Selective Incorporation of Docosahexaenoic Acid into lysobisphosphatidic Acid in Cultured THP-1 Macrophages

Nelly Besson[a], Francoise Hullin-Matsuda[a,b], Asami Makino[b], Motohide Murate[b], Michel Lagarde[a], Jean-Francois Pageaux[a], Toshihide Kobayashi[a,b], and Isabelle Delton-Vandenbroucke[a,*]

[a]Institut National de la Santé et de la Recherche Médicale (INSERM) UMR 585, Institut National des Sciences Appliquées (INSA)-Lyon, Institut Multidisciplinaire de Biochemie des Lipides, 69621 Villeurbanne, France, and [b]RIKEN, Saitama 351-0198, Japan

ABSTRACT: Lysobisphosphatidic acid (LBPA) is highly accumulated in specific domains of the late endosome and is involved in the biogenesis and function of this organelle. Little is known about the biosynthesis and metabolism of this lipid. We examined its FA composition and the incorporation of exogenous FA into LBPA in the human monocytic leukemia cell line THP-1. The LBPA FA composition in THP-1 cells exhibits an elevated amount of oleic acid (18:1n-9) and enrichment of PUFA, especially DHA (22:6n-3). DHA supplemented to the medium was efficiently incorporated into LBPA. In contrast, arachidonic acid (20:4n-6) was hardly esterified to LBPA under the same experimental conditions. The turnover of DHA in LBPA was similar to that in other phospholipids. Specific incorporation of DHA into LBPA was also observed in baby hamster kidney fibroblasts, although LBPA in these cells contains very low endogenous levels of DHA in normal growth conditions. Our results, together with published observations, suggest that the specific incorporation of DHA into LBPA is a common phenomenon in mammalian cells. The physiological significance of DHA-enriched LBPA is discussed.

Paper no. L9882 in *Lipids 41*, xxx–xxx (February 2006).

During atherogenesis, peripheral blood monocytes traverse the arterial endothelium and differentiate into macrophages. Much of the vessel wall lesion of atherosclerosis is composed of macrophages that have become engorged with cholesterol (1,2). Cells acquire cholesterol by receptor-mediated endocytosis of LDL or oxidized LDL. After endocytosis, LDL and oxidized LDL are first transported to early endosomes and then late endosomes/lysosomes (3). The molecular mechanism of cholesterol transport from late endosomes/lysosomes is not well understood. The characteristic feature of the late endosome is the accumulation of internal membranes (4). Recently it was shown that these internal membranes are highly enriched with a unique phospholipid (PL), lysobisphosphatidic acid (LBPA), also called bis(monoacylglycerol) phosphate (5,6). Antibodies against LBPA, when internalized from the medium, alter both the organization of internal membranes and the membrane traffic from late endosomes (7,8). These results suggest that LBPA-rich internal membrane domains contribute to membrane sorting and/or trafficking from late endosomes. Accumulation of cholesterol in late endosomes in Niemann–Pick type C (NPC) cells and in drug-treated cells that mimic NPC impaired membrane traffic from late endosomes (9–11). Treatment of the cells with anti-LBPA antibody also resulted in a massive accumulation of cholesterol in late endosomes, suggesting that the characteristic network of LBPA-rich membranes contained within multivesicular late endosomes regulates cholesterol transport (9).

LBPA is a structural isomer of phosphatidylglycerol. Whereas phosphatidylglycerol binds two acyl groups in one glycerol, LBPA contains one FA in each glycerol. In addition, LBPA has an *sn*-1, *sn*-1′ glycerophosphate backbone, instead of *sn*-3 stereoconfiguration, which is a common structure of all other PL (12,13). The FA composition of LBPA varies among cell types. In baby hamster kidney (BHK) cells, more than 90% of FA in LBPA is oleic acid (OA; 18:1) (6,14) whereas pulmonary alveolar macrophages efficiently incorporate arachidonic acid (AA; 20:4) (15–17). DHA (22:6) is selectively incorporated into LBPA in rat uterine stromal cells (18). DHA attracts special attention since epidemiological, animal, and clinical studies all indicate that DHA has potent anti-inflammatory and antiatherogenic properties (19,20). It is reported that membranes enriched with DHA-containing PL exclude cholesterol (21–24). Phorbol ester-treated THP-1 monocytes are a widely used model in studying cholesterol homeostasis in human macrophages (25–27). In the present study, we characterized the content, distribution, and FA composition of LBPA in THP-1 cells. Our results indicate that LBPA selectively incorporates DHA into THP-1 cells. Our results also suggest that the preferential incorporation of DHA is a common phenomenon in LBPA.

*To whom correspondence should be addressed at UMR585 INSERM, INSA (Institut National des Sciences Appliquées)-Lyon, 20 Ave A. Einstein, 69621 Villeurbanne, France. E-mail: isabelle.vandenbroucke@insa-lyon.fr

Abbreviations: AA, arachidonic acid; BHK, baby hamster kidney; EtnGpl, ethanolamineglycerophospholipid; INSA, Institut National des Sciences Appliquées; INSERM, Institut National de la Santé et de la Recherche Médicale; LBPA, lysobisphosphatidic acid; MUFA, monounsaturated FA; NPC, Niemann–Pick type C; OA, oleic acid; PL, phospholipids; PMA, phorbol myristate acetate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

MATERIAL AND METHODS

Materials. DHA and AA were from Sigma (Saint Quentin Fallavier, France). Tissue culture media were from Eurobio (Les Ulis, France). [9,10-^{3}H]OA (23 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol), [4,5-^{3}H]DHA (23 Ci/mmol), [1-^{14}C]DHA (54 mCi/mmol), and [1-^{14}C]EPA (20:5n-3) (54 mCi/mmol) were from PerkinElmer Life Science (Paris, France). All other lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Anti-Lamp-1 mouse monoclonal antibody was obtained from Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-EEA1 mouse monoclonal antibody was from Transduction Laboratories (Lexington, KY). Anti-CD63 mouse monoclonal antibody was obtained from Cymbus Biotechnology (Southampton, United Kingdom). Alexa Fluor 488 conjugated anti-mouse IgG was from Molecular Probes (Eugene, OR). Anti-LBPA mouse monoclonal antibody was obtained as described (7). Alexa Fluor 546 conjugated anti-LBPA antibody was prepared according to the manufacturer's instruction (Molecular Probes).

