# Inhibition of phosphatidylcholine synthesis via the phosphatidylethanolamine methylation pathway impairs incorporation of bulk lipids into VLDL in cultured rat hepatocytes

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**Abstract** **Inhibition of phosphatidylcholine (PC) synthesis via the phosphatidylethanolamine (PE) methylation pathway was shown to decrease the secretion of VLDL from primary rat hepatocytes (Nishimaki-Mogami et al. 1996. *Biochim. Biophys. Acta.* 1304: 21–31). To understand further the role of PE methylation, we determined the effect of bezafibrate, an inhibitor of PE methylation, on VLDL assembly within the microsomal lumen. Bezafibrate was shown to decrease VLDL (triacylglycerol) secretion only when cellular PE methylation was active in the presence of methionine. Pulse-chase experiments showed that bezafibrate treatment did not impair the movement of [$^{35}$S]apolipoprotein (apo)B-48 from microsomal membranes into the lumen. However, bezafibrate treatment resulted in reduced VLDL-[$^{35}$S]apoB-48 and increased [$^{35}$S]apoB-48-containing particles in the HDL density range (HDL-[$^{35}$S]apoB-48) within the lumen. Inhibition of PE methylation by bezafibrate or 3-deazaadenosine after the completion of HDL-[$^{35}$S]apoB-48 assembly effectively decreased VLDL-[$^{35}$S]apoB-48 secretion with a concomitant increase in HDL-[$^{35}$S]apoB-48 secretion. These findings suggest that inhibition of PC synthesis via the PE methylation pathway impairs the stage of bulk triacylglycerol incorporation during the assembly of VLDL.**—Nishimaki-Mogami, T., Z. Yao, and K. Fujimori. **Inhibition of phosphatidylcholine synthesis via the phosphatidylethanolamine methylation pathway impairs incorporation of bulk lipids into VLDL in cultured rat hepatocytes. *J. Lipid Res.* 2002.** 43: **1035–1045.**

**Supplementary key words** bezafibrate • 3-deazaadenosine • apolipoprotein B

VLDL are triacylglycerol (TG)-rich particles that are assembled from a single copy of apolipoprotein (apo)B with various lipids in the liver. The major factor that regulates hepatic VLDL assembly and secretion is the availability of lipids (1). The rate of synthesis of neutral lipids such as TG (2, 3) and cholesteryl ester (4–7), the major core lipid constituents of VLDL, has a profound effect on VLDL synthesis and secretion. In addition, active synthesis of phosphatidylcholine (PC), the major phospholipid component of VLDL, is also required for efficient secretion of hepatic VLDL. In hepatocytes of rats deficient in choline, decreased VLDL secretion was observed as a consequence of severely reduced PC synthesis (8).

PC is synthesized via the CDP-choline pathway and also via the phosphatidylethanolamine (PE) methylation pathway (9). Since the PE methylation pathway is quantitatively important only in the liver (9), it seems very likely that this pathway plays a significant role in VLDL secretion. In a previous study, we showed that inhibition of PC synthesis via the PE methylation pathway by bezafibrate or 3-deazaadenosine (DZA) decreases VLDL secretion from primary rat hepatocytes (10). Bezafibrate is found to potently inhibit the microsomal PE *N*-methyltransferase activity (11), and DZA is an inhibitor of *S*-adenosylhomocysteine hydrolase that blocks methylation reactions (12). The significant function of PE methylation in vivo has also been suggested by reduced plasma TG concentrations in rats that are treated with eritadenine (13), a compound similar to DZA (12). Furthermore, studies with transgenic

Abbreviations: APMSF, (*p*-amidinophenyl) methanesulfonyl fluoride-HCl; apo, apolipoprotein; DZA, 3-deazaadenosine; ER, endoplasmic reticulum; HDL-apoB, immunoaffinity purified HDL containing apoB; MTP, microsomal triglyceride transfer protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, PE *N*-methyltransferase; PMME, phosphatidylmonomethylethanolamine; PPAR, peroxisome proliferator-activated receptor; RIPA, radio-immunoprecipitation assay; TG, triacylglycerol; VLDL-apoB, immunoaffinity purified VLDL containing apoB.

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mice where the PE *N*-methyltransferase gene is inactived showed drastically decreased plasma lipid levels when the CDP-choline pathway was inactivated by feeding a choline-deficient diet (14). Combined, these experimental results strongly suggest that the PE methylation pathway in the liver may play a specific role in VLDL assembly and secretion. What remains to be determined are the mechanisms by which reduced PC synthesis via the PE methylation pathway impairs VLDL assembly and secretion.

Assembly of VLDL in the liver is a complex process. To date, mechanisms by which lipids are recruited during VLDL assembly have not been fully defined. Association of apoB polypeptide with lipids may occur at the stage of apoB translation and translocation across the rough endoplasmic reticulum (ER) membrane (15), resulting in a dense, primordial particle that serves as a precursor of mature VLDL (16–19). Several studies have suggested that translocation of apoB across the ER membrane is a crucial step in determining whether apoB is to be assembled into lipoproteins (when lipid supply is abundant) or to be degraded by the ubiquitin-proteasome pathway (when lipid supply is insufficient) (20–23). Lipids that have been shown to affect the efficiency of apoB translocation include TG (24), phosphatidylmonomethylethanolamine (PMME) (25), and other glycerolipids (26, 27). Conversion of the dense, primordial particle into mature VLDL is achieved post-translationally (16, 17, 19, 28) and is dependent upon the availability of bulk TG (16). Pulse-chase studies have shown that immediately after translation, apoB-48 forms particles of density resembling that of HDL. Conversion of the HDL-apoB-48 into VLDL-apoB-48 occurs at a delayed stage known as the second-step lipidation (16, 19, 28). These results support the two-step assembly model that was originally proposed for hepatic VLDL assembly on the basis of immunoelectron microscopy studies with the rat liver (29).

In a previous study, we observed reduced secretion of VLDL-apoB-48 and increased accumulation of HDL-apoB-48 in the medium during a 12 h treatment of cells with bezafibrate (10). To test the hypothesis that reduced PC synthesis via the PE methylation pathway disrupts the conversion of HDL-apoB-48 to VLDL-apoB-48, we determined the effect of inhibitors of PE methylation on various stages in VLDL assembly. We obtained evidence suggesting that reduced PE methylation impairs the late stage of assembly for the incorporation of bulk TG into VLDL-apoB-48.

## MATERIALS AND METHODS

### Materials

EXPRE$^{35}$S$^{35}$S Protein Labeling Mix (a mixture of [$^{35}$S]methionine and cysteine) and [2-$^{3}$H]glycerol was purchased from NEN, and [2-$^{14}$C]ethanolamine, [1-$^{3}$H]ethanolamine, and [methyl-$^{14}$C]choline chloride were obtained from Amersham. The culture medium and serum were purchased from GIBCO. (*p*-Amidinophenyl)methanesulfonyl fluoride-HCl (APMSF), triglyceride, and phospholipid assay kits were obtained from Wako Pure Chemical Industries Ltd., Japan. 3-Deazaadenosine was obtained from Southern Research Institutes (Birmingham, AL). Goat anti-human apoB antibodies were purchased from Chemicon International Inc. (Temecula, CA), and protein G-agarose was obtained from Boehringer Mannheim.

