The Assembly of Very Low Density Lipoproteins in Rat Hepatoma McA-RH7777 Cells Is Inhibited by Phospholipase A2 Antagonists*

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In McA-RH7777 cells, the oleate-stimulated assembly and secretion of very low density lipoproteins (VLDL) was associated with enhanced deacylation of phospholipids, which was markedly decreased by inactivation of the cellular phospholipase A2. Treatment of the cells with antagonists or antisense oligonucleotide of the hydrolase, or microsomal triglyceride transfer protein, significantly inhibited secretion of apoB100-VLDL and triglyceride. Similar inhibitory effect of the iPLA2 antagonists was observed on apoB48-VLDL secretion, but secretion of high density lipoprotein particles (such as apoAI- and apoB48-high density lipoprotein) or proteins in general was unaffected. The iPLA2 antagonist did not affect the synthesis of apoB100 or triglyceride, nor did it affect the activities of phospholipase D, phosphatidate phosphohydrolase, or microsomal triglyceride transfer protein. Inactivation of iPLA2 resulted in impaired apoB100-VLDL assembly as shown by decreased apoB100-VLDL and triglyceride within the microsomal lumen, with concomitant increase in apoB100 association with the microsomal membranes. The inhibitory effect of iPLA2 antagonists on apoB100-VLDL assembly/secretion could be abated by pretreatment of cells with oleate. Analysis of molecular species of microsomal phosphatidylcholine and phosphatidylethanolamine by electron spray tandem mass spectrometry revealed that the enrichment of oleoyl moieties was altered by the treatment of iPLA2 antagonist. These results suggest that the oleate-induced VLDL assembly/secretion may depend upon the establishment of membrane glycerolipids enriched in oleoyl chain, a process mediated by the iPLA2 activity.

Very low density lipoproteins (VLDL) are assembled in the liver and secreted as triglyceride (TG)-rich particles. Each VLDL particle contains various amounts of TG and a single copy of apoB100. The rat liver synthesizes a truncated form, apoB48 (collinear with the N-terminal 48% of apoB100), that also has the ability to assemble VLDL (1). The subcellular compartment where VLDL is assembled has not been unambiguously determined. Immunohistochemical and electronmicroscopic studies proposed that rat liver VLDL was assembled at the junction of rough and smooth endoplasmic reticulum (ER) (2). Although apoB immunostaining was observed within rough ER, VLDL-sized lipid staining entities lacking apoB were found within the lumen of smooth ER (2). Biochemical studies, however, suggested that association of lipid with apoB could occur not only post-translationally (3) but also during apoB100 translation (4–7). Recent experiments with improved techniques have shown that in McA-RH7777 cells, assembly of apoB100 (8, 9) and apoB48-VLDL (3, 10) are both achieved post-translationally and require the activity of microsomal triglyceride transfer protein (MTP). These studies support the model known as “two-step” assembly, which theorizes that the initial product is a primordial dense particle that is subsequently assembled with bulk TG to form a mature VLDL. The stepwise VLDL assembly also occurs in primary hepatocytes (7, 11).

The post-translational model for VLDL assembly is established on the basis of kinetic analysis that observes the time taken by apoB and TG to be assembled. It is noticed that there exists a temporal delay between the moment when apoB translation reaches completion and the time when VLDL is matured. In the case of apoB100, assembly of apoB100-VLDL is undetectable until 15–20 min after apoB synthesis (9). Such a lag period was also observed for TG incorporation into hepatic apoB48-VLDL (7). Since ER is a highly dynamic organelle that continuously undergoes vesiculation and fusion, the delayed apoB100-VLDL maturation may reflect the time needed for the movement of apoB100 (and TG as well) from the site of synthesis to the site of assembly. Data suggesting the involvement of ER vesiculation in VLDL assembly are obtained from studies with brefeldin A (10, 12). At a low dose of brefeldin A, assembly of VLDL is inhibited but assembly of dense particles remains normal. Thus, membrane trafficking mediated by vesiculation and fusion may be essential for bulk TG incorporation into VLDL. It has been recognized that changes in membrane lipid composition regulated by lipid catalyzing enzymes dramatically affect membrane vesiculation and fusion (13). Therefore, it is important to understand the role of enzyme activities that remodel biological membranes in VLDL assembly.

