatty acids (8, 9) which, when released by a calcium-independent, plasmalogen-specific, PLA_2 ($iPLA_2$), can form bioactive molecules such as prostaglandins and leukotrienes (10).

Consumption of n–3 polyunsaturated fatty acids, major constituents of marine fish oils, have been shown to have numerous beneficial effects, including improved cardiovascular health (11–13), reduction of carcinogenesis (14–16), as well as amelioration of certain autoimmune diseases, such as rheumatoid arthritis (17–19). The two most abundant of these fatty acids are eicosapentaenoic acid (EPA; 20:5 n–3) and docosahexaenoic acid (DHA; 22:6 n–3). Several studies have found that fatty acids such as EPA and DHA have a high affinity for acylation to the *sn*-2 position of plasmalogen phospholipids (20–22). The influ-

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ence of the plasmalogen status of a cell on n–3 fatty acids is not known and factors which direct any specificity of acylation have not been determined. Studies of patients with inborn errors of plasmalogen biosynthesis revealed decreased levels of DHA in several tissues (23–25). However, this has been attributed to the loss of peroxisomal functions which is also associated with these patients.

Previously, our laboratory described the isolation of the variant lines, RAW.12 and RAW.108, from the murine, macrophage-like cell line RAW 264.7 (26). These cell lines lack ether lipids, including plasmalogens. RAW.108 lacks dihydroxyacetonephosphate acyltransferase (DHAP-AT) activity, which catalyzes the first step in the biosynthetic pathway of all ether lipids including plasmalogens. RAW.12 is deficient in DHAP-AT as well as $\Delta 1'$-desaturase. This latter activity catalyzes the final step in plasmalogen biosynthesis, inserting the vinyl ether double bond. Addition of *sn*-1-hexadecylglycerol (HG), to the growth medium of RAW.108, bypasses the defect in DHAP-AT, allowing ether-lipids, including plasmalogens, to be restored to wild-type levels. Supplementation of RAW.12 with HG, however, allows for the formation of 1-alkyl-phospholipids, but not alk-1'-enyl-phospholipids (plasmalogens) as the $\Delta 1'$-desaturase lesion is not bypassed (26). Therefore, when RAW.12 cells are incubated with HG they accumulate plasmanylethanolamine, instead of plasmenylethanolamine.

Using these two variant cell lines and HG supplementation, we can differentiate between the importance of the ether and the vinyl ether linkages in cell function. Also, as they possess functional peroxisomes, we do not have to consider peroxisome loss as a contributing factor in altered phenotypes. We were interested in seeing whether these plasmalogen-deficient, peroxisome-competent cells displayed alterations in fatty acid composition of the phospholipid species, with emphasis on the polyunsaturated fatty acids. The present results show that while arachidonic acid (AA) levels are unchanged, DHA levels are reduced in the PE fraction of RAW cells lacking plasmalogens. Furthermore, we demonstrate that it is the ether bond that is crucial for the specificity of DHA incorporation into plasmalogens in RAW cells; the vinyl ether double bond is not essential. These results suggest that loss of plasmalogen synthesis may contribute to the reduced DHA levels reported in patients with disorders of peroxisome biogenesis.

MATERIALS AND METHODS

Materials

[4,5-^{3}H]docosahexaenoic acid ([^{3}H]DHA; 58 Ci/mmol), [9,10-^{3}H]oleic acid ([^{3}H]OA; 9.2 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid ([^{3}H]AA; 100 Ci/mmol), [^{32}P]inorganic phosphate ([32]P$_i$; 9000 Ci/mmol) and EN[3]HANCE spray were purchased from DuPont-New England Nuclear (Boston, MA). Ecoscint A scintillation fluid was purchased from National Diagnostics (Atlanta, GA), Ham's F12 medium, penicillin–streptomycin solution, and fetal bovine serum were obtained from BioWhittaker (Walkersville, MD). *sn*-1-Hexadecylglycerol and *sn*-1-octadecenylglycerol were obtained from Serdary Research Laboratories

(Engelwood Cliffs, NJ). Solvents were HPLC grade from Fisher Scientific (Pittsburgh, PA). All phospholipid standards and carrier lipids were acquired from Avanti Polar Lipids (Alabaster, AL). Boron trifluoride was purchased from Supelco (Bellefonte, PA). Fatty acid methyl ester (FAME) standards were purchased from Matreya (Pleasant Gap, PA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and culture conditions

RAW 264.7 (ATCC TIB71) was purchased from the American Type Culture Collection. RAW.108 and RAW.12 are plasmalogen-deficient variants of the RAW 264.7 cell line (26); RAW.108 displays a deficiency in DHAP-AT activity, while RAW.12 is deficient in both DHAP-AT and $\Delta 1'$-desaturase (plasmanylethanolamine desaturase) activities. Cells were maintained in suspension in petri dishes (Valmark Inc., Canada) at 37°C in a humidified atmosphere of 5% CO_2/95% air in Ham's F12 medium containing 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 units/ml). This medium is referred to as F12c. For most experiments, the cells were plated into tissue culture dishes (Corning, New York) or sterile, glass scintillation vials (United Scientific; Quincy, MA), to which they adhered.