### Culture of hepatocytes and metabolic labeling

Hepatocytes were prepared from female Wistar rats (120−170 g) that we fed with a standard laboratory diet as described previously (11). The cells were plated in 3 ml of DMEM containing 10% FBS, 100 μM ethanolamine, 0.1 μM insulin, and 100 μg/ml kanamycin. After 4–16 h, the medium was replaced with the experimental media as described in the figure legends. For [$^{35}$S] methionine/cysteine labeling, the medium was changed to a serum- and methionine-free medium and incubated for 1 h. The cells were pulse-labeled with [$^{35}$S]methionine/cysteine for 15 min and chased for up to 4 h in a medium containing 2.5 mM methionine, 1.0 mM cysteine, 1 μg/ml aprotinin, and 100 μM ethanolamine.

### Preparation of microsomal membranes and lumenal contents

After removal of the culture medium, the cells were washed once with PBS and permeabilized with 1 ml of buffer A [0.25 M sucrose, 10 mM Tris-HCl (pH7.4), 1 mM EGTA] containing 100 μg/ml digitonin for 10 min on ice. The extent of permeabilization was monitored by trypan blue. All solutions contained protease inhibitors: 0.1 mM leupeptin, 25 μM *N*-acetyl-leu-leu-norleucinal, 1 μM pepstatin, 50 μg/ml aprotinin, and 100 μM APMSF. After removal of the buffer, the cell ghosts were collected in 2 ml buffer A and centrifuged at 7,000 *g* for 10 min. The supernatant contained 95% activity of lactate dehydrogenase in cells. The pellet was suspended in 0.5 ml buffer A by homogenization and the homogenate was diluted with 0.5 ml of 0.2 M $Na_2CO_3$ and incubated for 30 min at room temperature. After adding 100 μl of 10% (w/v) BSA, the samples were centrifuged using a Beckman TLA-100.4 rotor at 70,000 rpm for 30 min. The supernatants (the lumenal contents) were collected and neutralized, and protease inhibitors were added. The pellets (membranes) were suspended in radio-immunoprecipitation assay (RIPA) buffer [0.05 M Tris-HCl (pH 8.0), 0.15 M NaCl, 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 1 mM EDTA, 1 mM dithiothreitol] containing 1% (w/v) SDS, and proteins were solubilized by sonication for 6 × 10 s and incubating for 1 h on ice. After dilution with 9 volumes of RIPA without SDS, the supernatant was used for immunoprecipitation.

### Density gradient centrifugation of lumenal content and medium

Cells were suspended in 0.5 ml buffer A and disrupted by 20 passages through a 22-gauge needle. The postnuclear supernatant was obtained by centrifugation at 500 *g* for 2 min and diluted with 0.5 ml of 0.2 M $Na_2CO_3$. The lumenal contents were separated from membranes by centrifugation at 400,000 *g* for 30 min. The medium and lumenal contents were fractionated by density gradient centrifugation as described previously (30). After centrifugation for 66 h at 38,000 rpm in a Hitachi P40ST rotor, the fractions were collected from the top of the tubes using an automatic liquid charger ALC-20 (Advantec, Japan).

### Immunoprecipitation of apoB and electrophoresis

Immunoprecipitation of apoB from membrane extracts or from the lumenal contents, medium, and gradient fractions after adding 1/10 volume of 10× RIPA was achieved using goat anti-human apoB antibodies and protein G-agarose as described previously (10). The apoB proteins were solubilized from the immunocomplexes and separated by electrophoresis on 3−15% gradient polyacrylamide gel containing 0.1% SDS (SDS-PAGE).

The radioactivity associated with [$^{35}$S]apoB was quantified by a Bio-Imaging Analyzer BAS-1500 (Fuji Film, Tokyo, Japan) using the photo-stimulated luminescence method.

### Lipid analysis

Lipids were extracted from the cells, culture medium, and the affinity purified apoB-containing lipoproteins (i.e., VLDL-apoB, HDL-apoB) with chloroform-methanol (2:1; v/v). Phospholipids were analyzed by TLC on Silica Gel G with a solvent system of chloroform-methanol-acetic acid-$H_2O$ (50:30:8:3; v/v/v/v) to resolve PC and lysoPE. Lipid radioactivity was directly quantified by a Bio-Imaging Analyzer as described above. The TG mass was determined using a TG assay kit, and phospholipid mass was quantified by measuring phosphorus content (for PC and PE) or using a phospholipid assay kit (for PC).

### Statistical analysis

Data was analyzed by using the Student's *t*-test for comparisons between two groups and using ANOVA followed by the Dunnett test for comparisons among multiple groups.

## RESULTS

### Reduced TG secretion by bezafibrate requires methionine

In a previous study, we showed that bezafibrate and DZA decrease VLDL secretion from cultured rat hepatocytes in a manner dependent on cellular PE methylation activities (10). To further ascertain that bezafibrate decreases VLDL secretion only when the cellular PE methylation pathway is active, we examined the effect of methionine depletion on bezafibrate action. When methionine, which provides methyl units for the PE methylation pathway, was depleted from the medium, treatment of cells with bezafibrate up to 12 h had no effect on TG secretion from rat hepatocytes (**Fig. 1A**), whereas bezafibrate elicited a 60% reduction of TG secretion after the same period of treatment in the presence of methionine (Fig. 1B). The majority (95%) of TG secreted into the medium was associated with VLDL ($d < 1.02$). Two hours after preincubation in a methionine-free medium, the incorporation of [$^3$H]ethanolamine into cellular PC (during a 3 h labeling in the same medium) was decreased to $3.41 \pm 0.03 \times 10^3$ dpm/dish compared with $15.1 \pm 0.01 \times 10^3$ dpm/dish in the methionine-supplemented control, while incorporation of label into cellular PE was $204 \pm 2 \times 10^3$ dpm/dish and $271 \pm 11 \times 10^3$ dpm/dish in methionine-depleted and supplemented cells. Addition of bezafibrate in methionine-free medium only slightly increased the reduction in [$^3$H]ethanolamine-labeled PC ($2.88 \pm 0.02 \times 10^3$ dpm/dish compared with $3.41 \pm 0.03 \times 10^3$ dpm/dish without bezafibrate), while label present in PE was $324 \pm 7 \times 10^3$ dpm/dish. These results indicate that the PE methylation pathway was rapidly inactivated by methionine depletion. Incorporation of [$^3$H]choline into cellular PC and aqueous metabolite during a 1 h labeling was unaffected by the a 2 h depletion of methionine. Reduced TG secretion by bezafibrate in methionine-supplemented cells was accompanied by an increase in cellular TG mass (from $107 \pm 5$ nmol/mg protein in the control to $131 \pm 4$ nmol/mg protein, $n = 3$, after a 12 h treatment), as reported previously (10). However, in the absence of methionine, the cell TG mass was unchanged by bezafibrate treatment ($112 \pm 8$ nmol/mg protein compared with $110 \pm 6$ nmol/mg protein in the control, $n = 3$). Pretreatment of cells with bezafibrate for 12 h marginally affected TG synthesis as determined by the incorporation of [$^{14}$C]oleate (during 2 h, $114 \pm 10\%$ of control, $n = 4$) or [$^3$H]glycerol (during 2 h, $84 \pm 6\%$, $n = 3$). These observations indicate that, at least within the time frame of 12 h, bezafibrate decreases VLDL secretion mainly through inhibiting PE methylation.