Cell culture. Human monocytic leukemia cell line THP-1 was obtained from RIKEN Bioresource Center (Tsukuba, Japan). Cells were grown in RPMI 1640 medium containing 10% (v/v) FCS, 2 mM ʌ-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in suspension in 75-cm^2 flasks at 37°C in an atmosphere of 5% CO_2 and subcloned periodically to maintain a cellular density around 2×10^5 cells/mL

Differentiation to a macrophage phenotype was induced by culturing 10–12 $\times$ 10^6 cells per 100-mm diameter dish in the presence of 100 nM phorbol myristate acetate (PMA) for 72 h. Where indicated, cells were differentiated in the presence of 5 μM AA or DHA. Medium was changed for the last 24 h. FA were added from ethanolic stock solutions, the final ethanol concentration being 0.025%. When radioactive FA were incorporated, cells were differentiated for 2 d in the presence of 100 nM PMA and then labeled with radioactive FA in complete medium for 24 h at 37°C in the presence of PMA. At the end of incubation, the medium was aspirated, cells were washed twice with PBS and scraped in PBS for lipid analysis. For immunofluorescence, cells were grown on glass-bottomed 35-mm dishes. Baby hamster kidney (BHK-21) cells were grown and maintained as described (28).

Lipid extraction and separation of lipids on TLC. Cells were collected by centrifugation and resuspended in methanol. Total lipids were extracted using the procedure of Folch *et al.* as described previously (18,29): 2 vol of chloroform were added to the methanolic cell suspension and after sonication and vortexing for 5 min, 0.9 vol of 0.88% KCl was added. Samples were vortexed and centrifuged for 10 min at 1500 $\times$ *g*. The chloroform layer was collected, and the aqueous phase was washed once with 2 vol of chloroform. The two chloroform layers were combined, dried under vacuum, and stored in chloroform at –20°C. Extracted lipids were spotted on a silica gel 60 TLC plate (20 $\times$ 20 cm, 0.25 mm; Merck AG, Darmstadt, Germany), which was developed in chloroform/methanol/40% methylamine (65:22:3, by vol). PL were revealed under UV light after spraying with 0.05% 2′,7′-dichlorofluorescein in methanol and were identified by comparison with authentic standards spotted on the same plate. For radioactive samples, the labeled compounds were detected and integrated using a Berthold LB 511 (Wildbad, Germany) TLC analyzer and identified by their R_f values as compared with those observed for standards. The silica gel containing lipids was scraped and lipids extracted using 2 $\times$ 2 mL chloroform/methanol/water (5:5:1, by vol). Water was added to the combined extracts to obtain 10:10:9 (by vol) chloroform/methanol/water mixture. The samples were vortexed and centrifuged, and the chloroform layer was collected. The solvent was then removed under nitrogen, and the dried lipids were resolubilized in chloroform.

HPLC. Purification of LBPA was carried out on a Nucleosil 5NH2, 250 $\times$ 4.6 mm column using a Hewlett-Packard 1100 HPLC system equipped with a quaternary pump and a diode array detector as previously described (18). The mobile phase was acetonitrile/methanol/water/50% methylphosphonic acid (730:250:15:0.3, by vol; pH adjusted to 6.2 with 28% NH_4OH) with a flow rate of 1.2 mL/min. The absorbance was monitored at 205 and 210 nm, and eluting lipids were identified by comparison with authentic standards. When radioactive lipids were analyzed, the detector was coupled with continuous-flow liquid scintillation counting using a Radiomatic Flow One β detector. The detector was operated with Ultima-Flow M (Packard) at a flow rate of 2.5 mL/min in a 400 μL cell. In some experiments, radioactive FAME were separated by reversed-phase HPLC using a 4.6 $\times$ 250 mm Ultrabase C18 column (SFCC-Shandon, Eragny, France) with 5 μm spherical particles as previously described (30,31). The mobile phase was acetonitrile/water (80:20 vol/vol), and the flow rate was 2.0 mL/min. The absorbance was monitored at 210 nm. Peaks were identified by comparing retention times with authentic standards.

GC. The FA composition of total lipids, PL classes, and purified LBPA was determined by GC after transmethylation using 5% H_2SO_4 in methanol (100°C, 90 min). FAME were analyzed with a Hewlett-Packard 5590 gas chromatograph. The capillary column was an SP2380 (0.32 mm, 30 m; Supelco, Bellefonte, PA). The temperature was programmed from 145 to 225°C at 1.2°C/min. Helium was used as a carrier gas. FAME were identified by comparison with the relative retention times of known standards. The percentage and mass of each FA were calculated using an internal standard (pentadecanoic acid methyl ester) as described previously (18,30).

Immunofluorescence. All manipulations were performed at room temperature. Cells were first washed with PBS and then were fixed with 3% paraformaldehyde in PBS for 20 min, quenched with 0.1 M NH_4Cl and then blocked with 0.2% gelatin in PBS. Cells were then permeabilized by treatment with 50 μg/mL digitonin for 5 min followed by incubation with the first monoclonal antibodies. After 30 min of incubation with Alexa Fluor 488 conjugated anti-mouse IgG, cells were washed with PBS and further incubated with Alexa Fluor 546 conjugated anti-LBPA monoclonal antibody. The specimens were mounted with Mowiol and examined under a Zeiss LSM

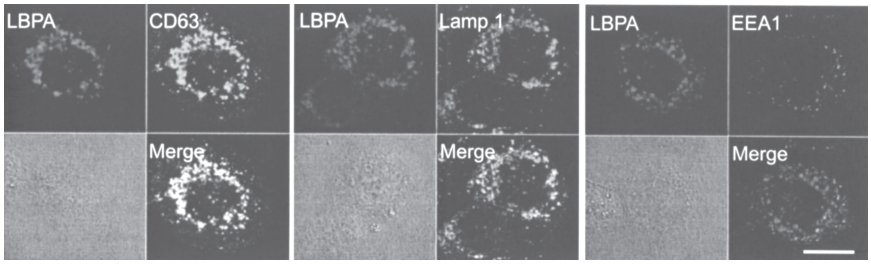

FIG. 1. Intracellular distribution of lysobisphosphatidic acid (LBPA) in THP-1 macrophages examined by immunofluorescence. THP-1 cells were grown for 3 d in the presence of 100 nM phorbol myristate acetate (PMA). Cells were then fixed and permeabilized as described in the Materials and Methods section. Cells were then doubly labeled with anti-LBPA antibody and various endosome markers as indicated. Bar, 10 μm.