Two families of cytosolic phospholipase A2 (PLA2, EC 3.1.1.4 and 3.1.1.6) have been identified in mammalian tissues. The most abundant enzyme is PLA2, EC 3.1.1.4 (PLA2, CA2⁺-independent phospholipase A2) that is present at high levels in the liver and kidney and is associated with the plasma membrane. The activity of this enzyme can be detected in microsomes and smooth ER fraction. The enzyme is responsible for the hydrolysis of phospholipids in the plasma membrane and the generation of arachidonic acid. In contrast, PLA2, EC 3.1.1.6 (PLA2, Ca2⁺-dependent phospholipase A2) is present at low levels in the liver and kidney and is associated with the endoplasmic reticulum. The enzyme is responsible for the hydrolysis of phospholipids in the endoplasmic reticulum and the generation of lysophospholipids. The enzyme is inhibited by the divalent cation Ca2⁺.

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3.1.1.4.) have been characterized, namely cPLA₂ (α, β, and γ forms) and iPLA₂. Although the β form of cPLA₂ is expressed in the liver (14), hepatic expression of the α (15) and γ (16) form is rather low. The iPLA₂ also consists of multiple isoforms, which are derived from alternative splicing of a single gene (17). Unlike cPLA₂, iPLA₂ does not have a Ca²⁺-dependent lipid-binding domain (18, 19), but carries repeating motifs homologous to the integral membrane protein-binding domain of ankyrin (20–22). The iPLA₂ displays broad substrate specificity and can be inhibited by a suicide substrate, bromoenol lactone (BEL) (23). Functional roles ascribed to iPLA₂ include phospholipid remodeling (24, 25) and Golgi membrane tubulation (26, 27), among others (28, 29). It is suggested that phospholipid turnover may be associated with VLDL secretion in rat liver cells treated with oleate (30). A potential role of phospholipid turnover in VLDL secretion was first suggested by a work (31) that showed ~50% of VLDL-TG secreted from rat liver might use fatty acids derived from phospholipid deacylation. However, it remains to be determined if phospholipid turnover plays a role in the process of bulk TG incorporation into VLDL. Here, we examined the role of iPLA₂-mediated phospholipid turnover in oleate-induced hepatic VLDL assembly/secretion.

EXPERIMENTAL PROCEDURES

Materials—[³⁵S]Methionine/cysteine (1000 Ci/mmol), 2-[³²P]Hpalmitoyl-phosphatidylcholine (PC) (89 Ci/mmol), [³H]oleic acid (9 Ci/mmol), [³H]glycerol (1.1 Ci/mmol), [³H]myristate acid (53 Ci/mmol), [¹⁴C]triolein (57 mCi/mmol), [³H]choline chloride (40 mCi/mmol), and [¹⁴C]phosphatidylcholine (GPC) was determined using cells labeled with [³H]choline-labeled cells were treated with iPLA₂, antagonists (100 μM) for 30 min, and accumulation of [³H]GPC in the cell and medium was quantified at the end of subsequent 2-h chase (with 0.4 mM oleate plus inhibitors).

Enzyme Assays—For PLA₂, phosphatidate phosphohydrolase (PAPH), and MTP activity assays, cells were treated with BEL (100 μM) for 30 min, followed by incubation with oleate (0.4 mM) plus BEL (100 μM) for 1 h. Cells that were incubated only with or without oleate were used as controls. The PLA₂ activity in cell homogenates was measured using [³H]palmitoyl-PC as substrate in the presence or absence of 10 μM CaCl₂ as described previously (33). Dithiothreitol (0.5 mM) was present when PLA₂ activity was determined in the absence of CaCl₂ (33).

The PAPH activity in cytosol or microsomes was determined by using N-ethylmaleimide to distinguish between PAPH and the membrane-bound lipid phosphatase phosphohydrolase (34, 35).

The TG transfer activity of MTP in cell homogenates was measured as described previously (36) using [¹⁴C]triolein as substrate. The assay of phospholipid remodeling (24, 25) and Golgi membrane tubulation (26, 27) was performed by transphosphatidylation assay (37). Cells were labeled with [³H]glycerol (1 Ci/mmol) for 18 h. After labeling, the cells were treated with iPLA₂, antagonists (100 μM) for 30 min, and incubated with butan-1-ol (0.5%) for an additional 30 min. Formation of [²H]Hspatididylbutanol at the end of incubation was quantified.