Phospholipid composition

Sterile 100-mm petri dishes containing 15 ml of F12c alone, or F12c containing 20 μm HG, were seeded with 7.5×10^5 cells. Three petri plates were used for each sample. After 48 h at 37°C, cells were harvested as a suspension, pelleted by centrifugation at 600 g for 7 min, and resuspended in PBS. The cells were washed twice with PBS in this manner and the final cell pellet was resuspended in 1 ml PBS. An aliquot from each sample was taken for determination of protein content by the method of Lowry et al. (27). The lipids were extracted by the method of Bligh and Dyer (28), spotted onto silica gel G plates (Analtech; Newark, DE), and developed using chloroform–methanol–acetic acid–H_2O 25:15:3:1.5 (v/v) with phospholipid standards run in parallel. Phospholipids were visualized by spraying with 50% sulfuric acid followed by heating. The areas corresponding to the various phospholipid species were scraped into acid-washed glass tubes and the phosphorus content of each was determined by the method of Rouser, Siakotos, and Fleischer (29).

Fatty acid analysis

Cells were plated and cultured, lipids were extracted, and TLC was performed as outlined above. Bands corresponding to the different phospholipids were scraped from the TLC plate and the lipids were eluted using chloroform–methanol 2:1. Samples were dried in a screw-capped test tube under a stream of nitrogen and trans-esterified with BF$_3$ in methanol (30) at 100°C for 1 h. Samples were cooled, 1.5 ml water was added, and the fatty acid methyl esters (FAME) were extracted twice with 3-ml portions of hexane. Samples were dried under a stream of nitrogen and resuspended in chloroform. FAME were separated using a SP-2330 fused silica capillary column (30 mm × 0.25 mm ID, 0.20 μm film; Supelco, Bellefonte, PA) in a Shimadzu 14A gas chromatograph with a flame ionization detector connected to a Shimadzu Chromatopac CR-501 data processing system. The column initial temperature was 150°C. The column temperature remained at 150°C for 2 min and then increased at the rate of 5.0°C per min until 240°C was attained. Individual FAME were identified by comparison of sample peak retention times to that of standard FAME mixtures.

Incorporation and distribution of radioactive fatty acids

Cells (2.5×10^5) were plated into sterile, glass scintillation vials in 1.0 ml F12c or F12c containing 20 μm HG. After 24 h at

37°C, medium was removed and replaced with 0.7 ml of F12c containing 2 μCi of carrier-free [3H]-labeled fatty acid, with or without 20 μm HG. Cells were incubated for 90 min at 37°C, labeling medium was removed, and the cell monolayers were washed twice with 3 ml of ice-cold F12c. Carrier lipid (200 μg of total heart extract) was added and lipids were extracted (28) directly from the monolayer while in the scintillation vial. Samples were dried under a stream of nitrogen and one half of each sample was loaded onto a silica gel 60 TLC plate (EM Science; Gibbstown, NJ). Phospholipid species were separated as described above. The plates were sprayed with EN³HANCE and exposed to X-ray film (GBX-2, Kodak; Rochester, NY) at −80°C. The bands of interest were scraped into scintillation vials containing 1 ml of methanol, 8 ml of scintillation fluid was added and radioactivity was determined by liquid scintillation spectroscopy. Parallel, unlabeled samples were used for protein determination (27).

To determine the distribution of fatty acids between ethanolamine phospholipid species, the remaining half of some samples were developed using 2-dimensional TLC to separate the plasmenyl and the diacyl species (31). Samples were loaded onto silica gel 60 TLC plates, run in the first dimension with chloroform–methanol–acetic acid–H_2O 25:15:3:1.5 (v/v), dried, and the area containing the phospholipids was sprayed with 10 mm $HgCl_2$ in acetic acid to cleave the vinyl ether bond. Once dry, the plates were developed in the second dimension with chloroform–methanol–formic acid 65:25:10 (v/v) to separate the unaffected diacyl species from the plasmalogen-derived sn-1-lyso compound. The plates were sprayed with EN³HANCE (DuPont-NEN) and exposed to X-ray film as above. Radioactive plasmenyl and diacyl bands were recovered from the plate and quantitated by liquid scintillation spectroscopy.

Phospholipid biosynthesis

Cells (5×10^5) were plated into sterile, glass scintillation vials in 1.0 ml F12c or F12c containing 20 μm HG. After 24 h at 37°C, medium was aspirated and 50 μCi of [$^{32}P_i$] in 1 ml F12c or F12c containing 20 μm HG was added. In some instances, HG was added only during labeling, while in other samples, HG was present both during labeling and the 24-h period prior to labeling. After 90 min at 37°C, labeling medium was removed, the monolayers were washed twice with 3 ml ice-cold F12c, and the lipids were extracted as above. The labeled phospholipids were separated using the two-dimensional TLC system described above, localized by autoradiography, and the radioactivity associated with each phospholipid was quantitated by liquid scintillation spectroscopy. Parallel, unlabeled samples were used for protein determination (27).