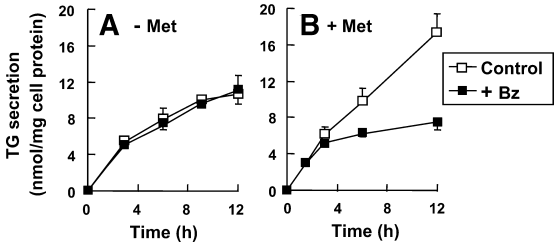


**Fig. 1.** Reduced TG secretion by bezafibrate requires methionine. Hepatocytes plated for 16 h in DMEM containing 10% FCS and 100 μM ethanolamine were further incubated in DMEM containing ethanolamine ± 200 μM bezafibrate (Bz) in the absence (A) or presence (B) of 400 μM methionine. At times indicate the amount of TG secreted into the medium was determined. Results are mean ± range from two independent experiments performed in duplicate.

### Reduced PE methylation by bezafibrate does not impair the movement of apoB-48 from the membrane to the lumen and medium

As the first step toward understanding the role of PE methylation in VLDL assembly, we examined the effect of bezafibrate on the movement of apoB from the membrane to the lumen and medium in a pulse-chase experiment. Rat hepatocytes were pretreated with bezafibrate for 12 h and pulse-labeled for 15 min with [$^{35}$S]methionine/cysteine. Incorporation of $^{35}$S-label into cell-associated apoB-100 and apoB-48 was slightly increased ($7 \pm 10\%$ and $16 \pm 13\%$, respectively; $n = 3$) by treatment with bezafibrate, but this was paralleled with increased $^{35}$S-label into total cellular proteins (by $17 \pm 12\%$; $n = 3$). After 15 min of chase, the amount of total [$^{35}$S]apoB-48 and [$^{35}$S]apoB-100 reached a maximum and declined thereafter (**Fig. 2**). The membranes-lumen ratio of [$^{35}$S]apoB-48 was 1.95 in control cells at this point (Fig. 2A), whereas the ratio was decreased to 0.87 by bezafibrate. A decrease of [$^{35}$S]apoB-48 in the membrane and the increases in lumen and medium in bezafibrate-treated cells were observed during the entire chase period, suggesting that the inhibition of PE methylation by bezafibrate does not impair, but rather accelerates, the movement of apoB-48 from the membrane to the lumen. In accordance with previous studies (10, 25, 30), the level of [$^{35}$S]apoB-100 in rat hepatocytes was very low compared with that of [$^{35}$S]apoB-48 (Fig. 2B). The treatment with bezafibrate exerted little effect on the distribution of [$^{35}$S]apoB-100 between the membrane and the lumen (Fig. 2B).

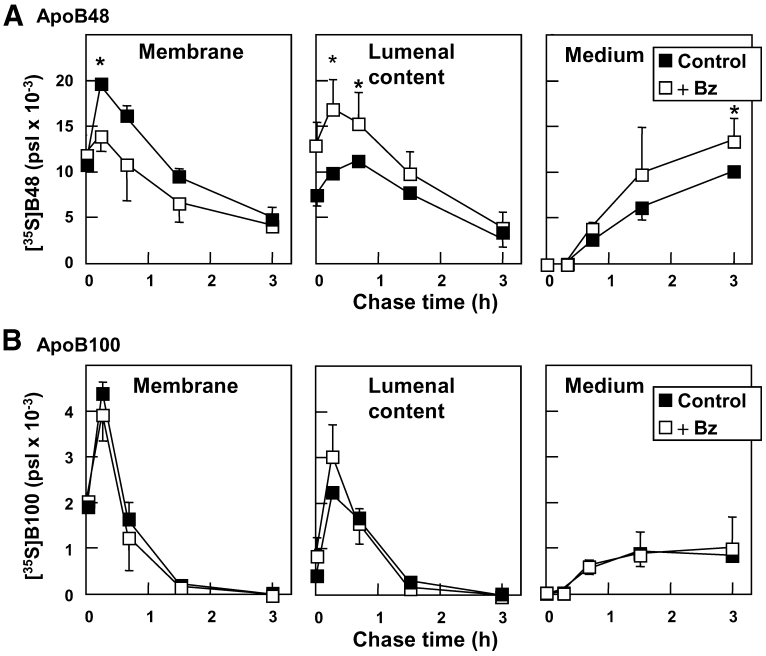


**Fig. 2.** Movement of [$^{35}$S]apoB from microsomal membranes into the lumen and medium is unimpaired in bezafibrate-treated cells. Hepatocytes cultured in DMEM containing 10% FCS and 100 μM ethanolamine ± 200 μM Bz for 12 h were pulse-labeled with [$^{35}$S]methionine/cysteine for 15 min. The cells were chased for up to 180 min in DMEM (containing methionine, cysteine, and ethanolamine) ± 200 μM Bz. At each time point during chase (i.e., 15, 45, 90, and 180 min), cells were permeabilized with digitonin and the lumenal contents were extracted with sodium carbonate. The [$^{35}$S]apoB-48 and [$^{35}$S]apoB-100 were immunoprecipitated from microsomal membrane, lumen, and medium, and were analyzed by SDS-PAGE. Radioactivity associated with [$^{35}$S]apoB-48 (A) and [$^{35}$S]apoB-100 (B) was quantified by a BAS-1500 image analyzer. Results are the mean ± SD (range) of two to five experiments. Values were normalized to control values (lumenal contents and membranes at 15 min chase and medium at 180 min chase) of a representative experiment. *Significantly different from respective controls ($P < 0.05$).