510 confocal microscope equipped with C-Apochromat 63XW Korr (1.2 n.a.) objective.

Data analysis. Data are expressed as mean ± SD. Statistically significant difference was tested using Student's *t*-test for paired samples.

RESULTS

LBPA in THP-1 cells. In the present study, we characterized LBPA in the cultured macrophage cell line THP-1. LBPA was purified from THP-1 total lipid extracts, using a first separation from total lipids by TLC, and a further separation by HPLC as described previously (18). The proportion of LBPA compared with other PL was determined on the basis of FA content in the different PL classes. Expressing the results as a percentage of total PL, based on the FA amount in each PL class, LBPA represents 1.4 ± 0.7% (mean ± SD) of the total PL, phosphatidylcholine (PtdCho) 51.7 ± 3.5%, phosphatidylethanolamine (PtdEtn) 23.8 ± 7.0%, sphingomyelin 6.0 ± 1.3%, and phosphatidylinositol/phosphatidylserine 16.3 ± 2.3%. The low proportion of LBPA was also found in THP-1 cells before differentiation into macrophages (data not shown).

The intracellular distribution of LBPA was then measured using a specific monoclonal antibody (7). In Figure 1, THP-1 macrophages were doubly labeled with anti-LBPA and anti-CD63 monoclonal antibodies, anti-LBPA and anti-Lamp 1 monoclonal antibodies, or anti-LBPA and anti-EEA1 monoclonal antibodies, as described in the Materials and Methods section. CD63 is a member of the four-time membrane-spanning proteins (tetraspanins) and is enriched in the internal membranes of multivesicular late endosomes (32,33). Lamp-1 is localized in the limiting membranes of late endosomes (7,34). LBPA was not co-localized with the early endosome marker, EEA1 (35). Co-localization of LBPA with late endosome markers indicates that, similar to other cell types (7,36), LBPA is enriched in late endosomes in THP-1 macrophages.

TABLE 1
FA Composition (%mol)of THP-1 Macrophages Unsupplemented and Supplemented with 5 μM Arachidonic Acid (AA) or DHA[a]

	PtdCho			PtdEtn			LBPA		
		Supplemented			Supplemented			Supplemented	
FA	Control	5 μM DHA	5 μM AA	Control	5 μM DHA	5 μM AA	Control	5 μM DHA	5 μM AA
16:0	35.6 ± 0.3	40.3 ± 3.6	43.2 ± 1.9*	14.6 ± 1.1	17.9 ± 1.5	25.7 ± 0.4**	23.1 ± 2.4	22.8 ± 2.15	28.6 ± 3.9
16:1	12.7 ± 3.3	10.4 ± 1.9	6.3 ± 0.6*	9.6 ± 1.4	7.5 ± 1.2	2.5 ± 0.3*	6.4 ± 1.2	8.7 ± 0.7	4.4 ± 1.3
18:0	4.5 ± 1.7	4.4 ± 0.8	5.0 ± 0.4	11.4 ± 4.6	13.2 ± 2.2	17.1 ± 2.3	24.1 ± 12.1	9.6 ± 2.1	16.4 ± 4.3
18:1	37.6 ± 2.5	32.8 ± 5.7	28.2 ± 0.9*	27.5 ± 0.7	25.2 ± 1.4	18.1 ± 0.4**	26.5 ± 6.8	17.5 ± 1.8	15.6 ± 6.0
18:2n-6	1.6 ± 0.2	1.6 ± 1.4	3.7 ± 3.1**	3.0 ± 0.9	3.6 ± 1.8	4.8 ± 2.1	4.7 ± 2.7	3.2 ± 1.1	4.1 ± 2.3
20:4n-6	1.5 ± 0.3	1.5 ± 0.4	5.1 ± 0.1**	9.7 ± 2.7	8.7 ± 1.0	18.7 ± 0.3*	7.2 ± 0.5	1.0 ± 1.3**	4.3 ± 3.1
20:5n-3	Trace	Trace	1.1 ± 0.2	2.1 ± 0.9	4.4 ± 1.5	4.0 ± 0.3	Trace	Trace	Trace
22:5n-3	Trace	Trace	Trace	1.2 ± 1.7	1.2 ± 0.2	1.6 ± 0.2	Trace	Trace	Trace
22:6n-3	0.8 ± 0.0	2.4 ± 1.4*	1.1 ± 0.2	5.2 ± 0.6	14.8 ± 1.7**	3.6 ± 0.1	6.6 ± 1.6	29.3 ± 2.1**	9.5 ± 2.3
Saturated	44.4 ± 1.4	49.4 ± 3.0	52.3 ± 2.8*	30.3 ± 3.6	32.4 ± 2.0	43.2 ± 3.3	47.2 ± 9.7	39.6 ± 5.4	54.9 ± 5.3
MUFA	50.8 ± 15	43.5 ± 7.1	34.7 ± 1.4	42.6 ± 5.2	33.1 ± 1.6	20.7 ± 0.1*	32.8 ± 5.6	24.8 ± 3.3	21.5 ± 5.8
PUFA (n-6)	3.6 ± 0.5	3.5 ± 2.1	10.7 ± 3.5*	16.4 ± 3.8	14.0 ± 2.1	26.9 ± 3.2*	13.4 ± 2.5	4.2 ± 2.2**	10.4 ± 2.1
PUFA (n-3)	1.1 ± 0.4	3.7 ± 2.0	2.5 ± 0.3**	9.8 ± 0.8	20.5 ± 2.5*	9.3 ± 0.0	6.6 ± 1.6	31.4 ± 2.6**	12.1 ± 3.6

[a]Total lipids were obtained from THP-1 macrophages [3 d of differentiation with 100 mM of phorbol myristate acetate (PMA)] without (control cells) or with 5 μM of FA. Phospholipids (PL) were separated by TLC and analyzed as described in the Materials and Methods section. The FA composition is expressed as mol% of total FA, and represents means ± SD from three separate experiments. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; LBPA, lysobisphosphatidic acid; MUFA, monounsaturated FA. Trace means <1% of total FA. Significant differences with respect to the values of unsupplemented cells were determined using Student's *t*-test and are indicated with asterisks. *$P < 0.05$; **$P < 0.01$.