RESULTS

Phospholipid composition

The major ether phospholipid species in RAW 264.7 cells is plasmenylethanolamine, which makes up 36% of the PE fraction (26). In the ether lipid-deficient variants, RAW.12 and RAW.108, this value is reduced to approximately 5% (26). As shown in **Table 1**, this reduction had no effect upon the headgroup composition; the level of all headgroup species, including PE, remained normal when compared to wild-type cells. The cells likely compensated for the loss of plasmenylethanolamine biosynthesis by increasing phosphatidylethanolamine synthesis, as observed in other ether lipid-deficient variants (32–34). In previous work, it was shown that supplementation of the growth medium with sn-1-hexadecylglycerol (HG) by-

TABLE 1. Phospholipid composition of RAW cell lines

| | Levels of Phospholipid Headgroup Species | | |
	RAW 264.7	RAW.12	RAW.108
	nmol P_i/mg cell protein		
SM	16.26 ± 3.10	17.16 ± 2.30	17.61 ± 1.31
PC	67.63 ± 6.65	69.59 ± 2.47	65.80 ± 3.34
PI/PS	19.47 ± 1.95	20.09 ± 1.01	19.69 ± 3.97
PE	32.10 ± 2.72	27.60 ± 2.46	29.77 ± 0.99
Other PLs	9.91 ± 1.26	9.77 ± 1.60	9.19 ± 1.63

Cells were grown for 48 h in F12c prior to harvest and extraction of the lipids. Phospholipid species were separated and quantitated as described in Materials and Methods. Values represent the mean ± standard deviation of three samples. Abbreviations: SM, sphingomyelin; PC, choline phospholipids; PI/PS, combined inositol/serine phospholipids; PE, ethanolamine phospholipids; other PLs, phosphatidic acid, cardiolipin, and phosphatidylglycerol.

passed the lesion in DHAPAT and restored ether lipids to the variants (26). In the case of RAW.108, the plasmalogen, plasmenylethanolamine, was restored while in RAW.12 only the ether-linked precursor, plasmanylethanolamine was restored, due to the additional lesion in the Δ1′-desaturase. Supplementation with 20 μm HG had no effect on the phospholipid composition of any of the strains (not shown).

Fatty acid composition of phospholipid headgroup species

Previous analysis (26) showed that the dominant PC species in RAW 264.7 cells was phosphatidylcholine (diacyl) with the remainder (10%) found as plasmanylcholine (alkylacyl). There was no detectable plasmenyl species. Consistent with these data, there were no dimethylacetals (generated from the vinyl ether-linked alkyl chains) detected in the PC fraction in any of the cell lines whether they were supplemented with HG or not (**Table 2**). There was little difference between wild-type and mutant cells with respect to fatty acid composition of this phospholipid headgroup species. There was a minor decrease in the n–3 fatty acids, DPA (22:5 n–3) and DHA (22:6 n–3), in RAW.12 and RAW.108 although the levels of these fatty acids are low in wild-type cells (1.67 and 2.38% of the total fatty acids, respectively). Culturing the cells in the presence of HG, to restore ether phospholipids, had no consistent effects on the levels of the DPA or DHA in the PC fraction.

Similar to PC, there was little or no plasmenyl species within the combined serine/inositol phospholipid fraction (PI/PS). Also, like PC, the fatty acid composition of the PI/PS fraction remained unaltered in the mutants and HG supplementation had no effect (data not shown).

There were three significant changes in the fatty acid composition of the PE fraction (**Table 3**). First, as expected, dimethyl acetals were detected in RAW 264.7 (primarily the 16:0 and 18:0 species), while no DMAs were detected in the variants. We were a bit surprised to find that there was no 18:1 DMA in wild-type cells as analysis of murine macrophages have shown this species to be a significant contributor to the DMA population (3). When cells were incubated with octadecenylglycerol, a putative 18:1 DMA peak appeared just ahead of 18:1 n–9 fatty acid methyl ester (not

TABLE 2. Fatty acid composition of choline phospholipids in RAW cell lines

	FAME and DMA Levels		
	RAW 264.7	RAW.12	RAW.108
	% total mass		
−HG			
14:0	2.12 ± 0.17	0.98 ± 0.20	1.63 ± 0.33
16:0 DMA	N.D.	N.D.	N.D.
16:0	30.27 ± 0.51	27.20 ± 0.57	34.23 ± 2.56
16:1	10.31 ± 0.83	8.05 ± 0.60	10.80 ± 1.4
18:0 DMA	N.D.	N.D.	N.D.
18:0	9.90 ± 0.16	9.45 ± 0.51	13.69 ± 3.07
18:1 n–9	26.73 ± 0.81	32.62 ± 0.33	24.88 ± 1.71
18:1 n–7	7.35 ± 0.31	9.67 ± 0.15	6.85 ± 0.48
18:2 n–6	2.32 ± 0.10	2.14 ± 0.09	1.74 ± 0.12
20:4 n–6	4.37 ± 0.50	4.24 ± 0.06	2.98 ± 0.56
20:5 n–3	0.43 ± 0.03	0.45 ± 0.02	N.D.
22:4 n–6	0.42 ± 0.07	0.24 ± 0.21	N.D.
22:5 n–3	1.67 ± 0.32	1.47 ± 0.04	0.81 ± 0.22
22:6 n–3	2.38 ± 0.13	1.51 ± 0.07	0.76 ± 0.22
+HG			
14:0	0.79 ± 0.73	1.33 ± 0.05	1.15 ± 0.30
16:0 DMA	N.D.	N.D.	N.D.
16:0	24.98 ± 0.34	20.90 ± 1.40	27.27 ± 1.79
16:1	10.25 ± 2.00	9.79 ± 0.71	11.84 ± 1.52
18:0 DMA	N.D.	N.D.	N.D.
18:0	9.89 ± 1.27	7.39 ± 0.45	10.85 ± 0.87
18:1 n–9	35.84 ± 2.79	38.96 ± 0.10	29.99 ± 1.74
18:1 n–7	7.04 ± 0.73	9.47 ± 0.42	5.98 ± 0.40
18:2 n–6	2.75 ± 0.10	2.35 ± 0.23	2.44 ± 0.19
20:4 n–6	3.10 ± 0.38	3.64 ± 1.14	4.76 ± 1.12
20:5 n–3	0.35 ± 0.08	0.12 ± 0.21	0.55 ± 0.48
22:4 n–6	0.56 ± 0.01	0.75 ± 0.40	0.19 ± 0.32
22:5 n–3	1.19 ± 0.25	1.63 ± 0.78	1.89 ± 0.62
22:6 n–3	0.98 ± 0.28	1.08 ± 0.61	1.82 ± 0.77