Density gradient centrifugation of the medium after 180 min chase showed that enhanced [$^{35}$S]apoB-48 secretion by bezafibrate treatment resulted from a large increase (by 138 ± 108%; n = 3) in secretion of apoB-48 in the density range of HDL (HDL-apoB-48) (**Fig. 3A, B**; fractions 3–6). In contrast, the secretion of VLDL-[$^{35}$S]apoB-48 (fraction 1) was significantly diminished by bezafibrate by 30 ± 10% ($P < 0.05$; n = 3). Radioactivity

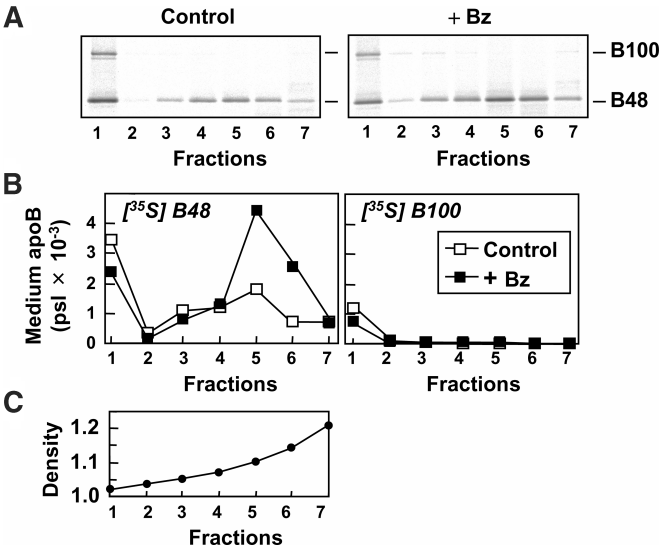


**Fig. 3.** Secretion of VLDL-[$^{35}$S]apoB is reduced in Bz-treated cells. Hepatocytes were cultured and subjected to pulse-chase analysis as described in the legend to Fig. 2. After 180 min chase, the medium was collected and subjected to density gradient centrifugation. A: The [$^{35}$S]apoB proteins in each fraction were immunoprecipitated, resolved by SDS-PAGE, and visualized by a BAS-1500 image analyzer. B: Quantification of radioactivity associated with [$^{35}$S]B-48 (left panel) or [$^{35}$S]B-100 (right panel). The results are representative of three independent experiments. C, density of each fraction.

associated with VLDL-[$^{35}$S]apoB-100 was relatively low, and a small reduction of VLDL-[$^{35}$S]apoB-100 (by 19 ± 10%; n = 3) was not significant ($P < 0.05$).

### Reduced PE methylation by bezafibrate impairs VLDL assembly within the microsomal lumen

To further understand the mechanism by which reduced PE methylation decreased VLDL secretion, we performed pulse-chase experiments to analyze VLDL assembly within the microsomal lumen. As shown in **Fig. 4A**, in control cells the majority of [$^{35}$S]apoB-48 that appeared in the lumen after 15 min of chase had a density resembling that of HDL (fractions 3–6). The amount of these HDL-like particles decreased with time, which was accompanied by an increase in the amount of VLDL-[$^{35}$S]apoB-48 (fraction 1). The amount of VLDL-[$^{35}$S]apoB-48 reached a maximum after 60 min of chase, accounting for 60% of total [$^{35}$S]apoB-48 presented in the lumen. In bezafibrate-treated cells, the majority of [$^{35}$S]apoB-48 was present as HDL-like particles throughout the entire chase period (Fig. 4B). At 60 min of chase, VLDL-[$^{35}$S]apoB-48 accounted for only 27% of total [$^{35}$S]apoB-48 in the lumen. The radioactivity in VLDL-[$^{35}$S]apoB-48 was significantly reduced by 33 ± 10% (n = 3; $P < 0.05$) compared with the control by bezafibrate treatment. This was accompanied by a large increase in HDL-apoB-48 (170 ± 60%; n = 3). The appearance of VLDL-[$^{35}$S]apoB-100 within the lumen at 60 min chase was decreased by 26 ± 8% (n = 2) (Fig. 4B). The prolonged appearance of [$^{35}$S]apoB-48 associated with HDL-like particles in bezafibrate-treated cells suggested that conversion of these primordial particles into mature VLDL was impaired.

### Reduced PE methylation impairs the late stage of assembly for the addition of bulk core lipids into VLDL-apoB-48

To determine if reduced PE methylation impairs the conversion of primordial HDL-apoB-48 to VLDL-apoB-48,

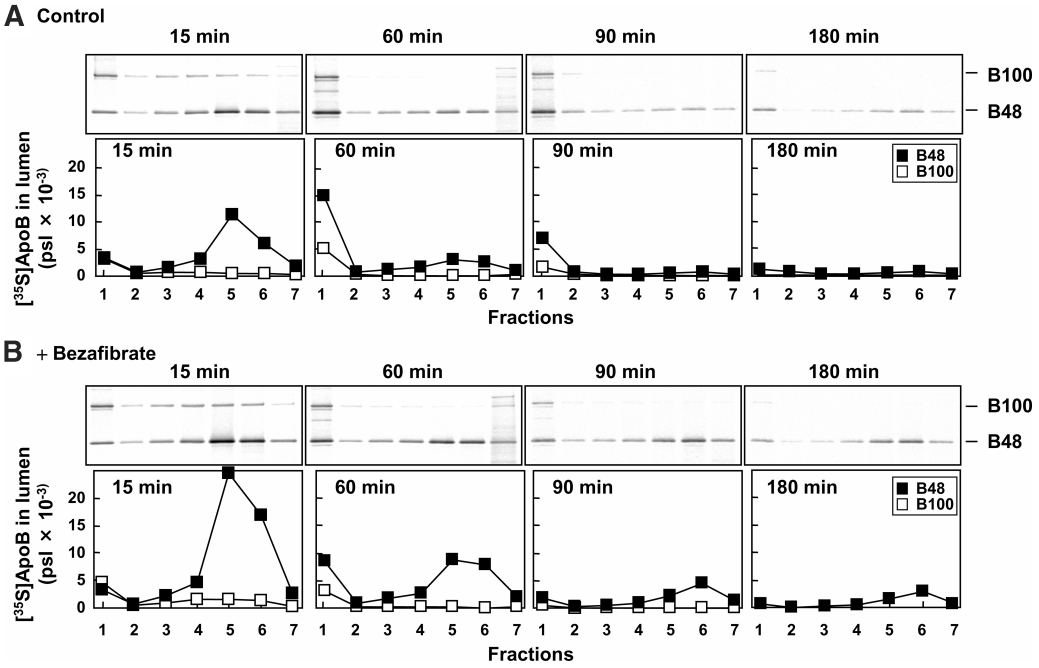


**Fig. 4.** Bz treatment decreases VLDL assembly in microsomal lumen. Hepatocytes were cultured with or without 200 μM Bz and pulse-labeled as described in the legend to Fig. 2. The cells were chased for 15 to 180 min in DMEM supplemented with methionine, cysteine, ethanolamine, and 0 μM (A) or 200 μM bezafibrate (B). At each chase time, cells were harvested, and the lumenal contents obtained were subjected to density gradient centrifugation. Gel image of [$^{35}$S]apoB in each fraction (top) and the quantified radioactivity associated with [$^{35}$S]apoB (bottom) are shown in each panel. The results are representative of three independent experiments.