TABLE 2
Percent Distribution[a] of Radioactivity in Lipids from THP-1 Macrophages Labeled for 24 h with [^{3}H]AA, [^{3}H]OA, [^{14}C]DHA, or[^{14}C]EPA

	Percentage of total radioactivity in PL			
Radioactive FA	ChoGpl	EtnGpl	PtdIns/PtdSer/Cer*P*Cho	LBPA
DHA	19.5 ± 4.2	66.3 ± 4.1	6.5 ± 3.0	6.4 ± 1.1
AA	19.0 ± 2.7	69.4 ± 7.5	13.6 ± 0.8	0.8 ± 0.3
EPA	16.1 ± 2.7	72.7 ± 8.5	9.7 ± 2.2	1.6 ± 0.4
OA	63.8 ± 0.1	21.8 ± 0.1	4.2 ± 0.3	7.5 ± 0.1
DHA/AA	1.0	1.0	0.5	8.0

[a]Values refer to averages ± SD of three different experiments. Total lipids were obtained from THP-1 cells differentiated 2 d with 100 nM of PMA and the last 24 h, with 100 nM PMA plus 0.5 μCi/mL of radioactive FA. PL classes were separated by TLC and analyzed as described in the Materials and Methods section. OA, oleic acid; ChoGpl, cholineglycerophospholipids; EtnGpl, ethanolamineglycerophospholipids; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; Cer*P*Cho, sphingomyelin; for other abbreviations see Table 1.

LBPA is enriched with DHA in THP-1 cells. Table 1 shows the FA composition of LBPA and two major PL classes, PtdCho and PtdEtn, in THP-1 macrophages. The FA composition of PtdCho and PtdEtn obtained in this experiment is close to that reported by others (37). PtdEtn contains a high level of monounsaturated FA (MUFA) and is enriched with PUFA compared with PtdCho, which is mainly constituted of saturated FA and MUFA. The major FA in LBPA were saturated FA and MUFA, especially 18:1, which represents 26% of the total FA. However, the PUFA content in LBPA was relatively high. n-3 and n-6 PUFA together account for 20% of the total FA in LBPA, whereas this proportion was 26% in PtdEtn and less than 5% in PtdCho. The main PUFA of LBPA were AA (20:4n-6) and DHA (22:6n-3), which were found in similar proportions. It is noteworthy that DHA accounts for 33% of the total PUFA in LBPA. This percentage is higher than those in PtdEtn and PtdCho (20 and 17%, respectively) whereas AA represents around 35% of the total PUFA regardless of the PL class. Moreover, DHA was the only n-3 PUFA representative in LBPA. In contrast, PtdEtn contained both EPA (20:5n-3) and docosapentaenoic acid (22:5n-3) in addition to DHA. We have also analyzed PL from undifferentiated THP-1 cells (data not shown) and found similar FA compositions, indicating that PMA treatment and the differentiation of monocytes into macrophages had no significant effect on cellular FA composition.

DHA is selectively incorporated into LBPA in THP-1 cells. We then asked whether exogenously added FA alter FA composition of LBPA, PtdCho, and PtdEtn. As shown in Table 1, after DHA supplementation, the proportion of this FA was significantly increased in the three PL compared with their respective controls. Interestingly, the highest increase of DHA was found in LBPA with a 4.5-fold increase vs. a 3- and 2.8-fold increase, respectively, in PtdCho and PtdEtn. This increase of DHA in LBPA was compensated for by a significant decrease in the proportion of AA and a less marked decrease of stearic acid (18:0) and OA. In contrast, incorporation of DHA did not significantly decrease the percentage of AA in PtdCho and PtdEtn. In supplemented cells, DHA accounted for more than 90% of the total n-3 PUFA in LBPA as compared with 70% in PtdEtn. Determination of the FA amounts revealed that the cellular LBPA content was not affected by incubation with DHA (0.26 ± 0.12 vs. 0.27 ± 0.12 nmol/10^6 cells, in control and supplemented cells, respectively). After AA supplementation, the proportion of AA was significantly increased in PtdCho and PtdEtn, and levels of linoleic acid were also increased, with a compensatory decrease in MUFA. In contrast, no increase of AA and its retroconversion products was observed in LBPA.

TABLE 3
Percent Distribution[a] of [^{14}C]DHA, or[^{3}H]DHA in PL from THP-1Macrophages Coincubated with 5 μM AA

	Percentage of total radioactivity in PL			
FA	ChoGpl	EtnGpl	PtdIns/PtdSer/Cer*P*Cho	LBPA
[^{14}C]DHA 5 μM	23.6 ± 4.2	64.5 ± 4.7	4.7 ± 0.7	7.2 ± 0.6
+ AA 5 μM	24.0 ± 2.7	63.7 ± 3.1	4.2 ± 0.2	8.0 ± 0.1
[^{3}H]DHA 10 nM	22.3 ± 0.7	61.9 ± 0.8	7.0 ± 0.3	8.8 ± 0.8
+ AA 5 μM	25.0 ± 1.6	58.8 ± 1.5*	6.9 ± 0.3	9.3 ± 0.7

[a]Values refer to averages ± SD of triplicates. Total lipids were obtained from THP-1 cells differentiated 2 d with 100 nM of PMA and the last 24 h, with 100 nM PMA plus 5 μM [^{14}C]DHA or 10 nM [^{3}H]DHA in the absence or presence of 5 μM AA. PL were separated by TLC and analyzed as described the in Materials and Methods section. For abbreviations see Tables 1 and 2.

TABLE 4
Percent Distribution[a] of Radioactivity in PL from BHK Cells Labeled for 24 h with [^{3}H]AA, [^{3}H]OA, or [^{3}H]DHA

	Percentage of total radioactivity in PL			
Radioactive FA	ChoGpl	EtnGpl	PtdIns/PtdSer/Cer*P*Cho	LBPA
DHA	5.8 ± 0.8	76.1 ± 4.2	13.9 ± 3.6	3.0 ± 0.6
AA	7.0 ± 0.6	70.3 ± 0.9	21.3 ± 0.1	0.4 ± 0.1
OA	38.7 ± 2.6	45.8 ± 3.7	10.7 ± 2.1	4.5 ± 0.5
DHA/AA	0.8	1.1	0.7	7.5

[a]Values refer to averages ± SD of three different experiments. Total lipids were obtained from baby hamster kidney (BHK) cells labeled for 24 h with 0.5 μCi/mL of radioactive FA. PL were separated by TLC and analyzed as described in the Materials and Methods section. For other abbreviations see Tables 1 and 2.

