Head Group Specificity in the Requirement of Phosphatidylcholine Biosynthesis for Very Low Density Lipoprotein Secretion from Cultured Hepatocytes*

Zemin Yao and Dennis E. Vance†

From the Lipid and Lipoprotein Research Group and the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

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We have demonstrated that hepatic very low density lipoprotein (VLDL) secretion requires active phosphatidylcholine (PC) synthesis via either the CDP-choline pathway or phosphatidylethanolamine (PE) methylation pathway (Yao, Z., and Vance, D. E. (1988) J. Biol. Chem. 263, 2998–3004). In the present work, the head group specificity of phospholipid synthesis required for lipoprotein secretion was investigated in cultured hepatocytes isolated from choline-deficient rats. When N-monomethylethanolamine (0.1 mM) or N,N-dimethylethanolamine (0.1 mM) was added to the culture medium, the cells synthesized correspondingly phosphatidylmonomethylethanolamine (PMME) or phosphatidyl(dimethylethanolamine) (PDME). However, the synthesis of PDME could correct the impaired VLDL secretion only to a limited extent, whereas the synthesis of PMME inhibited VLDL secretion. Although dimethylethanolamine did not promote VLDL secretion as well as choline, dimethylethanolamine altered the increased triacylglycerol synthesis in the choline-deficient cells as effectively as choline. Supplementation of the culture medium with ethanolamine (0.1 mM) had little effect on cellular PE or PC levels, nor was normal VLDL secretion resumed. However, the amounts of cellular PC and PE were both decreased when the medium was supplemented with N-monomethylethanolamine or N,N-dimethylethanolamine. These results suggest that the choline head group moiety of PC is specifically required for normal VLDL secretion and cannot be replaced with ethanolamine, monomethyl ethanolamine, or dimethylethanolamine. In addition, the impaired VLDL secretion from the choline-deficient hepatocytes could also be corrected by supplementation of betaine (0.2 mM) and homocysteine (0.2 mM), indicating the utilization of a methyl group from betaine for PC formation via methylation of PE.

The liver is the major organ for the synthesis and secretion of plasma VLDL and HDL apolipoproteins (1) and also a major site for the formation and export of PC (2). Mammalian plasma lipoproteins are coated with a monolayer of phospholipids, of which the choline-containing phospholipids (PC, PC, and sphingomyelin) comprise more than 80% (3). The importance of choline phospholipid formation has been implicated in lipoprotein metabolism since Best and Huntsman (4) first described the phenomenon of choline deficiency. Recently, we have studied the role of PC synthesis in lipoprotein secretion by cultured hepatocytes from choline-deficient rats and demonstrated that active synthesis of PC is required for TG-rich lipoprotein (VLDL) secretion (5). In addition, we have shown that either of the two major PC synthetic pathways, the CDP-choline pathway or PE methylation pathway, is sufficient to resume normal VLDL lipoprotein secretion from the choline-deficient hepatocytes. Since pre-existing PE could not substitute for PC biosynthesis in VLDL secretion, the specific requirement of PC synthesis for VLDL secretion has been suggested (5).

Three stepwise methylations of hepatic PE catalyzed by PE N-methyltransferase (EC 2.1.1.17), lead to the sequential formation of PMME, PDME, and PC (6). These metabolic intermediates, PMME and PDME, are normally present in very small amounts (7). However, in the absence of choline and methionine, rat hepatocytes synthesize significant amounts of phospholipids containing N-monomethylethanolamine or N,N-dimethylethanolamine from the corresponding free bases (7), presumably via the cytidine diphosphate pathway used for PC and PE synthesis (8). It has been speculated that the formation of CDP esters of the N-monomethylated and N,N-dimethylated bases is catalyzed by different enzymes, probably phosphoethanolamine cytidylyltransferase (EC 2.7.7.14) and phosphocholine cytidylyltransferase (EC 2.7.7.15), respectively (9). The effect of accumulation of these partially methylated phospholipids on membrane properties and cell functions has been studied in mouse fibroblast cells (LM cells). The polar head group modulation does not affect the activities of seven membrane-bound enzymes (10) or the characteristic transition temperatures of plasma membranes and intracellular membranes (11).

In rat hepatocytes, choline can either be used for PC synthesis (12) or oxidized to betaine (12, 13), and the betaine is rapidly released into the culture medium (14). Oxidation of choline in the liver has been suggested as a major mechanism for diminishing free choline concentration (15). The enzymes involved in the formation of betaine were reduced by 40% in livers from choline-deficient rats (16). On the other hand, DuVigneaud and his co-workers (17) demonstrated that betaine behaves similarly to choline in permitting growth of rats when homocysteine is provided. In addition, it has been reported that betaine can replace choline and prevent fatty liver formation in choline-deficient rats (18). In the present study, betaine was added at various concentrations to examine its effect on VLDL secretion from choline-deficient hepatocytes. The results are presented below.

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† Medical scientist of the Alberta Heritage Foundation for Medical Research.