we examined the effect of inhibitors on this late stage of assembly. Two sets of pulse-chase experiments with [$^{35}$S] methionine/cysteine were performed by using cells that had been preincubated in a choline- and methionine-free medium (without inhibitors) for 16 h, and subsequently the effect of inhibitors was examined in methionine-supplemented medium. This protocol was used to obtain rapid effect elicited by reduced PE methylation. While the reduced incorporation of [$^{3}$H]ethanolamine into cellular PC can be observed as early as 20 min after initiating bezafibrate treatment (11), reduction in TG secretion became evident after a 6 h treatment (Fig. 1B) in normal (choline- and methionine-supplemented) medium. We hypothesized that this lag-time resulted from the compensation of PC synthesis via the CDP-choline pathway. Since cultured rat hepatocytes have a huge pool of phosphocholine (31), we depleted this precursor pool for the CDP-choline pathway by preincubating cells in choline- and methionine-free medium as previously reported (32). Upon labeling with a tracer amount of [$^{3}$H]choline (2.5 μM) for 1 h, depletion of choline and methionine for 16 h decreased incorporation of label into aqueous metabolites (mainly phosphocholine) (37 ± 12% of total cellular label compared with 89 ± 5% in normal cells) without affecting total uptake of [$^{3}$H]choline, indicating partial depletion of the aqueous precursor pool. Under these conditions, TG secretion was rapidly inhibited by 49 ± 8% ($P < 0.01$; n = 4) by a 3 h treatment with bezafibrate [**Fig. 5A** (a)], but was unaltered in cells preincubated in normal medium [Fig. 5A(b) and Fig. 1B (up to 3 h)], indicating that depletion of the phosphocholine pool did effectively reduce lag time. In parallel, PC synthesis via the PE methylation pathway as determined by incorporation of [$^{3}$H]ethanolamine into PC (for 3 h) was decreased by choline- and methionine-depletion (3.29 ± 0.13 × $10^3$ dpm/dish compared with 15.1 ± 0.01 × $10^3$ dpm/dish in methionine- and choline-supplemented control) and was not further reduced by bezafibrate (3.23 ± 0.11 × $10^3$ dpm/dish in PC). Addition of methionine (2.5 mM) to the medium during labeling restored incorporation of label into PC (to 11.6 ± 0.4 × $10^3$ dpm/dish) but did not in the presence of bezafibrate (3.35 ± 0.15 × $10^3$ dpm/dish in PC).

In the first pulse-chase experiments, cells were pulse-labeled for 15 min with [$^{35}$S]methionine/cysteine and treated with or without bezafibrate only during the chase period. Secretion of VLDL-[$^{35}$S]apoB-48 was reduced by bezafibrate by 43 ± 2% (average ± range; n = 2) during the first 90 min chase and by 64 ± 9% ($P < 0.05$; n = 5) during the 90–240 min chase (Fig. 5B, C; compare fraction 1 in left and middle panels). Similarly, DZA, a compound that inhibits general methylation reactions, decreased secretion of VLDL-[$^{35}$S]apoB-48 by 29 ± 4% (n = 2) during the first 90 min chase and by 51 ± 11% ($P < 0.05$; n = 3) during the 90–240 min chase (Fig. 5B, right panels). In contrast to VLDL-[$^{35}$S]B-48, secretion of dense particles containing [$^{35}$S]apoB-48 (d > 1.04 g/ml, fractions 3–6) was increased by bezafibrate by 68 ± 30% (n = 2) during the 0–90 min chase and by 90 ± 44% ($P < 0.05$; n = 5) during the 90−240 min chase. Similarly enhanced

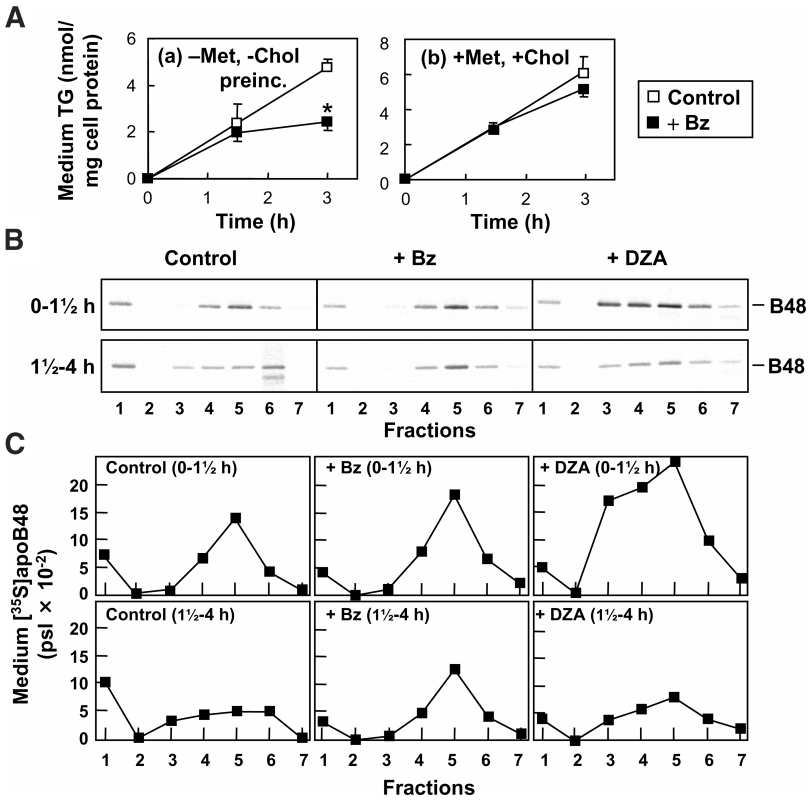


**Fig. 5.** Inhibition of PE methylation by Bz and DZA exclusively in the chase period decreases VLDL−[$^{35}$S]apoB-48 secretion. A: Hepatocytes were cultured for 16 h in a choline- and methionine-free DMEM (a) or normal (containing choline and methionine) DMEM (b) containing 18% FCS and 100 μM ethanolamine. The effect of Bz (200 μM) on TG secretion was examined in choline-free but methionine-supplemented DMEM (a) or normal DMEM (b) containing ethanolamine for up to 3 h. Results are the mean ± SD from four independent experiments. *Significantly different from control ($P < 0.01$). B, C: The cells cultured in choline- and methionine-free DMEM as described above were pulse-labeled for 15 min with [$^{35}$S]methionine/cysteine and chased for up to 4 h in serum-free DMEM (containing methionine, cysteine, and ethanolamine) ± Bz (200 μM) or DZA (10 μM). Density distribution of medium [$^{35}$S]apoB-48 (during a 0–1.5 h or 1.5–4 h chase) visualized by a BAS-1500 image analyzer (B). Quantification of radioactivity associated with [$^{35}$S]apoB-48 in each fraction (C). Results are the representative of two to five independent experiments.

secretion of HDL-[$^{35}$S]apoB-48 was observed by DZA treatment, by 116 ± 64% (n = 2) during the 0–90 min, and by 35 ± 14% (n = 3; $P < 0.05$) during the 90–240 min chase.