Cells were cultured for 48 h in F12c medium in the absence (−HG) or the presence (+HG) of *sn*-1-hexadecylglycerol. Lipids were extracted and the phospholipids were isolated and transmethylated as described in Materials and Methods. Dimethylacetal (DMA) species resulting from the vinyl ether-linked alkyl chains are designated as such. Numbers represent the molar percent of total identified fatty acids as the mean ± standard deviation of three samples with the exception of RAW 264.7 −HG, in which 5 samples were used. The values for other fatty acids, which together accounted for less than 2% of the total fatty acid mass, were not included. These were 18:3, 20:1, 20:2, and 20:3. The levels of these fatty acids did not vary between cell lines; N.D. = not detectable.

TABLE 3. Fatty acid composition of ethanolamine phospholipids in RAW cell lines

	FAME and DMA Levels		
	RAW 264.7	RAW.12	RAW.108
	% total mass		
−HG			
16:0 DMA	5.59 ± 0.99	N.D.	N.D.
16:0	7.79 ± 1.15	10.36 ± 0.18	11.90 ± 0.55
16:1	4.17 ± 0.75	N.D.	N.D.
18:0 DMA	0.99 ± 0.18	1.26 ± 0.11	1.34 ± 0.08
18:0	22.84 ± 0.66	29.00 ± 1.27	32.82 ± 1.68
18:1 n–9	12.96 ± 1.45	20.25 ± 0.17	18.25 ± 0.28
18:1 n–7	3.80 ± 0.36	4.79 ± 0.05	4.06 ± 0.18
18:2 n–6	0.89 ± 0.08	1.26 ± 0.04	1.38 ± 0.01
20:4 n–6	18.57 ± 1.09	17.36 ± 0.46	17.60 ± 1.39
20:5 n–3	1.52 ± 0.18	1.30 ± 0.07	1.36 ± 0.13
22:4 n–6	1.53 ± 0.11	1.34 ± 0.05	0.72 ± 0.06
22:5 n–3	7.70 ± 0.45	4.76 ± 0.28	3.63 ± 0.36
22:6 n–3	10.11 ± 0.80	7.16 ± 0.49	5.95 ± 0.77
+HG			
16:0 DMA	10.28 ± 2.05	3.96 ± 1.62	12.17 ± 2.46
16:0	7.98 ± 0.56	7.83 ± 21.41	13.84 ± 5.62
16:1	0.63 ± 0.21	N.D.	1.02 ± 0.29
18:0 DMA	3.04 ± 0.07	N.D.	N.D.
18:0	19.10 ± 2.71	23.39 ± 5.10	19.04 ± 2.02
18:1 n–9	10.78 ± 0.03	15.34 ± 0.90	9.62 ± 0.49
18:1 n–7	2.81 ± 0.07	3.43 ± 0.57	2.75 ± 0.11
18:2 n–6	0.73 ± 0.04	0.24 ± 0.42	0.42 ± 0.36
20:4 n–6	18.41 ± 0.18	20.10 ± 1.48	17.26 ± 2.12
20:5 n–3	1.80 ± 0.12	1.33 ± 0.17	1.39 ± 0.28
22:4 n–6	3.01 ± 0.02	4.17 ± 0.64	2.87 ± 0.37
22:5 n–3	9.31 ± 0.11	9.31 ± 1.74	9.04 ± 1.70
22:6 n–3	10.76 ± 0.44	10.47 ± 2.50	10.40 ± 2.35

Cells were cultured for 48 h in F12c medium in the absence (−HG) or the presence (+HG) of *sn*-1-hexadecylglycerol. Lipids were extracted, and the phospholipids were isolated and transmethylated as described in Materials and Methods. Dimethylacetal (DMA) species resulting from the vinyl ether-linked alkyl chains are designated as such. Numbers represent the molar percent of total identified fatty acids as the mean ± standard deviation of three samples with the exception of RAW 264.7 − HG, in which 5 samples were used. The values for other fatty acids, which together accounted for less than 2% of the total fatty acid mass, were not included. These were 14:0, 18:3, 20:1, 20:2, and 20:3. The levels of these fatty acids did not vary between cell lines; N.D. = not detectable.

shown). Therefore, it seems unlikely that this DMA species was lost within other peaks. In RAW.108 supplemented with HG, wild-type levels of 16:0 DMA were present, demonstrating that HG bypassed the DHAP-AT deficiency phenotype in these cells. The loss of Δ1′-desaturase activity in RAW.12 cells prevented HG from recovering plasmenylethanolamine and, therefore, only a small amount of 16:0 DMA was observed in the HG-supplemented RAW.12 cells. Second, the relative levels of the saturated fatty acids, palmitate (16:0) and stearate (18:0), and the monounsaturated fatty acid, oleate (18:1 n–9; OA), were elevated in the variants. Finally, in both mutant cell lines there was a 50% reduction of the n–3 fatty acids DPA and DHA whereas levels of another n–3 fatty acid, eicosapentaenoate (20:5 n–3, EPA), appeared unchanged. Surprisingly, there were no changes in the amount of arachidonate (20:4 n–6; AA).