‡ The abbreviations used are: VLDL, very low density lipoprotein; LDI, low density lipoprotein; HDL, high density lipoprotein; BF, bottom fraction of salt gradient (d > 1.18 g/ml); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; PDME, phosphatidyl(dimethylethanolamine); TG, triacylglycerol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPTLC, high performance thin-layer chromatography; TLC, thin-layer chromatography.
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Liver in choline-deficient animals (18). The lipotropic effect of betaine might result from methionine formation catalyzed by betaine-homocysteine methyltransferase (EC 2.1.1.5) (19), an important reaction for methionine conservation in the liver.

To gain further understanding of the requirement of phospholipid synthesis for hepatic VLDL secretion, we examined the effect of various choline analogs or methyl group donors on hepatic lipogenesis and lipoprotein secretion. The present results indicate that PC synthesis is specifically required for VLDL secretion, and this requirement cannot be replaced by the synthesis of PE, PMME, or PDME. Second, betaine and homocysteine could effectively substitute for methionine in permitting normal VLDL secretion in the hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—Choline-deficient diet was obtained from ICN Biochemicals, Canada. [methyl-3H]Choline (12 Ci/mmol), L-[4,5-3H]Leucine (45 Ci/mmol), [9,10-3H]oleic acid (4.2 Ci/mmol), and aqueous solutions were obtained from New England Nuclear, United Kingdom. Choline oxidase (15 units/mg protein), catalase (from bovine liver, 45,000 units/mg protein), choline chloride, ethanamine, N-mono-methy ethanolamine, N,N-dimethylethanolamine, and homocysteine were obtained from Sigma, and the purities of these chemicals were examined by TLC on Silica Gel 60 plates by the Sigma method. The betaine was visualized with iodine, scraped, and eluted 15 times with 2 ml of methanol. The methanol was evaporated, and the sample was redissolved in 3 ml of H2O and stored at −20°C.

Analysis of Lipoproteins Secreted by Hepatocytes—Male Sprague-Dawley rats (initially weighing 45-50 g) were fed a choline-deficient diet for 3 days. Hepatocytes were isolated, by collagenase perfusion, from a single rat and cultured in a choline- and methionine-free medium as described previously (5). The conditions of supplementation of various choline analogs are indicated in the figure legends. The viability of the cells incubated under different conditions was identical (85-90%) as examined by trypan blue exclusion.

Preparation of [methyl-3H]Betaine—[methyl-3H]Betaine was prepared enzymatically from [methyl-3H]choline by treatment with choline oxidase and catalase (20). Choline oxidase (1 mg of protein) and catalase (300 units) were incubated with 200 μCi of [methyl-3H]choline at 37°C for 1 h. The reaction was terminated by boiling the mixture for 1 min. [methyl-3H]Betaine, plus 2 μmol of betaine as carrier, was separated by TLC on a Silica Gel 60 plate (20 × 20 cm) developed in methanol, 0.6% (w/v) NaCl, 28% (w/v) NH4OH (10:1:1, v/v). The TLC plates were developed from BDH Chemicals. Culture medium, serum, and solutions for hepatocyte culture were all obtained from GIBCO, and reagents for electrophoresis were purchased from Bio-Rad.

Preparation and Treatment of Hepatocytes—Male Sprague-Dawley rats (initially weighing 45-50 g) were fed a choline-deficient diet for 3 days. Hepatocytes were isolated, by collagenase perfusion, from a single rat and cultured in a choline- and methionine-free medium as described previously (5). The conditions of supplementation of various choline analogs are indicated in the figure legends. The viability of the cells incubated under different conditions was identical (85-90%) as examined by trypan blue exclusion.

RESULTS

Effect of Phospholipid Bases on the Concentrations of Major Phospholipids in Hepatocytes—Hepatocytes obtained from the choline-deficient rats were incubated with various phospholipid bases, and the concentrations of four major cellular phospholipids (PC, PE, PMME, and PDME) were estimated by two-dimensional TLC on Silica Gel 60 plates. The plates were developed in chloroform, methanol, 28% (w/v) NaCl, 28% (w/v) NH4OH, and the cellular choline derivatives were separated in methanol, 0.6% (w/v) NaCl, 28% (w/v) NH4OH, and each dish of hepatocytes was incubated with 2 ml of the medium. Cellular lipids were extracted with chloroform/methanol (2:1, v/v).

Analysis of Lipid Synthesis—The synthetic rate of cellular TG and phospholipids (PC, PE, PMME, and PDME) were estimated by [9,10-3H]oleic acid incorporation. [3H]Oleic acid was mixed with 0.01 N NaOH in methanol, and the methanol was removed by evaporation under N2. The oleate was resuspended in a serum-free medium (2 μCi/ml) containing 0.16 mg/ml bovine serum albumin as a fatty acid carrier, and each dish of hepatocytes was incubated with 2 ml of the medium. Cellular lipids were extracted with chloroform/methanol (2:1, v/v).