Thin Layer Chromatography (TLC)—Lipids in total cells, subcellular fractions, or enzyme assay mixtures (e.g. PLA₂ and PLD) were extracted with chloroform/methanol/acidic acid/saturated NaCl/H₂O (4:2:0.01:1:2; by volume), and resolved by TLC using Silica Gel 60 plates. The solvent system chloroform/methanol/acidic acid/formic acid/H₂O (70:30:12:4:2; by volume) was used for separation of PC and phosphatidylethanolamine (PE), whereas hexane/diethyl ether/acidic acid (70:20:10; by volume) was used for separation of PC and phosphatidylcholine. The iPLA₂ was resolved by thin layer chromatography (26, 38). For phosphatidylbutanol separation, the TLC was performed sequentially by first developing with chloroform/methanol/acidic acid (65:15:7.5, by volume) to two-thirds of the plate and then with chloroform/methanol/acidic acid/water (75:45:5:1, by volume) to the top. For separation of water-soluble metabolites such as GPC, the aqueous phase of the lipid extract was resolved using methanol/0.6% sodium chloride/ammonium hydroxide (5:100) from IDL/LDL and HDL that were synthesized from VLDL.

Oligonucleotide Treatment—An antisense oligonucleotide of the rat iPLA₂ cDNA was designed starting from the initiation site: 5'-AGGCCTCCAAGAAGCTCAT-3'. Control sense oligonucleotide was 5'-ATGCAGTTCTTTGGACGCCT-3'. Both were synthesized by Life Technologies, Inc. Cells (20% confluence) were incubated with 10 μM oligonucleotides (freshly prepared prior to use) in serum-free DMEM for 4 h. A final concentration of 20% fetal bovine serum was then added (38), and the cells were cultured for 48 h prior to experiments.

Tandem Mass Spectrometry—Equal aliquots of lipid extracts were dissolved in methylene chloride/methanol/H₂O (45/45/10, by volume) and applied to a Micromass Quattro II triple quadruple mass spectrometer (Micromass) at a flow rate of 8 μl/min (39). Data were acquired using MassLynx NT software (Micromass). A mixture of di-14:9, di-16:0, 16:0–18:1, and di-20:4 PC standards and a separate mixture of di-16:0, di-18:0, and 18:0–22:6 PE standards of equal molar concentration was used to determine instrument settings and concentration for optimal signal intensities. Standards and samples contained 1% of formic acid for positive ion analysis. The PC species were detected by precursor scanning for molecules generating an ion fragment of 184 mass units (phosphocholine moiety) in the positive ion mode at collision energy of 25 V. The PE species were detected by scanning for molecules with a loss of neutral fragment 141 (phosphoethanolamine moiety) in the positive ion mode at collision energy of 20 V. The fatty acid composition of each molecular species was determined by daughter ion analysis in the negative ion mode.

RESULTS

Oleate-induced VLDL Assembly/Secretion and Phospholipid Decacylation—We used cumulative flotation ultracentrifugation technique (40) to resolve B100-VLDL₁ (S₉>100) and B100-VLDL₂ (S₂–100) from IDL/LDL and HDL that were synthesized by hB100-transfected McA-RH7777 cells. The high level

HCl, pH 8.0), and the samples were diluted to 0.2% (w/v) SDS prior to immunoprecipitation of hB100.

Lipid Labeling—Cells in six-well dishes were pulse-labeled with [³H]glycine (2 μCi/ml) or [³H]glycerol (3 μCi/ml) for 3 h, and the labeled lipids were chased for indicated times in the presence or absence of 0.4 mM oleate. The effects of phospholipid remodeling of glycerophosphocholine (GPC) was determined using cells labeled with [³H]choline-labeled cells were treated with iPLA₂, antagonists (100 μM) for 30 min, and accumulation of [³H]GPC in the cell and medium was quantified at the end of subsequent 2-h chase (with 0.4 mM oleate plus inhibitors).