The second pulse-chase experiment introduced a variation where the addition of bezafibrate into the chase medium was delayed 0 to 90 min prior to the onset of chase (**Fig. 6A**). This delay was introduced to ensure that inhibition of PE methylation was initiated after the completion of HDL-[$^{35}$S]apoB-48 formation (preliminary experiments showed that the appearance of HDL-[$^{35}$S]apoB-48 in the microsomal lumen peaked between 15 and 40 min of chase). In comparison to control cells (i.e., no bezafibrate treatment), secretion of VLDL-[$^{35}$S]apoB-48 from cells treated with bezafibrate immediately (i.e., 0 min) or 15–40 min after the pulse was decreased by 50−65% during the subsequent 2.5 h chase (chase II) (Fig. 6B, left two columns in left panel). Concomitantly, secretion of HDL-[$^{35}$S]apoB-48 during chase II was increased compared with the control (Fig. 6B, left two columns in right panel). These results suggest that PE methylation indeed is required for VLDL-apoB-48 secretion after the completion of apoB-48 synthesis and HDL-B-48 formation. The inhibitory effect of bezafibrate treatment on VLDL-[$^{35}$S]apoB-48 secretion was no longer observed after a 90 min delay time.

### Lack of a correlation between levels of cellular PE and TG secretion

Inhibition of PE methylation was shown to cause elevation in cellular PE concentration (10). Thus, consideration was given to the possibility that the impaired VLDL secretion upon bezafibrate treatment was a consequence of altered intracellular PE level. To test this possibility, we determined cell-associated PE concentration and the cells' ability to secrete TG under different treatment conditions. Incubation of hepatocytes in ethanolamine-free DMEM resulted in a 2-fold reduction in cell PE concentration compared with fresh cells (data not shown). Supplementation of cells with ethanolamine increased cellular PE (**Fig. 7A**, top, first and third columns) and restored it to levels comparable to those observed in vivo (10). Secretion of TG was unaltered by ethanolamine depletion (Fig. 7B; first and third columns). Treatment with bezafibrate

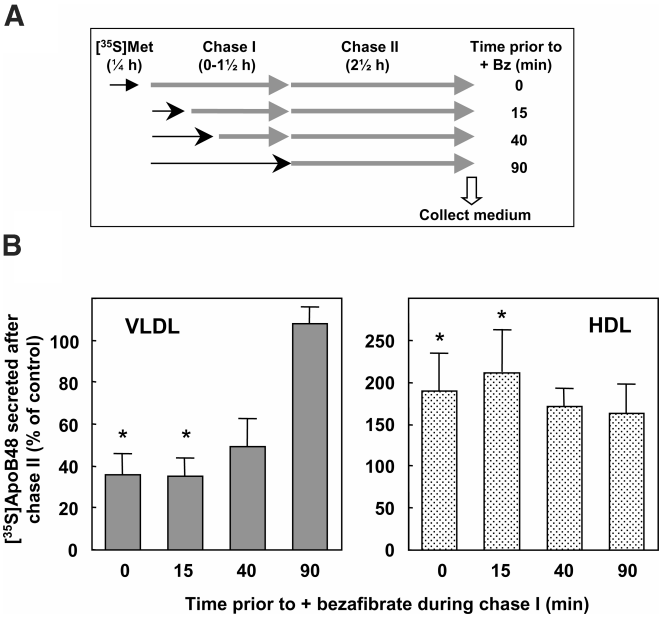


**Fig. 6.** Bz efficiently reduces VLDL-[$^{35}$S]B-48 secretion after the completion of HDL-apoB-48 assembly. A: The experiments were performed essentially the same as in Fig. 5B and C, except that after pulse-labeling the cells were incubated in a chase medium for 0−90 min (represented by thin arrows in chase I) prior to the addition of 200 μM Bz (represented by thick arrows in chase I). At the end of chase I, the medium was changed and the cells were incubated for another 2.5 h (chase II). Medium of control cells did not contain bz in chase I or chase II period. Medium was collected at the end of chase II, and subjected to fractionation to isolate VLDL. B: Secretion of VLDL-[$^{35}$S]B-48 and HDL-[$^{35}$S]B-48 under different conditions was quantified. Results are expressed as a percentage of control (i.e., cells treated with no Bz), which are the mean ± SD (or range) of independent experiments (n = 5 for 0 min, n = 3 for 15 min, and n = 2 for 40 min and 90 min prior to the addition of Bz). *Significantly different from control ($P < 0.05$).

increased cellular PE levels by 50% in ethanolamine-depleted cells (Fig. 7A; first and second columns) and also by 30% in ethanolamine supplemented cells (third and fourth columns). However, whereas bezafibrate decreased TG secretion by 60% in ethanolamine-supplemented cells (Fig. 7B; third and fourth columns), the reduction was not significant in ethanolamine-depleted cells (first and second columns). Under no circumstances were the cell PC concentrations altered (Fig. 7A; bottom). These results show that altered TG secretion is not always correlated with cellular PE levels. Thus, the impaired VLDL secretion by bezafibrate treatment is not simply attributable to an altered cellular PE concentration.

### Newly made PC is secreted as VLDL rather than HDL-apoB-48 particles

Studies have shown that PC derived from PS decarboxylation and PE methylation is preferentially used for lipoproteins rather than cellular PC (33, 34). We examined the distribution of newly synthesized PC among apoB-containing lipoprotein particles secreted into the medium. Cells were metabolically labeled with [$^{14}$C]ethanolamine for 4 h (**Fig. 8**). The specific radioactivity of [$^{14}$C]ethanolamine-labeled PC associated with VLDL ($d < 1.02$) was higher (by 100%) than that of high-density apoB particles (HDL-apoB; $d > 1.04$, fractions 3−7) (Fig. 8A). Elevated specific activity of PC associated with VLDL compared with HDL-apoB (by 130%) was also observed when cells were labeled with [$^{14}$C]choline for 4 h (Fig. 8B). Inhibition of PE methylation by bezafibrate for 4 h resulted in a decrease (65%) in the specific activity of cellular [$^{14}$C]ethanolamine-labeled PC and an increase (29%) in that of [$^{14}$C]choline-labeled PC. Still, the specific radioactivity of PC associated with VLDL was higher than that of HDL-apoB particles regardless of [$^{14}$C]ethanolamine or [$^{14}$C]choline label (270% and 80%, respectively). These results show that newly synthesized PC derived either from ethanolamine or choline was secreted as mature VLDL rather than HDL-apoB particles. During a 4 h treatment with bezafibrate, PC mass associated with VLDL was not significantly reduced (1.64 ± 0.93 nmol/mg cell protein in bezafibrate-treated cells vs. 1.91 ± 0.61 nmol/mg protein in the control). Within this time frame, specific radioactivities of [$^{14}$C]choline-labeled PC associated with VLDL and HDL-apoB, in parallel to the cellular value, were elevated by inhibiting PE methylation (Fig. 8B), indicating the compensation of reduced PC synthesis via PE methylation by the CDP-choline pathway.