Restoration of ether-linked species to the ethanolamine phospholipids by supplementation with HG restored the fatty acid profile to near wild-type. Of particular note was that both DPA and DHA were returned to wild-type levels. As there were no changes in the phospholipid composition of the mutants, the differences in fatty acid composition of the ethanolamine phospholipids represent differences in mass.

Uptake of fatty acids into phospholipids

We measured the uptake and distribution of three fatty acids into the different phospholipid headgroup classes. The choice of these fatty acids was based upon changes within the PE fraction of the mutants: DHA (decreased), AA (remained unchanged), and OA (increased).

In wild-type cells, the majority of the radioactivity from [3H]DHA was found associated with the PE fraction over a 90-min labeling period (**Fig. 1**) and there was little redistribution of label among phospholipid classes after a 24-h incubation with unlabeled medium (not shown). Uptake of [3H]DHA into PE was reduced by 40–45% in the variants and restoration of ether-linked PE with HG supple-
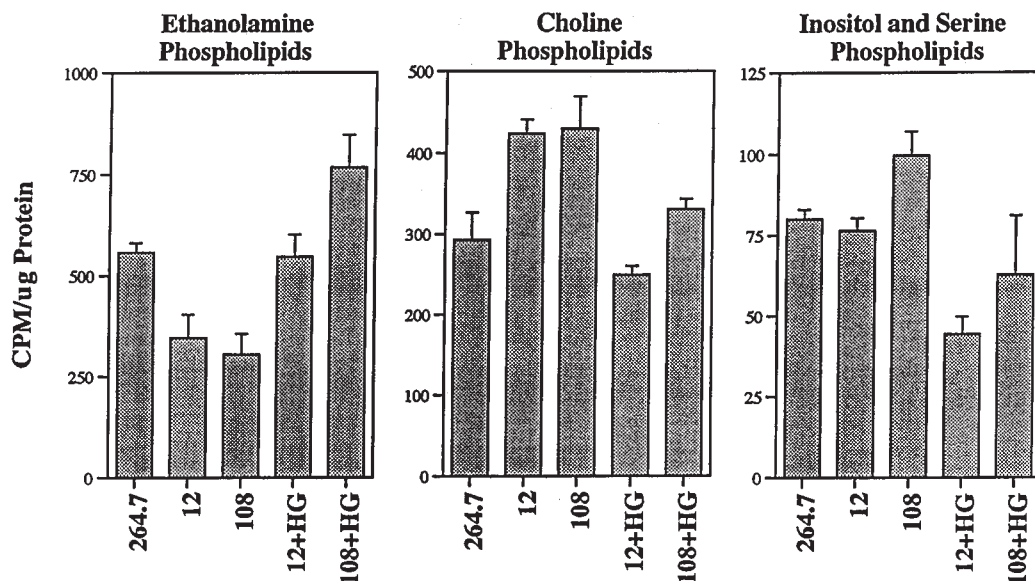
Fig. 1. Incorporation of [³H]DHA into phospholipids. Cells were labeled for 90 min with the radioactive fatty acid and its distribution into the different phospholipid classes was measured as described in Materials and Methods. In the HG-treated cells (+HG), the cells were incubated with medium containing 20 μm HG for 24 h prior to and during the 90 min of labeling. All values represent the average ± standard deviation of three samples.

mentation reversed this. The opposite was observed with respect to uptake into PC; DHA uptake was increased in the variants and this was reversed with HG supplementation, although the differences were not as dramatic. Incorporation into the PI/PS fraction was low and there were no consistent changes relating to the presence of ether lipids although HG supplementation did reduce the labeling in both RAW.12 and RAW.108.

When the PE subspecies were separated, DHA displayed a preference for distribution into the plasmenyl species (**Table 4**). In wild-type cells, 55% of the [³H]DHA was found associated with plasmenylethanolamine even though the ether-linked species represents 36% of the PE mass (26). In RAW.108 cells, 4.9% of PE was plasmenylethanolamine (26), yet 28% of [³H]DHA incorporated into PE entered plasmenylethanolamine. When RAW.108 cells

were supplemented with HG, 73% of DHA entering PE was associated with plasmenylethanolamine.

In the RAW cells, the distribution of [³H]AA was not greatly affected by the ether lipid status of the cell (**Fig. 2**). The majority of label from [³H]AA after a 90-min pulse was associated with PC. Although there was a slight reduction of the amount of AA incorporated into the PE fraction of the mutant cells, it was not restored upon supplementation with HG.

Separation of the PE subspecies revealed that the distribution of [³H]AA was more in concert with the relative amounts of plasmenylethanolamine (Table 4). In wild-type cells, 42% of the label was associated with plasmenylethanolamine, closer to the 36% value obtained for relative mass contribution obtained earlier (26). Also, the plasmenylethanolamine-deficient RAW.108 showed only 12% of the label associated with plasmenylethanolamine.