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expression of the recombinant protein makes hB100 more readily detectable than the limited amount of endogenous rat apoB. In these cells, VLDL1 assembly/secretion and phospholipid turnover. A, hB100-expressing cells were labeled with [35S]methionine/cysteine in DMEM ≥ 0.4 mM oleate (OA) for 2 h. The content of microsomal lumen (left) and the medium (right) were fractionated, and 35S-B100 in each fraction was immunoprecipitated and analyzed by SDS-PAGE. B and C, cells were labeled with either [3H]oleic acid (B) or [3H]glycerol (C) for 3 h, incubated with unlabeled medium for 30 min, and chased in the presence (open circle) or absence (closed circle) of 0.4 mM oleate for 2 and 4 h. Data are means (n = 2, some error bars are within symbols) from a representative experiment performed at least three times.

The [3H]oleoyl moiety released from phospholipid is either in free fatty acid form (from the action of phospholipase A (PLA)) or in glycerolipid form such as diacylglycerol (from the action of PLD followed by PAPH). Two pieces of evidence suggested that the PLD pathway might play a limited role. First, oleate exerted no effect on the change of radioactivity of [3H]glycerol-labeled PC or PE during chase (Fig. 1C, top panels), although it did stimulate incorporation of [3H]glycerol into TG and increase secretion of [3H]glycerol-labeled PC, PE, and TG (Fig. 1C, bottom panels). Second, transphosphatidylidylation assay showed that PLD activity was unchanged by oleate in cells treated with or without phospholipid myristate acetate (a compound known to stimulate PLD activity (Refs. 25 and 37)) (Table I, left two columns). Thus, the observed phospholipid turnover is likely catalyzed by a PLA-type activity.

**PLA Antagonists Inhibited Phospholipid Turnover and VLDL Secretion**—Phospholipid turnover was determined by monitoring the release of free fatty acid from [3H]oleate-labeled cells and the formation of GPC in [3H]choline-labeled cells. In both cases, oleate increased the release of [3H]oleoyl acid into the medium by >10-fold (Fig. 2A) and the accumulation of [3H]GPC within the cells by >20-fold (Fig. 2B), indicative of enhanced PC deacylation. Treatment with 100 μM PLA antagonists (i.e. BEL or MAFP) blunted (by 55%) secretion of [3H]oleoyl acid (Fig. 2A) and abolished (by 93%) accumulation of [3H]GPC in the cells (Fig. 2B).

Treatment with PLA antagonists also inhibited VLDL secretion. Preliminary experiments showed that the IC50 of BEL to inhibit VLDL-hB100 and VLDL-TG secretion was ~60 μM (data not shown). At 100 μM, treatment with BEL decreased secretion of 35S-B100 as VLDL and VLDL1 by 70% and 50%, respectively, but secretion of IDL/LDL was less affected (Fig. 3A, BEL + OA). Similar decreases in 35S-B100 secretion as VLDL1 (by 60%) or VLDL2 (by 45%) were observed in MAFP-treated cells (Fig. 3A, MAFP + OA). The inhibited VLDL secretion was confirmed by lipid labeling experiments, where treatment with BEL or MAFP diminished [3H]glycerol-labeled TG secretion by more than 50% (Fig. 3B). Most of the secreted [3H]TG was associated with VLDL1 and VLDL2 (data not shown). The inhibitory effect of BEL on VLDL-TG secretion was also manifest in pulse-chase experiments where TG was labeled with [3H]oleate (Fig. 3B, inset). At 100 μM, BEL did not affect secretion of endogenous apoA-I as HDL (Fig. 3C), nor did it affect secretion of total choloracetic acid-insoluble 35S-labeled proteins (data not shown). The inhibitory effect of BEL treatment on hB100-VLDL secretion was also observed semiquantitatively by immunoblot analysis (Fig. 3D). Moreover, similar inhibitory effect of BEL occurred to the secretion of endogenous rat 35S-B100-VLDL (Fig. 3E). Pulse-chase analysis showed that the secretion of total 35S-B100 was inhibited by ~15% at the end of 2-h chase (Fig. 3F).

The effect of BEL treatment on VLDL secretion was also observed in cells expressing hB48 that secreted both hB48-VLDL and hB48-HDL, where increasing dose of BEL (0–100 μM) decreased hB48-VLDL but not hB48-HDL secretion (Fig. 4, A and B). Thus, BEL preferentially inhibits the secretion of TG-rich particles such as B100-VLDL1 and B48-VLDL with little effect on the general secretory pathway. A point of note is that, although iPLA activity is essential for VLDL secretion, it does not appear to be limiting nor is it sufficient. Transfection of iPLA2 (20) either transiently or stably into McA-RH7777 cells resulted in a 2-fold increase in iPLA2 activity (50 versus 25 units/mg of cell protein), but it did not enhance B100-VLDL1 secretion (data not shown). Similarly, expression of the iPLA2 in HepG2 cells also failed to induce B100-VLDL1 secretion, even though the iPLA activity was increased in transfected cells (88 versus 1.3 units/mg of cell protein) to a level comparable to that in McA-RH7777.