Altogether, these results show that DHA is preferentially incorporated into LBPA.

The apparent selectivity of LBPA for DHA was further examined by comparing the incorporation of several radioactive FA into PL classes. Radiolabeled OA, AA, EPA, and DHA were incubated with THP-1 macrophages for 24 h, and the distribution of cellular radioactivity was determined after separation of the lipid and PL classes by TLC (Table 2). For OA and DHA, nearly 5% of the incorporated radioactivity was found associated with LBPA, which represents less than 1% of the total PL in terms of mass. This is indicative of a high rate of incorporation of these FA in LBPA, when compared with AA. Indeed, DHA was eightfold more efficiently incorporated than AA into LBPA (Table 2). After incubation with EPA, a precursor of DHA, LBPA was only slightly labeled. EPA was metabolized to DHA, with 2% of radioactive EPA being converted to DHA as shown in total cellular lipids. This proportion increased up to 40% in LBPA (data not shown), indicating that DHA, which was derived from EPA, was preferentially esterified into LBPA. These results, together with those presented in Table 1, show that regardless of the source of DHA (i.e., formed either by elongation/desaturation or by direct addition), DHA was incorporated selectively into LBPA in THP-1 macrophages.

We next examined whether the selectivity for DHA still would be observed if DHA was coincubated with AA. This condition mimics the *in vivo* situation where both FA are present at comparable levels and could therefore compete for uptake and incorporation mechanisms. THP-1 macrophages were incubated with 5 μM [^{14}C]DHA (0.3 μCi/mL) in the absence or presence of 5 μM AA. Total incorporation of radioactive DHA was not affected by the presence of exogenous AA (92.8 ± 0.06% of initially added radioactivity vs. 92.9 ± 0.21% in the control experiment). In addition, the distribution of incorporated radioactivity to total PL was not significantly altered (85.3 ± 5.1% of cellular radioactivity vs. 87.6 ± 4.4% in the control). As shown in Table 3, the percentage of DHA incorporated into LBPA was not modified in the presence of 5 μM AA. We have also incubated macrophages with 10 nM [^{3}H]DHA (0.3 μCi/mL) together with a 500 times higher concentration of AA (5 μM). In this condition, coincubation with AA reduced the cellular uptake of DHA (83.6 ± 2.5% of initially added radioactivity vs. 95.2 ± 5.2% in the control, $P \leq 0.05$) but not its distribution in total PL (82.2 ± 1.8% of cellular radioactivity vs. 85.3 ± 3.7% in controls). Of interest, the percentage of DHA incorporated into ethanolamineglycerophospholipids (EtnGpl) was slightly decreased whereas it was not changed into LBPA (Table 3). These results indicate the LBPA selectivity for DHA even when a high level of AA is available for incorporation.

DHA is selectively incorporated into LBPA in BHK cells. Selective incorporation of DHA into LBPA has been reported in several cell types (18,38). The results in pulmonary macrophages are controversial. Whereas Huterer and Wherrett (38) showed the efficient incorporation of DHA, Waite and colleagues (17) reported that LBPA is a reservoir of AA. The FA composition of LBPA varies among cell types. In BHK cells, more than 90% of FA in LBPA is OA (18:1) (6). To determine whether selective incorporation of DHA into LBPA is a common phenomenon, we measured the incorporation of radioactive FA in BHK cells. Radioactive DHA and OA were efficiently incorporated into LBPA whereas, as observed in THP-1 cells, AA was not significantly incorporated with a DHA/AA ratio of around 8, as shown in Table 2 for THP-1 cells (Table 4).

DHA turns over similarly in PtdCho, PtdEtn, and LBPA in THP-1 cells. The present results, together with previous reports, confirm the selective ability of LBPA to incorporate DHA and suggest that this selectivity is extended to most cell types, regardless of their DHA content in LBPA. The reason for the preferential incorporation of DHA into LBPA is not understood. It has been suggested that the turnover of certain FA, such as AA and OA, is high in LBPA of pulmonary alveolar macrophages (15). Conversely, one might suggest that the turnover of DHA in LBPA is particularly slow compared with that in other PL, allowing its accumulation in that pool. This possibility was examined in THP-1 cells by following the removal of DHA from PL after the cells had been supplemented with DHA. As shown in Figure 2, after 3 d of supplementation, the DHA content was increased by 400% in LBPA, 280% in PtdEtn, and 220% in PtdCho compared with nonsupplemented control cells. After DHA removal, the DHA content decreased progressively back to the initial value in all PL. The data show no significant difference of DHA turnover in LBPA compared with PtdCho and PtdEtn.

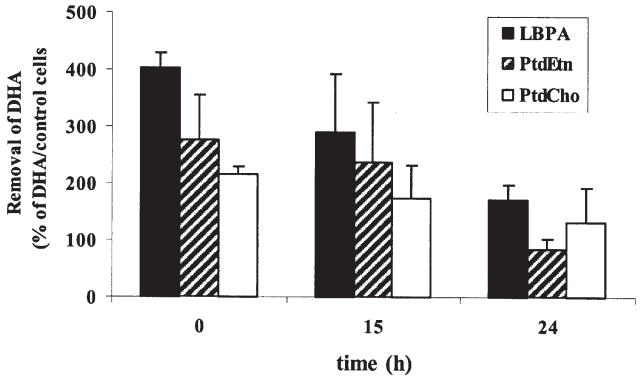

FIG. 2. Turnover of DHA in various phospholipids in THP-1 macrophages. THP-1 cells were grown for 3 d in the presence of 100 nM PMA and 5 μM DHA (time 0). DHA was then removed and cells were further incubated for various intervals in normal medium. Lipids were then extracted, and DHA composition was measured by GC. DHA content without supplementation represents 100%. PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; for other abbreviations see Figure 1.