Labeling of the cells with [³H]OA resulted in the majority of the label associated with the PC fraction. We could find no consistent differences between variant and wild-type cells with respect to the amount of [³H]OA incorporated into any of the phospholipid fractions (**Fig. 3**). There was, however, a 41–53% decrease in the amount of label incorporated into PE in all three cell lines which had been supplemented with HG compared to unsupplemented cells. There were no consistent changes upon addition of HG in the PC and PI/PS fractions.

DHA uptake is influenced by de novo plasmenylethanolamine biosynthesis

We were able to rapidly stimulate the rate of synthesis of plasmenylethanolamine in RAW.108 cells by adding HG to the medium (**Fig. 4**). While plasmenylethanolamine biosynthesis in RAW.108 was only 23% that of wild-

TABLE 4. [³H]DHA and [³H]AA recovered in plasmenylethanolamine

| | Plasmalogen Labeling | | |
	[³H]DHA	[³H]AA	Plasmalogen Mass
	% PE-associated radioactivity		*% of total PE mass*
RAW 264.7	55.16 ± 1.86	42.89 ± 8.29	36
RAW.108	28.55 ± 1.78	12.60 ± 2.22	5
RAW.108 + HGz	73.18 ± 4.01	38.97 ± 2.36	—

Cells were labeled with [³H]DHA or [³H]AA and the labeled phospholipids were separated as described in Materials and Methods. These values are presented as the percent of PE-associated label that was found in the plasmenyl fraction. The remainder was found associated with the diacyl fraction. In the HG-treated cells (+HG), the cells were incubated with medium containing 20 μm HG for 24 h prior to and during the 90 min of labeling. Values represent the mean ± SD of three samples. The plasmalogen mass was determined previously (26).
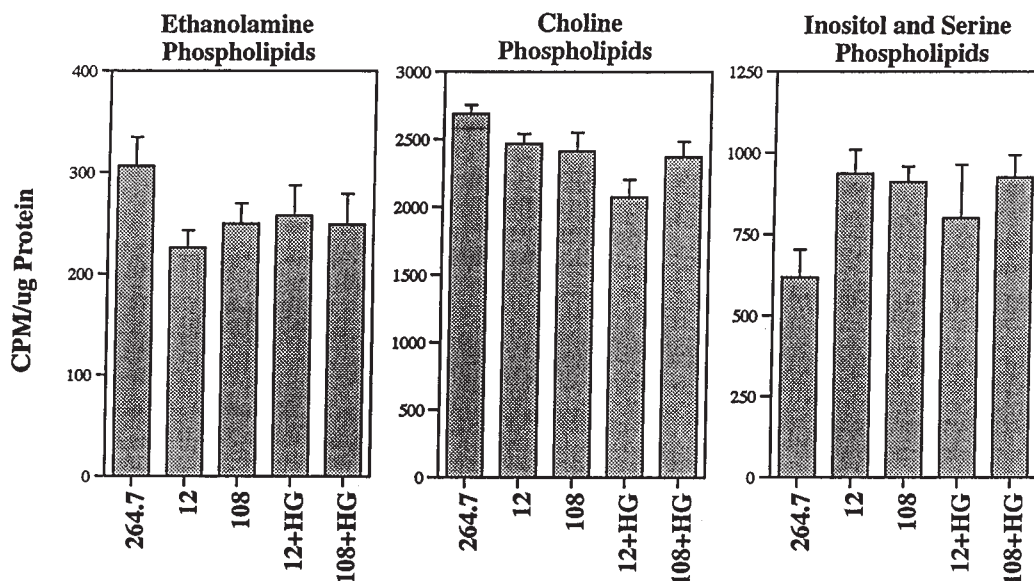
Fig. 2. Incorporation of [³H]AA into phospholipids. Cells were labeled for 90 min with the radioactive fatty acid and its distribution into the different phospholipid classes was measured as described in Materials and Methods. In the HG-treated cells (+HG), the cells were incubated with medium containing 20 μm HG for 24 h prior to and during the 90 min of labeling. All values represent the average ± standard deviation of three samples.

type cells in unsupplemented medium (−/− HG), supplementation with HG prior to and during the labeling (+/+ HG) resulted in complete recovery of plasmenylethanolamine biosynthesis. Importantly, we could almost completely restore plasmenylethanolamine biosynthesis by the addition of 20 μm HG (−/+ HG) only at the time of $^{32}P_i$ addition.