Since BEL inhibits preferably iPLA over cPLA and other secreted forms of PLAs, (41), the above data suggest an effect more attributable to iPLA2. However, BEL was reported to
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Table I

| Effect of oleate and BEL on the enzyme activities in McA-RH7777 cells |
|--------------------------|--------------------------|
| PLD<sup>+</sup> | PAPH<sup>+</sup> | PLA₂<sup>+</sup> | MTP<sup>+</sup> |
| - PMA | + PMA | Cytosol | Microsome | - Ca<sup>2+</sup> | + Ca<sup>2+</sup> | pmol/min/mg |
| none | none | 0.56 ± 0.03 | 5.60 ± 0.54 | 9.59 ± 0.47 | 0.16 | 3.01 ± 0.18 | 7.50 ± 1.65 | 7.94 ± 0.47 | 4.26 ± 0.13 |
| + oleate | + oleate | 6.03 ± 0.03 | 4.79 ± 0.14 | 6.37 ± 1.07 | 0.19 | 5.32 ± 0.32 | 8.50 ± 0.50 | 10.17 ± 0.23 | 2.30 ± 0.17 |
| + oleate + BEL | + oleate + BEL | 6.01 ± 0.01 | 5.54 ± 0.62 | 8.16 ± 0.12 | 0.32 | 5.18 ± 0.32 | 0.60 ± 0.05 | 0.84 ± 0.23 | 2.27 ± 0.25 |

*Cells were prelabeled with [3H]myristic acid (1 μCi/ml) for 18 h and then incubated with oleate ± BEL for 60 min as indicated. Butanol-1 (final concentration of 0.5%) was present in the media during the last 30 min. One set of cells were treated 2 μM PMA. Radioactivity associated with [3H]phosphatidylbutanol in the cells was quantified and used as a measure of PLD activity.

Cytoplasmic and microsomes were prepared from cells treated with or without BEL for 30 min. The PAPH activity was measured using [3H]phosphatidic acid as substrate (34), and expressed as nanomoles of diacylglycerol formed/min/mg of cell protein. Inclusion of BEL (up to 100 μM) in the assay did not affect PAPH activity.

The PLA₂ activity in cell homogenates was assayed using 2-[3H]palmitoyl-PC as substrate (10 mM CaCl<sub>2</sub> (33), and presented as nanomoles of fatty acid release/min/mg of cell protein.

The MTP activity, presented as picomoles of [3H]triolein transferred/min/mg of cell protein, was determined using cell homogenates (9, 36). Including 0.2 μM MTP inhibitor BMS-197636 (9) entirely abolished the TG transfer activity of the samples (data not shown).

![Fig. 2](image-url) Effect of PLA₂ antagonists on fatty acid release and GPC generation. Cells expressing hB100 were labeled with [3H]oleic acid (A) or [3H]choline (B) in DMEM (20% serum) for 4 h and 22 h, respectively. After labeling, the cells were washed, treated with 100 μM BEL or MAFP for 15 min, and chased in different conditions (i.e. in the absence or presence of 0.4 mM oleate (OA) or with or without BEL or MAFP) for up to 4 h for [3H]oleic acid release (A) and for 2 h for [3H]GPC analysis (B) (25).

![Fig. 3](image-url) Effect of PLA₂ antagonists on apoB100-VLDL secretion. Cells expressing hB100 were treated with 100 μM BEL or MAFP for 30 min prior to labeling with [35S]methionine/cysteine (A and C) or with [3H]glycerol (B) for 2 h in the presence of 0.4 mM oleate (OA). Analysis of medium 35S-B100 was the same as in Fig. 1A, and medium [3H]TG as in Fig. 1C. Inset, after incubation with or without BEL for 30 min, the cells were labeled with [3H]oleic acid for 3 h, washed, and chased under indicated conditions for 2 and 4 h. Medium [3H]TG was analyzed as in Fig. 1B. C, density distribution of medium rat 35S-apoA-I. D, hB100-expressing cells were treated with BEL for 30 min followed by incubation with oleate for 2 h. Medium hB100 was detected by immunoprecipitation with anti-apoB antibody 1D1. E, analysis of medium rat 35S-B100 from non-transfected McA-RH7777 cells was similar to that in panel A. F, hB100-expressing cells were pulse-labeled with [3H]methionine/cysteine in the absence or presence of BEL for 1 h, and 35S-B100 was chased with oleate ± BEL for up to 4 h. Medium 35S-B100 was analyzed by immunoprecipitation followed by SDS-PAGE/fluorography.