DISCUSSION

The present study was undertaken to characterize LBPA in THP-1 macrophages in the context of related investigations on the putative role of LBPA in cholesterol homeostasis. We first showed that LBPA is almost exclusively localized in late endosomes in THP-1 macrophages, which is important regarding its involvement in LDL-derived cholesterol transport. Lipid composition of monocytic THP-1 cells already has been reported, but no data were available for differentiated macrophages, in particular for LBPA content and FA composition. Our data indicate that LBPA represents 1–2% of total PL in THP-1 macrophages and accumulates in late endosomes. This proportion is not different from peritoneal macrophages in which LBPA was also reported to be a minor PL (15). In contrast, in pulmonary alveolar macrophages, LBPA constitutes roughly 15–18% of the total cellular PL (15,16). It has been postulated that the presence of a large quantity of the precursor phosphatidylglycerol in lung surfactant could account for such a high level of LBPA in alveolar macrophages (39). Most other mammalian cell types, including human fibroblasts (39), polymorphonuclear leukocytes (16), BHK cells (6,14), PC12 cells (40), and rat uterine stromal cells (18) contain low amounts of LBPA. A striking accumulation of LBPA, however, is found in tissues from patients suffering from genetic lipidosis such as Niemann–Pick disease (41) as well as rat organs treated with drugs that induced lysosomal lipid storage (14,42–44).

The FA composition of LBPA in THP-1 macrophages resembles that of other cell types in terms of the elevated amount of OA (18:1n-9) and enrichment in PUFA. The proportion of OA was found to constitute 30–50% of total FA of LBPA in uterine stromal cells (18) and pulmonary alveolar macrophages (15–17), reaching more than 80% in BHK cells (6,14). With respect to PUFA, our data suggest an enrichment in DHA, as reported for PC12 cells (40) and uterine stromal cells (18). This is strikingly different from the situation observed in pulmonary alveolar macrophages. The latter cells contain mostly n-6 FA, mainly linoleic acid (18:2n-6) and AA, but have only a trace amount of DHA in their LBPA (15–17). In rat testis, 22:5n-6 accounts for more than 70% of the total FA of LBPA (18). It thus appears that while PUFA are consistently reported as being substantial constituents of LBPA, the specific composition of the PUFA varies depending on the cell type. This supports the idea that FA composition of LBPA could depend on the cellular function of this PL, as described for AA-containing LBPA in rabbit pulmonary macrophages (17).

Our results show that both THP-1 and BHK cells efficiently incorporated DHA into LBPA by comparison with other FA such as OA and AA. It should be noticed that although the percentage of DHA incorporation was not significantly different from that of OA in BHK cells, OA represents more than 80% of the endogenous FA in LBPA of those cells. In contrast to DHA, incorporation of AA into LBPA was very inefficient in both THP-1 and BHK cells. Preferential incorporation of DHA into LBPA has been reported in other cell types. In pulmonary alveolar macrophages, DHA was more efficiently incorporated than AA into LBPA despite a much lower endogenous content of DHA (0.5 vs. 7%) (38). In uterine stromal cells, where AA makes up about 6% of the total FA in LBPA (18), no labeling was detected after incubation with radioactive AA, while DHA was readily incorporated. Our present data further show that the high incorporation of DHA into LBPA was not affected by the presence of a high level of AA, which brings out further evidence for selectivity of LBPA toward DHA. One interesting point is that, whereas AA and DHA were found in similar amounts in LBPA in THP-1 macrophages, only exogenous DHA, but not AA, was incorporated in this PL. This suggests that DHA turnover in LBPA is much more rapid than that of AA. The specific localization of LBPA in late endosomes may also be important in this respect. It is possible that in these acidic compartments, FA metabolism differs from what is typically described for cellular membrane PL. For example, it is not known whether all FA can equally reach these compartments or whether classical acyltransferases are efficient. This is an important difference with EtnGpl that are enriched in both n-3 and n-6 PUFA and can also incorporate AA at a high rate. It should be noted that both EtnGpl and LBPA show high affinity for DHA, which raises the question of a potential relationship between these PL. Preliminary data using plasmalogen EtnGpl-deficient human skin fibroblasts showed that the DHA content in LBPA was not changed in these cells compared with controls, arguing against a role of plasmalogens as a reservoir of DHA during LBPA remodeling. From our present data, we can exclude a difference in turnover of DHA in LBPA compared with other PL to explain DHA enrichment and high incorporation. It may be alternatively proposed that the selective incorporation of DHA into LBPA reflects a DHA-specific transacylase activity (13,45) or is related to the unique cellular localization of LBPA. Further experiments will be necessary to elucidate the molecular mechanisms of the preferential incorporation of DHA into LBPA.

The physiological relevance of DHA enrichment of LBPA must also be addressed. LBPA is almost exclusively localized in membranes of late endosomes that are essential compartments of the endocytic pathway and participate in LDL-derived cholesterol distribution. The content of DHA in biological membranes has been shown to influence membrane properties such as fluidity, fusion, and raft formation (24). Importantly, it has been reported that membranes enriched with DHA-containing PL exclude cholesterol (21–23). These properties may be advantageous for the removal of cholesterol from late endosomes. DHA has interesting medical implications since its dietary presence has been positively linked to the prevention of numerous human afflictions including cancer and heart disease (46–49). However, little is known about the molecular mechanisms of action of DHA. Future study of DHA in LBPA may shed a revealing light on the biological role of this unique FA.

ACKNOWLEDGMENTS

This work was supported by grants from INSERM, RIKEN Frontier Research System, and the International HDL Award Program.