Similar to the short-term $^{32}P_i$-labeling, the addition of HG only during the 90-min labeling with [³H]DHA resulted in a correction of the labeling pattern to wild-type (**Fig. 5**). Therefore, the recovery of plasmalogen biosynthesis was sufficient for directing DHA to plasmenylethanolamine; pre-existing plasmenylethanolamine pools were not required.
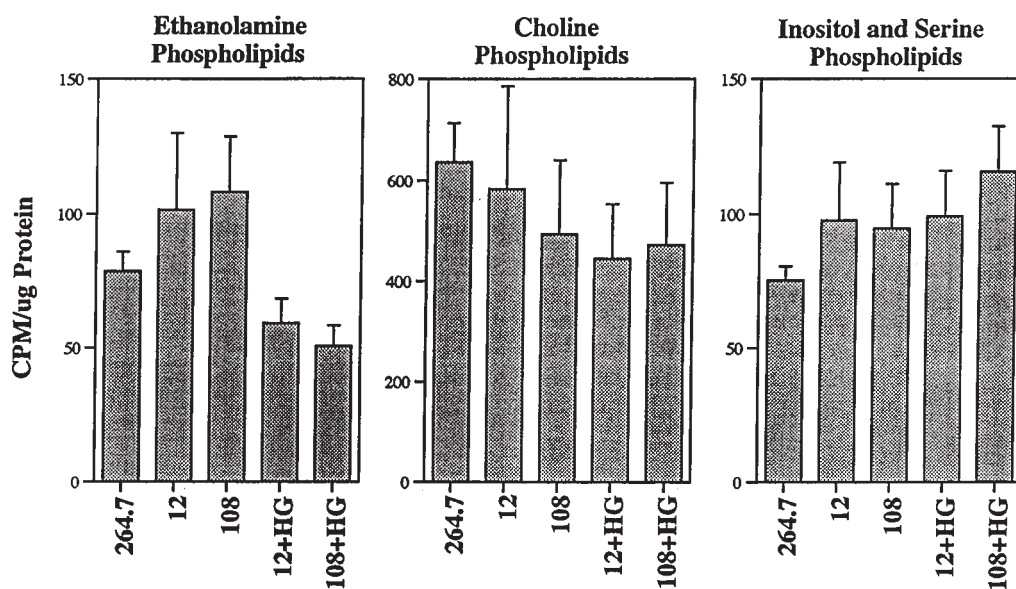

Fig. 3. Incorporation of [³H]OA into phospholipids. Cells were labeled for 90 min with the radioactive fatty acid and its distribution into the different phospholipid classes was measured as described in Materials and Methods. In the HG-treated cells (+HG), the cells were incubated with medium containing 20 μm HG for 24 h prior to and during the 90 min of labeling. All values represent the average ± standard deviation of three samples.
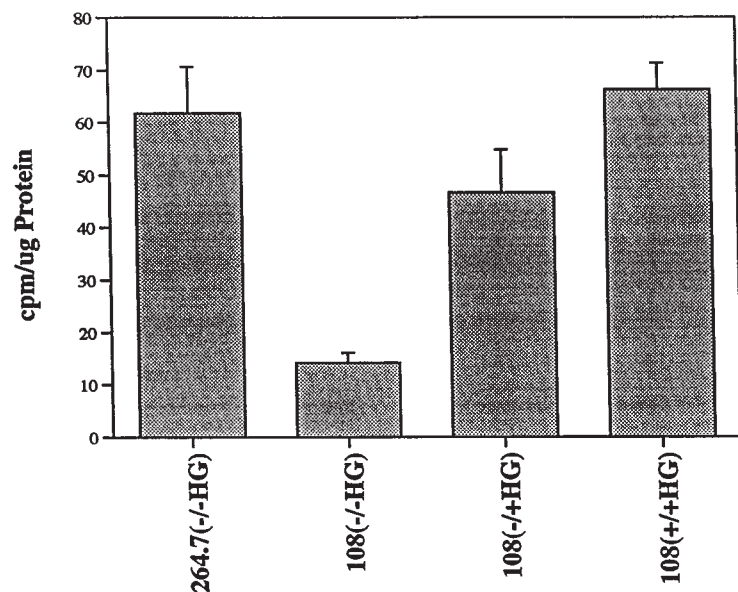
Fig. 4. Plasmalogen biosynthesis in RAW 264.7 and RAW.108. Cells were labeled for 90 min with [$^{32}P_i$] at 37°C and the labeling of plasmenylethanolamine was measured as described in Materials and Methods. HG was not present in the medium ($-/-$ HG), added only during the labeling period ($-/+$ HG), or present for 24 h prior to and during the 90 min of labeling ($+/+$ HG). All values represent the average ± standard deviation of three samples.

DISCUSSION

Many studies examining the fatty acid composition of plasmalogen phospholipids have demonstrated high levels of polyunsaturated fatty acids when compared with their diacyl-counterparts (8, 9). Other studies have demonstrated that both n–6 and n–3 fatty acids are incorporated preferentially into plasmalogens (35–41). These observations have caused speculation that one of the functions of plasmalogens is to serve as a sink for polyunsaturated fatty acids and to maintain high levels of these fatty acids in some tissues.

Our data support this notion, at least with respect to certain n–3 fatty acids. The only phospholipid head group class in which plasmalogens were a major contributor, PE, was enriched in polyunsaturated fatty acids in the RAW cells; the loss of plasmalogens in two, independently isolated mutants led to a reduction of DPA and DHA levels in this head-group class. Labeling studies demonstrated preferential distribution of DHA into plasmenylethanolamine and incorporation of DHA into PE was reduced in the mutants. Finally, restoration of plasmalogens, or ether lipids, through supplementation with HG, completely reversed these changes so that the variants resembled wild-type cells.
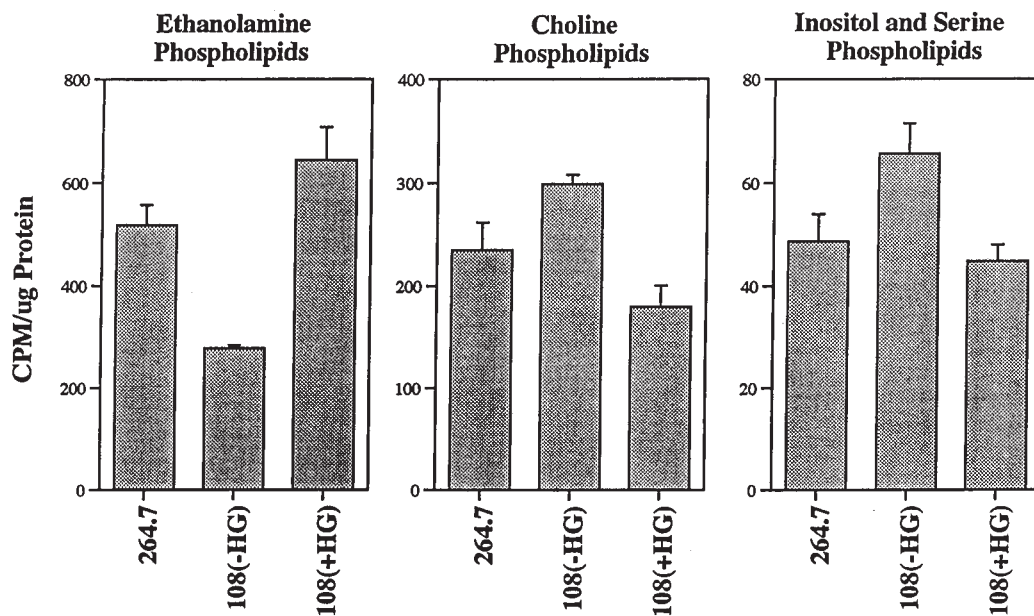