The decreased VLDL assembly by PLA₂ antagonists was concurrent with diminished accumulation of [3H]TG within microsomal lumen. In comparison with control (i.e. no oleate supplementation), cells treated with oleate exhibited increased inhibitory PLA₂ activity as well (42). Hence, we measured PAPH activity in McA-RH7777 cells and found that neither the cytosolic nor the microsomal-associated PAPH activity was inhibited by treatment of 100 μM BEL (Table I, third and fourth columns from left) or MAFP (data not shown). Additionally, addition of BEL at various concentrations up to 100 μM directly into the enzyme assay mixture did not affect PAPH activity (data not shown). In contrast, the PLA₂ activity was potently inhibited by BEL in a manner independent of Ca²⁺ (Table I, fifth and sixth columns from left). Furthermore, treating cells with antisenase oligonucleotides of iPLA₂ decreased iPLA₂ activity by 50% as compared with controls (Fig. 5A, and decreased secretion of B100-VLDL₁ and B100-VLDL₂ by 50% and 60%, respectively (Fig. 5B). These results combined argue convincingly for a requirement of iPLA₂ in hepatic phospholipid turnover and in VLDL secretion.

Inactivation of iPLA₂ Impairs VLDL Assembly—Assembly of VLDL₁ is achieved post-translationally in McA-RH7777 cells (9), which can be demonstrated by pulse-chase experiments with [35S]methionine/cysteine (20-min pulse and 50-min chase in the presence of oleate) (Fig. 6A, left). Treatment of BEL did not affect the synthesis of total B100 or the association of B100 with IDL/LDL particles, but decreased B100-VLDL₁ assembly by 60% at the end of chase (Fig. 6A, arrows in +BEL versus control).

The decreased VLDL₁ assembly by iPLA₂ antagonists was concurrent with diminished accumulation of [3H]TG within microsomal lumen. In comparison with control (i.e. no oleate supplementation), cells treated with oleate exhibited increased cellular [3H]TG (29.95 ± 1.50 versus 22.26 ± 0.05 cpm × 10⁵/ dishes). The oleate-stimulated incorporation of [3H]glycerol into microsomal membrane TG and cytosolic TG was little altered by BEL or MAFP (Fig. 6B, middle and right). However, the PLA₂ antagonists decreased accumulation of [3H]TG in the microsomal lumen by 50% (Fig. 6B, left). The decreased [3H]TG in the microsomal lumen was not a result of compromised MTP
The inhibited VLDL assembly by BEL within microsomal lumen was not associated with impairment in translocation of apoB across the ER membranes. Trypsin digestion assay of microsomes showed that there was no difference between control (Fig. 6C, control, lanes 2, 5, and 8) and BEL-treated cells (Fig. 6C, +BEL, lanes 2, 5, and 8) in gaining trypsin resistance of 35S-B100 during the entire chase period (Fig. 6C).