REFERENCES

1. Stary, H.C., Chandler, A.B., Dinsmore, R.E., Fuster, V., Glagov, S., Insull, W., Jr., Rosenfeld, M.E., Schawrtz, C.J., Wagner, W.D., and Wissler, R.W. (1995) A Definition of Advanced Types of Atherosclerotic Lesions and a Histological Classification of Atherosclerosis. A Report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association, *Arterioscler. Thromb. Vasc. Biol. 15*, 1512–1531.
2. Tabas, I. (2002) Consequences of Cellular Cholesterol Accumulation: Basic Concepts and Physiological Implications, *J. Clin. Invest. 110*, 905–911.
3. Brown, M.S., and Goldstein, J.L. (1986) A Receptor-Mediated Pathway for Cholesterol Homeostasis, *Science 232*, 34–47.
4. Gruenberg, J. (2001) The Endocytic Pathway: A Mosaic of Domains, *Nat. Rev. Mol. Cell Biol. 2*, 721–730.
5. Kobayashi, T., Startchev, K., Whitney, A.J., and Gruenberg, J. (2001) Localization of Lysobisphosphatidic Acid-rich Membrane Domains in Late Endosomes, *Biol. Chem. 382*, 483–485.
6. Kobayashi, T., Beuchat, M.H., Chevallier, J., Makino, A., Mayran, N., Escola, J.M., Lebrand, C., Cosson, P., and Gruenberg, J. (2002) Separation and Characterization of Late Endosomal Membrane Domains, *J. Biol. Chem. 277*, 32157–32164.
7. Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R.G., and Gruenberg, J. (1998) A Lipid Associated with the Antiphospholipid Syndrome Regulates Endosome Structure and Function, *Nature 392*, 193–197.
8. Galve-de Rochemonteix, B., Kobayashi, T., Rosnoblet, C., Lindsay, M., Parton, R.,G., Reber, G., de Maistre, E., Wahl, D., Kruithof, E.K., Gruenberg, J., and de Moerloose, P. (2000) Interaction of Anti-phospholipid Antibodies with Late Endosomes of Human Endothelial Cells, *Arterioscler. Thromb. Vasc. Biol. 20*, 563–574.
9. Kobayashi, T., Beuchat, M.H., Lindsay, M., Frias, S., Palmiter, R.D., Sakuraba, H., Parton, R.G., and Gruenberg, J. (1999) Late Endosomal Membranes Rich in Lysobisphosphatidic Acid Regulate Cholesterol Transport, *Nat. Cell Biol. 1*, 113–118.
10. Mukherjee, S., and Maxfield, F.R. (2004) Lipid and Cholesterol Trafficking in NPC, *Biochim. Biophys. Acta 1685*, 28–37.
11. Sturley, S.L., Patterson, M.C., Balch, W., and Liscum, L. (2004) The Pathophysiology and Mechanisms of NP-C Disease, *Biochim. Biophys. Acta 1685*, 83–87.
12. Brotherus, J., and Renkonen, O. (1974) Novel Stereoconfiguration of Lyso-bis-phosphatidic Acid of Cultured BHK Cells, *Chem. Phys. Lipids 13*, 178–182.
13. Amidon, B., Schmitt, J.D., Thuren, T., King, L., and Waite, M. (1995) Biosynthetic Conversion of Phosphatidylglycerol to *sn*-1:*sn*-1′ Bis(monoacylglycerol) Phosphate in a Macrophage-like Cell Line, *Biochemistry 34*, 5554–5560.
14. Brotherus, J., Niinioja, T., Sandelin, K., and Renkonen, O. (1977) Experimentally Caused Proliferation of Lysosomes in Cultured BHK Cells Involving an Increase of Biphosphatidic Acids and Triglycerides, *J. Lipid Res. 18*, 379–388.
15. Huterer, S., and Wherrett, J. (1979) Metabolism of Bis(monoacylglycero)phosphate in Macrophages, *J. Lipid Res. 20*, 966–973.
16. Mason, R.J., Stossel, T.P., and Vaughan, M. (1972) Lipids of Alveolar Macrophages, Polymorphonuclear Leukocytes, and Their Phagocytic Vesicles, *J. Clin. Invest. 51*, 2399–2407.
17. Cochran, F.R., Roddick, V.L., Connor, J.R., Thornburg, J.T., and Waite, M. (1987) Regulation of Arachidonic Acid Metabolism in Resident and BCG-Activated Alveolar Macrophages: Role of Lyso(bis)phosphatidic Acid, *J. Immunol. 138*, 1877–1883.
18. Luquain, C., Dolmazon, R., Enderlin, J.M., Laugier, C., Lagarde, M., and Pageaux, J.F. (2000) Bis(monoacylglycerol) Phosphate in Rat Uterine Stromal Cells: Structural Characterization and Specific Esterification of Docosahexaenoic Acid, *Biochem. J. 351*, 795–804.
19. Pakala, R., Sheng, W.L., and Benedict, C.R. (2000) Vascular Smooth Muscle Cells Preloaded with Eicosapentaenoic Acid and Docosahexaenoic Acid Fail to Respond to Serotonin Stimulation, *Atherosclerosis 153*, 47–57.
20. De Caterina, R., Liao, J.K., and Libby, P. (2000) Fatty Acid Modulation of Endothelial Activation, *Am. J. Clin. Nutr. 71*, 213S–223S.
21. Pal., S., and Davis, P.J. (1990) n-3 Polyunsaturated Fatty Acids Enhance Cholesterol Efflux from Human Fibroblasts in Culture, *Biochem, Biophys. Res. Commun. 173*, 566–570.
22. Dusserre, E., Pulcini, T., Bourdillon, M.C., Ciavatti, M., and Berthezene, F. (1995) Omega-3 Fatty Acids in Smooth Muscle Cell Phospholipids Increase Membrane Cholesterol Efflux, *Lipids 30*, 35–41.
23. Stillwell, W., Shaikh, S.R., Zerouga, M., Siddiqui, R., and Wassall, S.R. (2005) Docosahexaenoic Acid Affects Cell Signaling by Altering Lipid Rafts, *Reprod. Nutr. Dev. 45*, 559–579.
24. Stillwell, W., and Wassall, S.R. (2003) Docosahexaenoic Acid: Membrane Properties of a Unique Fatty Acid, *Chem. Phys. Lipids 126*, 1–27.
25. Auwerx, J. (1991) The Human Leukemia Cell Line, THP-1: A Multifaceted Model for the Study of Monocyte-Macrophage Differentiation, *Experientia 47*, 22–31.
26. Kritharides, L., Christian, A., Stoudt, G., Morel, D., and Rothblat, G.H. (1998) Cholesterol Metabolism and Efflux in Human THP-1 Macrophages, *Arterioscler. Thromb. Vasc. Biol. 18*, 1589–1599.
27. Llaverias, G., Lacasa, D., Vasquez-Carrera, M., Sanchez, R.M., Laguna, J.C., and Alegret, M. (2005) Cholesterol Regulation of Genes Involved in Sterol Trafficking in Human THP-1 Macrophages, *Mol. Cell. Biochem. 273*, 185–191.
28. Gruenberg, J., Griffiths, G., and Howell, K.E. (1989) Characterization of the Early Endosome and Putative Endocytic Carrier Vesicles *in vivo* and with an Assay of Vesicle Fusion *in vitro*, *J. Cell Biol. 108*, 1301–1316.
29. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues, *J. Biol. Chem. 226*, 497–509.