## DISCUSSION

### Decreased VLDL secretion by bezafibrate is dependent on cellular PE methylation activities

In the previous study, we demonstrated that impaired VLDL secretion by bezafibrate or DZA is dependent on cellular PE methylation activities (10). Inhibition of PE methylation by these agents efficiently decreases VLDL secretion in the presence of ethanolamine but not in the absence of ethanolamine. Ethanolamine maintains cellular PE levels and PE methylation activities at levels comparable to those in vivo, whereas depletion of ethanolamine (culture of hepatocytes in conventional medium) adversely affects results in impaired PE methylation pathway (10). In the present study, we further confirmed the PE methylation-dependent action of bezafibrate by depleting methionine from the medium. We showed that depletion of methionine rapidly decreased incorporation of [$^{3}$H]ethanolamine into PC and, in parallel, abolished the reduction in TG secretion elicited by bezafibrate (Fig. 1). Furthermore, both PE methylation activity and the inhibitory effect of bezafibrate on TG secretion were restored by the addition of methionine to the medium (Fig. 5A). These results clearly indicate that reduced TG secretion by bezafibrate requires PC synthesis via the PE methylation pathway. The possibility exists that the lack of inhibitory effect of bezafibrate upon methionine depletion is due to impaired protein synthesis. However, this is unlikely because a study shows that decreased VLDL secretion by 3 days depletion of choline and methionine can be restored by the supplementation with choline even without methionine (8).

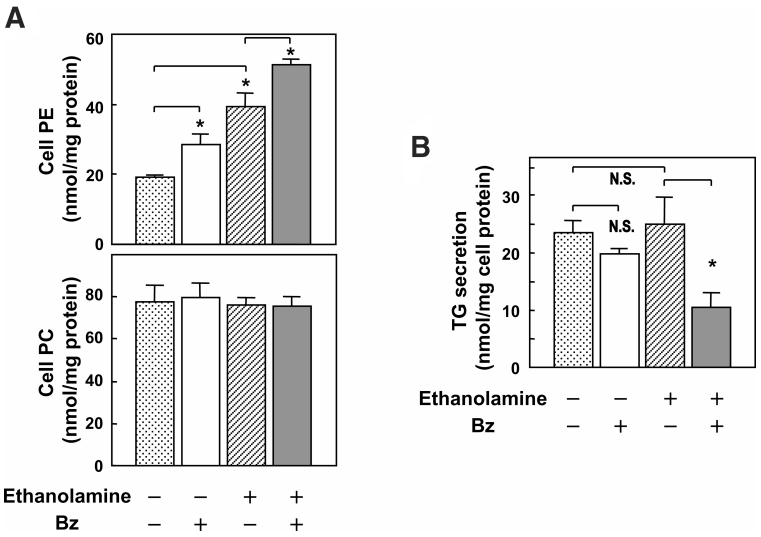


**Fig. 7.** Lack of correlation between levels of cellular phosphatidylethanolamine (PE) and triacylglycerol (TG) secretion. Hepatocytes that had been cultured for 15 h in DMEM containing 10% FCS ± ethanolamine (100 μM) were further incubated for 12 h in serum-free DMEM ± ethanolamine (100 μM) and/or Bz (200 μM). At the end of incubation, the levels of cellular phosphatidylcholine (PC) and PE (A) and the amount of TG secreted into the medium (B) were determined. Results are average ± SD of three to eight independent experiments. *Significantly different from respective values ($P < 0.05$). NS, difference from between two values was not significant ($P < 0.05$).

### Inhibition of PE methylation is associated with impaired bulk TG incorporation into VLDL-apoB-48

In the present study, we investigated the mechanism by which reduced PC synthesis via the PE methylation pathway disrupts VLDL-apoB-48 secretion. We examined the effect of the inhibitors at several stages in VLDL assembly and secretion. Administration of monomethylethanolamine to hepatocytes is known to cause accumulation of PMME in the membrane, which results in reduced translocation of apoB into the ER (27, 35, 36). However, we showed that reduced PE methylation and an accompanying increase in cellular PE, unlike accumulation of PMME (27, 35), do not impair the movement of apoB from the membrane to the lumen. In parallel, the normal or enhanced formation and secretion of apoB-containing HDL-like particles in bezafibrate-treated cells exclude the possibility that translocation of apoB or an early lipidation stage, which is mediated by microsomal triglyceride transfer protein (MTP) (37), is impaired. In support of our observations, a study with choline-deficient rat liver showed that severe impairment of PC synthesis does not affect translocation of apoB (38).

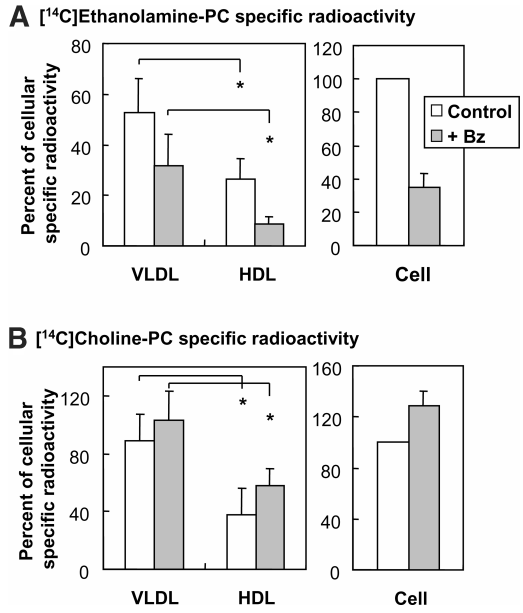


**Fig. 8.** Specific radioactivity of [$^{14}$C]ethanolamine- or [$^{14}$C]choline-labeled PC associated with VLDL, HDL-apoB, or cell. Hepatocytes plated for 4 h in DMEM containing 10% FCS and 100 μM ethanolamine were incubated in DMEM containing either [$^{14}$C]choline or [$^{14}$C]ethanolamine for 4 h in the presence or absence of 200 μM bezafibrate. The concentration of ethanolamine was adjusted to 100 μM. At the end of incubation, the conditioned medium was fractionated into VLDL (fraction 1, d < 1.02 g/ml) and HDL (fractions 3–7, d > 1.04 g/ml) (see Fig. 3 legend), and apoB-containing lipoproteins in each fraction were immunoprecipitated. Lipids were extracted from the immunocomplexes and the cells, respectively. The radioactivity associated with lipids was directly quantified by a Bio-Imaging Analyzer. Specific radioactivity represents radioactivity/PC mass and is expressed as percentage of specific radioactivity of cellular PC. Results are the mean ± SD of three independent experiments that were performed in duplicate. *Significant difference between VLDL and HDL ($P < 0.05$).

In the present study, we showed that the inhibition of PC synthesis via the PE methylation pathway results in a diminished formation of mature VLDL-apoB-48 in microsomal lumen whereas the appearance of HDL-like apoB-48 containing particles was elevated. Furthermore, we showed that an acute inhibition of PE methylation by either bezafibrate or DZA after the completion of HDL-apoB-48 assembly efficiently decreases VLDL-apoB-48 secretion. These findings clearly indicate that the defects reside in the conversion of primordial particles to VLDL-apoB-48, and are consistent with a two-step model of VLDL assembly (16, 19, 28). Our present study thus highlights the importance of the PE methylation pathway in the late stage of TG recruitment during VLDL assembly.