**Inactivation of iPLA₂ Results in Accumulation of ApoB on Microsomal Membranes**—Treatment with BEL resulted in a small, but reproducible, increase (about 5–10%) in cell-associated 35S-B100, as determined by pulse-chase analysis (Fig. 7A). Since previous studies suggested that VLDL was assembled from apoB precursors attached to the microsomal membranes (8, 43), we sought to determine if BEL treatment resulted in increased membrane association of apoB. The cells were labeled with [35S]methionine/cysteine for 2 h, and the association of 35S-B100 with membranes (Fig. 7B) and within the lumen (Fig. 7C) was examined. In BEL-treated cells, there was indeed an increased (by 10%) association of 35S-B100 with membranes over the controls (Fig. 7B, lanes marked by arrows). At the end of 2 h of labeling, 35S-B100 associated with luminal VLDL₁ and VLDL₂ was not as abundant as that seen in pulse-chase experiments (Fig. 6A). However, the inhibitory effect of BEL on luminal 35S-B100-VLDL₁ was still observable (Fig. 7C, lanes marked by arrows). It was reported that the membrane-associated 35S-B100 could be dislodged from membranes by deoxycholate (8). We confirmed this observation and found that deoxycholate removed approximately 75% of the membrane associated 35S-B100 (Fig. 7B, lanes labeled Doxy/KCl) and increased luminal 35S-B100 (Fig. 7C). Comparison of luminal 35S-B100 derived from sodium carbonate treatment in the absence (Fig. 7C, top) or presence of deoxycholate (Fig. 7C, bottom) showed that the recovery of luminal 35S-B100 from deoxycholate-treated samples was markedly increased in IDL/LDL and HDL fractions. Quantitative analysis (Fig. 7D) showed that there was at least 2-fold increase in 35S-B100 (mostly in IDL/LDL and HDL form) that were associated with microsomal membranes in BEL-treated cells. Thus, the activity of iPLA₂ may play a role in the release of membrane-associated apoB precursor (i.e. in IDL/LDL forms) into microsomal lumen to form mature VLDL.

**Effect of Pretreatment with Oleate and iPLA₂ Antagonists on VLDL Assembly and Microsomal Phospholipid Species**—To gain insight into the mechanism by which iPLA₂ antagonist inhibits oleate-induced VLDL assembly, we tested the effect of
iPLA₂ inhibition on cells that had been pretreated with oleate. When cells were incubated with oleate for 30 min prior to iPLA₂ inactivation, the inhibitory effect of BEL treatment on apoB₁₀₀-VLDL₁ assembly (Fig. 8A, left) or secretion (Fig. 8A, right) was no longer observable. Likewise, secretion of [³⁵S]TG, induced by oleate (Fig. 8B, left, +OA versus −OA) was not affected by BEL treatment from cells that had been incubated with oleate prior to BEL treatment (+OA→+BEL). However, in cells that had been pretreated with the antagonist, the oleate-induced [³⁵H]TG secretion was attenuated significantly (+BEL→+OA). The altered BEL treatment protocol had little effect on the oleate-stimulated incorporation of [³²P]glycerol into cell TG (Fig. 8B, right). Thus, the iPLA₂ activity becomes nonessential for VLDL assembly/secretion in cells that have been treated with oleate. To examine if this effect was oleate-specific, we compared oleate with eicosapentaenoic acid, an n-3 fatty acid. Although treatment with eicosapentaenoic acid (0.4 mM) resulted in accumulation of cell-associated [³²P]glycerol-labeled TG to a level similar to that treated with oleate as shown in Fig. 8B, eicosapentaenoic acid did not prevent inhibition of VLDL secreted exerted by BEL treatment (data not shown).

The above results showing differential effect between oleate and eicosapentaenoic acid suggest that VLDL assembly is dependent of proper lipid composition, which may be affected by inactivation of iPLA₂. We therefore determined molecular species of microsome-associated PC and PE under different treatment conditions with oleate and/or iPLA₂ antagonists. Electron spray tandem mass spectrometry analysis showed that incubation with oleate increased total cellular PC and PE masses by 60% and 110%, respectively. However, oleate did not increase the mass of PC (oleate, 1.40×10⁴; control, 1.42×10⁴; arbitrary units/mg of protein) or PE (oleate, 0.90×10⁴; control, 1.12×10⁴; arbitrary units/mg of protein) associated with the microsomal membranes. This result derived from mass spectrometry analysis agrees with our previous chemical measurement (9) that oleate increased incorporation of [³²P]glycerol into PC (an indication of increased synthesis) but did not affect PC mass in the microsomal membranes. However, oleate treatment resulted in enrichment of species with 18:1/18:1 or 18:1/18:2) were less affected by oleate treatment (130%) and 18:1/18:1 in PE (by 24%) as compared with controls (Fig. 9, A and B). Species of PC and PE with other diacyl chains (e.g. 16:0/18:1 or 18:1/18:2) were less affected by oleate treatment. Treatment of cells with BEL caused a moderate decrease in microsomal PC species with oleoyl chain and markedly decreased the oleoyl-containing PE species (BEL→+OA). Preincubation of cells with oleate prevented the decrease in oleoyl-containing PC and PE species caused by BEL treatment (OA→BEL versus BEL→OA). These results suggest that oleate pretreatment may have primed the microsomal phospholipids that are essential for VLDL assembly and have achieved an effect that is otherwise dependent upon iPLA₂ action. The effect of oleate and iPLA₂ antagonist on other microsomal phospholipids (such as phosphatidylserine or phosphatidylinositol) was rather specific to the assembly and secretion of TG-rich VLDL (e.g. B₁₀₀-VLDL₁ and B₄₈-VLDL) but not the secretion of small lipoproteins such as HDL. The specific inhibitory effect of iPLA₂ antagonists toward VLDL assembly is reminiscent of observations derived from studies under many other pathophysiological or pharmacological conditions where VLDL secretion is impaired but HDL secretion is normal. Several notable examples related to phospholipid me-