Fig. 5. Incorporation of [^{3}H]DHA into phospholipid classes during formation of nascent plasmalogens. Cells were labeled for 90 min with the radioactive fatty acid and its distribution into the different phospholipid classes was measured as described in Materials and Methods. In the HG-treated cells (+HG), the cells were incubated with medium containing 20 μm HG only during the 90 min of labeling. All values represent the average ± standard deviation of three samples.

We expected plasmalogen loss to severely affect the levels and distribution of AA in the variants. Surprisingly, this was not the case. While AA levels are high in PE in wild-type cells, the loss of plasmalogens had no effect on the levels in PE or any head-group class. Short-term labeling studies showed no tendency of AA to distribute preferentially into plasmenylethanolamine versus phosphatidylethanolamine and AA uptake into PE was actually increased in the variants. These results are probably not extendable to all cell types. Kidney and plasma of patients with peroxisome/plasmalogen deficiency displayed reduced arachidonate levels (23–25).

The unique lesions associated with these mutants, particularly RAW.12, have allowed us to make two observations concerning a mechanism for directing DHA to plasmenylethanolamine. First, the HG-supplementation studies revealed that the ether bond was the crucial structural element associated with specificity; the vinyl ether double bond was not required for recognition. Second, the labeling studies demonstrated that the preferential uptake of DHA into plasmenylethanolamine did not require the existence of established plasmalogen pools; DHA labeling of PE in RAW.12 and RAW.108 was restored when HG was present only at the time of labeling, pre-supplementation was not required. We can, therefore, speculate that specificity occurs at an early stage during plasmenylethanolamine biosynthesis, such as at the acylation of 1-alkyl-2-lyso-*sn*-glycero-3-phosphate or shortly after plasmenylethanolamine biosynthesis, on nascent pools, through rapid deacylation/reacylation or transacylation events. Evidence exists for both possibilities; Fleming and Hajra (42) presented evidence for a putative 1-alkyl-*sn*-glycero-3-phosphate:acyl-CoA acyltransferase in rat brain microsomes that demonstrated a preference for polyunsaturated fatty acyl-CoA, while Pageaux et al. (43) demonstrated a redistribution of radioactive DHA into plasmenylethanolamine after initial uptake into diacyl phospholipids.

Our findings may help to explain the decreases in the levels of certain polyunsaturated fatty acids in tissues from patients with inborn errors in peroxisome biogenesis and function such as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease (23–25). In all tissues examined from these patients, the levels of DHA are decreased, particularly in the ethanolamine phospholipids. The fact that peroxisomes are important for the biosynthesis of DHA from precursor fatty acids in animal cells has led to the suggestion that the loss of peroxisomal function is the major factor associated with the decrease in DHA levels in these patients (44). However, other factors may be important. Both DHAP-AT and alkyl-DHAP synthase, which catalyze the first two steps in plasmalogen biosynthesis, are peroxisomal (45, 46). These activities are lost in the patients and a severe reduction in plasmalogen levels results (47, 48). Our findings, using the peroxisome-competent, plasmalogen-deficient mutants suggests that the plasmalogen deficiency associated with these disorders may also contribute to decreased DHA levels.

Because DHA has a wide range of biological effects, some of the pathology associated with peroxisomal disorders has been attributed to reduced levels of this fatty acid (49, 50). Although dietary supplementation with DHA or fish oil raised patients' DHA levels, this therapy yielded mixed results in alleviating their symptoms (51), suggesting that increasing the levels of DHA alone may not be sufficient. Plasmalogens probably play important roles in other cell functions. For example, the presence of DHA or other polyunsaturated fatty acids, within plasmalogen pools, may be important for their use in biological processes. A number of investigators have reported the existence of plasmalogen-specific phospholipase A_2 activities (10, 52–54). It is possible that DHA and/or other fatty acids require release through activation of a plasmalogen-specific phospholipase as a requisite for biological activity. Plasmalogens have also been linked to signaling processes (4, 54, 55). Finally, several studies have suggested that plasmalogens protect cells, lipoproteins, and membranes against reactive oxygen species (7, 56, 57). It is possible that these functions have a bearing on some of the pathology associated with the plasmalogen-deficiency disorders.

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