Quantitative analysis, however, showed that an increase in HDL-apoB-48 by inhibiting PE methylation was far more than a decrease in VLDL-apoB-48. A small reduction (by 30−60%) in VLDL-apoB-48 was accompanied by a large increase (70−170%) in HDL-apoB-48, eventually leading to enhanced secretion of total apoB-48 and an elevated ratio of HDL-apoB-48-VLDL-apoB-48 (Figs. 3–6). A possible explanation for this is that secretion of primor-

dial HDL-apoB-48 is accelerated by reduced PE methylation. The resulting depletion in the precursor pool may cause a reduction in VLDL assembly. We found that reduced PE methylation resulted in a decrease in membrane-associated apoB-48 and an increase in lumenal apoB-48 (Fig. 2). Several reports have suggested that apoB polypeptides associated with microsomal membranes may serve as a precursor of lumenal apoB-containing lipoproteins (17, 39). Remarkably, membrane-associated apoB-100 was suggested to be the precursor of VLDL-apoB-100 in hepatoma cells (17).

**The requirement of PE methylation is similar to that of the CDP-choline pathway for VLDL assembly**

As the major component of the surface of VLDL particles, PC was thought to play a role in maintaining the structure of particles (38). Chronic and severe depletion of choline in the diet causes a severe impairment of PC biosynthesis in rat liver and results in a marked reduction in VLDL secretion (8). This reduction was thought to be attributable to a blockage in the ER-to-Golgi trafficking, but not a defect in the assembly of VLDL in the ER (38, 40). However, the present results showed that inhibition of PC synthesis via the PE methylation pathway impairs the bulk TG incorporation into VLDL. Thus, the possibility exists that the two pathways of PC synthesis have distinct functions at different stages of VLDL assembly/secretion. However, for the following reasons we do not think this is likely. First, our data shows the importance of newly synthesized PC either from the PE methylation pathway or the CDP-choline pathway in the late maturation stage in VLDL assembly (Fig. 8). Second, studies with choline-deficient rats also suggest that the defect, like that in reduced PE methylation, could exist in the conversion from the primordial particle to mature VLDL. Indeed, the lumenal content TG-(PC+PE) ratio increased from 1.0 in the ER to 1.75 in the Golgi in hepatocytes (40), indicating that a significant amount of TG was loaded in the post ER compartment. Furthermore, secretion of VLDL-B-48 was impaired but secretion of HDL-B-48 was relatively normal from choline-deficient hepatocytes (8). Third, many model systems suggest that impaired VLDL secretion caused by reduced PE methylation is apparently compensated by the CDP-choline pathway. They include *i*) apparently normal serum lipid profile in PEMT-deficient mice fed with choline-containing diet (41); *ii*) restored plasma TG levels in eritadenine-treated rats by feeding with excess amounts of choline (13); *iii*) re-established VLDL secretion from choline-deficient hepatocytes by methionine (8); and *iv*) unimpaired VLDL secretion upon deceasing PE methylation from rat hepatocytes by ethanolamine depletion (10). These findings indicate that cellular PC supply is coordinately regulated and maintained by the two synthetic pathways. Indeed, a line of evidence has accumulated showing that cellular PC levels are coordinately regulated through two pathways (41−43).

However, our previous (10) and present results have shown that inhibition of PE methylation diminishes the secretion of TG-rich VLDL even when the CDP-choline pathway is functional. A question therefore arises as to why the functional CDP-choline pathway cannot compensate the inhibited PE methylation under the current experimental conditions. Our explanation for this is that compensation by the CDP-choline pathway could be sustained only when the supply of substrates (e.g., choline or diacylglycerol) is abundant. Inhibition of PE methylation does not immediately cause reduced TG secretion. There is a 3 h lag before the reduction becomes significant [Figs. 1B and 5A(b)]. In contrast, when cellular phosphocholine pool that serves as precursor pool for the CDP-choline pathway is depleted by incubating cells in the choline- and methionine-free medium for 16 h, the effect of inhibition of PE methylation on VLDL-TG secretion becomes rapidly manifest (i.e., within 3 h after bezafibrate treatment) [Fig. 5A(a)]. This treatment modestly depletes phosphocholine pool but does not affect PC levels in cells, in contrast to the hepatocytes prepared from rats maintained on choline-deficient diet for 3 days (i.e., choline-deficient rats), where phosphocholine pool is severely depleted (only 4% of total label is present in aqueous metabolite upon 30 min pulse labeling with tracer amount of [$^3$H]choline) and PC levels decreased by 40% compared with normal cells (44). It is noteworthy that both inhibition of PE methylation (10) and depletion of choline and methionine (conditions used in Figs. 5 and 6) decrease VLDL secretion without affecting cellular PC levels. This probably makes teleological sense because the cells would preserve adequate cellular PC levels at the expense of secretion thus the secretion of VLDL is more susceptible than the cellular PC contents to the inhibition of PE methylation.

**New mechanism for hypolipidemic effect of bezafibrate**

The present study also points out a new mechanism for the clinically known hypolipidemic agent, bezafibrate. Several studies have suggested that fibrates, including bezafibrate, exert their TG lowering effects via activation of the α form of peroxisome proliferator-activated receptor (PPAR) (45). Induced lipoprotein lipase gene expression and decreased apoC-III gene expression by fibrate treatment have been observed (46), which attribute to enhanced lipolysis of TG-rich VLDL. In addition, decreased production of VLDL by fibrates is explained by enhanced oxidation of fatty acids (45). However, in the present study, we found that bezafibrate acutely inhibits (within 50 min) the formation of VLDL-apoB-48 (Fig. 6). This observation, together with ethanolamine- (10) and methionine-dependent reduction in VLDL production (Fig.1), indicate that within the time frame of 12 h bezafibrate exerts its effect at post-transcription levels by inhibiting PE methylation. Indeed, we have found that 24 h treatment of cells with bezafibrate (200 μM) only slightly induce peroxisomal fatty acid oxidation (by 1.3-fold) and does not affect the activity of 2,4-dienoyl-CoA reductase, which plays an essential role in mitochondrial β-oxidation of unsaturated fatty acids (data not shown). Peroxisomal fatty acid oxidation is greatly induced (10-fold) in the liver of rats given bezafibrate for several days (47). Possibly, enhanced fatty

acid oxidation contributes to decreased VLDL secretion in these animals, although robust induction of enzymes involved in peroxisomal fatty acid oxidation is not observed in humans but restricted to rodents (48). To date, only bezafibrate, but not other fibrates, potently reduces VLDL secretion through inhibiting PE methylation (T. Nishimaki-Mogami, unpublished observations). We propose that reduced PE methylation contributes to the prominent TG lowering effect of bezafibrate, in addition to the PPARα-mediated transcriptional regulation of apoC-III and lipoprotein lipase that is common to all fibrates.

In summary, we have shown that PE-derived PC is preferentially utilized for VLDL assembly and secretion in rat primary hepatocytes. Inhibition of PE methylation impairs the incorporation of bulk TG into VLDL after the primordial precursor lipoprotein is synthesized. Our studies have provided new evidence that hepatic phospholipid metabolism is closely associated with VLDL assembly/secretion.

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