**DISCUSSION**

Multiple lines of evidence from the current work suggest that the assembly of VLDL requires iPLA₂ activity. The inhibitory effect of iPLA₂ antagonists (chemical inhibitors or antisense oligonucleotides) is rather specific to the assembly and secretion of TG-rich VLDL (e.g. B₁₀₀-VLDL₁ and B₄₈-VLDL) but not the secretion of small lipoproteins such as HDL. The specific inhibitory effect of iPLA₂ antagonists toward VLDL assembly is reminiscent of observations derived from studies under many other pathophysiological or pharmacological conditions where VLDL secretion is impaired but HDL secretion is normal. Several notable examples related to phospholipid me-
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**FIG. 9. Analysis of PC and PE species.** Cells expressing hB100 were incubated with 100 μM BEL (black bars) or 0.4 mM oleate (OA) (gray bars) for 30 min, followed by oleate + BEL for 2 h. White bars, non-treated controls; hatched bars, oleate-treated only. Microsomal lipids were extracted and subjected to electrospray tandem mass spectrometry for the analysis of PC (A) and PE (B) species as described under “Experimental Procedures.” Values are expressed as percentages of non-treated controls. The analysis was performed twice with identical results.

It is now clear that bulk TG incorporation into VLDL is achieved post-translationally (3, 9, 10, 47). However, less clear is the mechanism by which bulk TG is incorporated. Abundant TG availability is essential, but it alone is not sufficient to drive VLDL assembly, as exemplified by studies with hepatic cells treated with n-3 fatty acids (48–50) or insulin (51, 52), where active ER-to-Golgi trafficking remains functional. Thus, the current study of iPLA2-mediated phospholipid turnover, together with previous observations, highlight the existence of a possible vesicular transport system that is specialized for TG-rich VLDL assembly/secretion.

Changes in phospholipid composition render significant biological consequences. Alteration in membrane shape (56) and membrane curvature (57) is associated with membrane vesiculation and tubulation (13, 58). Glycerolipids containing monounsaturated acyl chains (e.g. oleoyl chain) are shown to activate proteins essential for the coating of budding vesicles (59) and membrane fusion (60). In addition to their direct effect on membrane curvature, distinct phospholipid species can regulate membrane fusion by changing the secondary structure of peptide in fusion protein (61). It is therefore conceivable that deacylation/reacylation of phospholipid, induced by oleate, provides a means for oleoyl chain to be incorporated into phospholipid species and for subsequent generation of lysophospho-
lipid, diacylglycerol, and phosphatidic acid. Generation and proper localization of these oleyl-rich glycerolipid species may be important for the event of bulk TG incorporation into VLDL. Once such a primed membrane is established (e.g. in the case of pretreatment of cells with oleate), inhibition of iPLA₂ activity with antagonists may not exert an acute effect on bulk TG incorporation into VLDL. The activity of iPLA₂, including iPLA₂ present in cytosol of cells, is shown to participate in endosome fusion of macrophages (62), in the Golgi tubule-mediated retrograde trafficking to the ER, and in the maintenance of Golgi complex architecture in rat hepatocytes (26, 27).

Our current results, although inconclusive, reveal a possible function of iPLA₂-mediated phospholipid remodeling in VLDL assembly/secretion. However, whether the impaired VLDL assembly/secretion by iPLA₂ inhibition is the results of perturbed membrane movement essential for bulk TG incorporation remains to be determined.

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