30. Pageaux, J.F., Bechoua, S., Bonnot, G., Fayard, J.M., Cohen, H., Lagarde, M., and Laugier, C. (1996) Biogenesis and Metabolic Fate of Docosahexaenoic and Arachidonic Acids in Rat Uterine Stromal Cells in Culture, *Arch. Biochem. Biophys. 327*, 142–150.
31. Bernoud, N., Fenart, L., Benistant, C., Pageaux, J.F., Dehouck, M.P., Moliere, P., Lagarde, M., Cecchelli, R., and Lecerf, J. (1998) Astrocytes Are Mainly Responsible for the Polyunsaturated Fatty Acid Enrichment in Blood-Brain Barrier Endothelial Cells *in vitro*, *J. Lipid Res. 39*, 1816–1824.
32. Escola, J.M., Kleijmeer, M.J., Stoorvogel, W., Griffith, J.M., Yoshie, O., and Geuze, H.J. (1998) Selective Enrichment of Tetraspan Proteins on the Internal Vesicles of Multivesicular Endosomes and on Exosomes Secreted by Human B-Lymphocytes, *J. Biol. Chem. 273*, 20121–20127.
33. Kobayashi, T., Vischer, U.M., Rosnoblet, C., Lebrand, C., Lindsay, M., Parton, R.G., Kruithof, E.K., and Gruenberg, J. (2000) The Tetraspanin CD63/Lamp3 Cycles Between Endocytic and Secretory Compartments in Human Endothelial Cells, *Mol. Biol. Cell 11*, 1829–1843.
34. Griffiths, G., Hoflack, B., Simons, K., Mellman, I., and Kornfeld, S. (1988) The Mannose 6-Phosphate Receptor and the Biogenesis of Lysosomes, *Cell 52*, 329–341.
35. Mu, F.T., Callaghan, J.M., Steele-Mortimer, O., Stenmark, H., Parton, R.G., Campbell, P.L., McCluskey, J., Yeo, J.P., Tock, E.P., and Toh, B.H. (1995) EEA1, an Early Endosome-Associated Protein. EEA1 Is a Conserved α-Helical Peripheral Membrane Protein Flanked by Cysteine "Fingers" and Contains a Calmodulin-Binding IQ Motif, *J. Biol. Chem. 270*, 13503–13511.
36. Schaible, U.E., Schlesinger, P.H., Steinberg, T.H., Mangel, W.F., Kobayashi, T., and Russell, D.G. (1999) Parasitophorous Vacuoles of *Leishmania mexicana* Acquire Macromolecules from the Host Cell Cytosol *via* Two Independent Routes, *J. Cell Sci. 112*, 681–693.
37. Galella, G., Marangoni, F., Rise, P., Colombo, C., Galli, G., and Galli, C. (1993) n-6 and n-3 Fatty Acid Accumulation in THP-1 Cell Phospholipids, *Biochim. Biophys. Acta 1169*, 280–290.
38. Huterer, S., and Wherrett, J.R. (1986) Incorporation of Polyunsaturated Fatty Acids into Bis(monoacylglycero)phosphate and Other Lipids of Macrophages and of Fibroblasts from Control and Niemann–Pick Patients, *Biochim. Biophys. Acta 876*, 318–326.
39. Waite, M., Roddick, V., Thornburg, T., King, L., and Cochran, F. (1987) Conversion of Phosphatidylglycerol to Lyso(bis)phosphatidic Acid by Alveolar Macrophages, *FASEB J. 1*, 318–325.
40. Holbrook, P.G., Pannell, L.K., Murata, Y., and Daly, J.W. (1992) Bis(monoacylglycero)phosphate from PC12 Cells, a Phospholipid That Can Comigrate with Phosphatidic Acid: Molecular Species Analysis by Fast Atom Bombardment Mass Spectrometry, *Biochim. Biophys. Acta 1125*, 330–334.
41. Weisz, B., Spirer, Z., and Reif, S. (1994) Niemann–Pick Disease: Newer Classification Based on Genetic Mutations of the Disease, *Adv. Pediatr. 41*, 415–426.
42. Yamamoto, A., Adachi, S., Ishikawa, K., Yokomura, T., and Kitani, T. (1971) Studies on Drug-Induced Lipidosis. 3. Lipid Composition of the Liver and Some Other Tissues in Clinical Cases of "Niemann–Pick-like Syndrome" Induced by 4,4′-Diethylaminoethoxyhexestrol, *J. Biochem. (Tokyo) 70*, 775–784.
43. Yamamoto, A., Adachi, S., Kitani, T., Shinji, Y., and Seki, K. (1971) Drug-Induced Lipidosis in Human Cases and in Animal Experiments. Accumulation of an Acidic Glycerophospholipid, *J. Biochem. (Tokyo) 69*, 613–615.
44. Hostetler, K.Y., Reasor, M., and Yazaki, P.J. (1985) Chloroquine-Induced Phospholipid Fatty Liver. Measurement of Drug and Lipid Concentrations in Rat Liver Lysosomes, *J. Biol. Chem. 260*, 215–219.
45. Heravi, J., and Waite, M. (1999) Transacylase Formation of Bis(monoacylglycerol)phosphate, *Biochim. Biophys. Acta 1437*, 277–286.
46. Carroll, K.K. (1991) Dietary Fats and Cancer, *Am. J. Clin. Nutr. 53*, 1064S–1067S.
47. McLennan, P., Howe, P., Abeywardena, M., Muggli, R., Raederstorff, D., Mano, M., Rayner, T., and Head, R. (1996) The Cardiovascular Protective Role of Docosahexaenoic Acid, *Eur. J. Pharmacol. 300*, 83–89.
48. Wijendran, V., and Hayes, K.C. (2004) Dietary n-6 and n-3 Fatty Acid Balance and Cardiovascular Health, *Annu. Rev. Nutr. 24*, 597–615.
49. Nordoy, A., Marchioli, R., Arnesen, H., and Videbaek, J. (2001) n-3 Polyunsaturated Fatty Acids and Cardiovascular Diseases, *Lipids 36* (*Suppl.*), S127–S129.

[Received October 10, 2005; accepted January 30, 2006]