Analysis of Lipid Synthesis—The synthetic rate of cellular TG and phospholipids (PC, PE, PMME, and PDME) were estimated by two-dimensional TLC and quantitated as described (23). In this work, labeling experiments, cellular phospholipids were extracted with chloroform/ methanol (2:1, v/v) and separated by developing TLC (Silica Gel 60) plates in chloroform/methanol/acetic acid/formic acid/H2O (70:30:12:4:2; v/v), and cellular choline derivatives were separated in methanol, 0.6% (w/v) NaCl, 28% (w/v) NH4OH, and the sample was developed in chloroform/methanol/acetic acid/formic acid/H2O (70:30:12:4:2; v/v). In this solvent system, PE and PMME co-migrated. Lipid spots on the TLC plates were visualized with iodine vapor.

Other Methods—Phospholipids were quantitated by phosphorus analysis (24). Protein was determined according to Lowry et al. (25) using bovine serum albumin as a standard.

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concentration increased about 25 nmol/mg cell protein after 20-h incubation) but had no significant effect on cellular PE levels. The addition of ethanolamine, however, did not change the cellular PE levels significantly and had no effect on cellular PC concentrations. There were also no detectable amounts of PMME and PDME in the cells supplemented with choline or ethanolamine. Exposure of the cells to N-monomethylethanolamine led to an active synthesis of PDME (but not N,N-dimethylethanolamine presumably results from a divergence of the isotope into actively synthesized [3H]phospholipids. The total incorporation of [3H]oleate into cellular chloroform-soluble materials was identical in the cells supplemented with these head groups (data not shown). The rate of hepatic TG synthesis in the absence or presence of choline was also measured using [2,3H]glycerol (1 Ci/mmol, 15 μCi/dish) as a precursor, and similar results as that shown in Fig. 2 were obtained.

Effect of Phospholipid Bases on the Secretion of TG from Hepatocytes—Knowing that the hepatocytes could synthesize significant amounts of PMME and PDME from the corresponding bases, we examined whether or not the synthesis of these partially methylated phospholipids could correct the impaired VLDL secretion from the choline-deficient cells. The mass secretion of TG from the hepatocytes incubated with 100 μM of each of the free bases were quantitated to estimate the hepatic VLDL secretion (Fig. 3). Results obtained clearly showed that the secretion of TG from the cells was low in the presence of phospholipid bases, except when choline was added to the culture medium. Addition of ethanolamine had no effect on TG secretion; N,N-dimethylethanolamine had a small stimulatory effect in the first 14 h, whereas N,N-dimethylethanolamine was inhibitory. Similar amounts of TG were secreted from the hepatocytes supplemented with N,N-dimethylethanolamine at higher concentrations (up to 200 μM; data not shown). N-Monomethylethanolamine had no effect, and N,N-dimethylethanolamine caused a small reduction in hepatic TG synthesis from [3H]oleic acid (Fig. 2). Hence, the lack of a restoring effect on TG secretion by supplementing with these free bases (Fig. 3) suggests a specific requirement of phospholipid, mainly PC, synthesis for hepatic TG-rich lipoprotein secretion.

**Fig. 2. Effect of phospholipid bases on the incorporation of [9,10-3H]oleate into TG, PC, PE/PMME, and PDME in hepatocytes.** The experiment was performed as described in the legend of Fig. 1, except that [9,10-3H]oleate (4 μCi/dish) was included in the culture medium and ethanolamine was omitted. The conditions are: no addition (X), 100 μM choline (■), 100 μM N,N-dimethylethanolamine (▲). The formation of [3H]PDME in the presence of N-monomethylethanolamine was not determined. Each point represents the average of two individual dishes which do not differ by more than 5%. The experiment was repeated and similar results were obtained.

**Fig. 3. Effect of phospholipid bases on the secretion of TG from hepatocytes.** The experiment was performed as described in the legend of Fig. 1. At the indicated times, lipids were extracted from culture medium. TG was separated by TLC and quantitated as described under "Experimental Procedures." The conditions are: no addition (X), 100 μM choline (■), 100 μM ethanolamine (▲), 100 μM N,N-dimethylethanolamine (▲), 100 μM N,N-dimethylethanolamine (▲). The experiment has been repeated three times, and each value is the mean of three individual measurements. The average standard error of the mean is 18%.
Effect of Phospholipid Bases on the Secretion of Lipoproteins from Hepatocytes—The above studies were extended to examine the effect of base supplementation on lipoprotein secretion. Lipoproteins secreted from the cells in a 12-h period were fractionated into VLDL, LDL, HDL, and BF (d > 1.18 g/ml) fractions in a salt gradient by ultracentrifugation. Since the amounts of lipoproteins secreted by cultured hepatocytes were small, lipids in each of the four fractions were semi-quantitatively analyzed by HPTLC (Fig. 4). Compared to the cells maintained in choline- and methionine-free condition, the amount of VLDL and LDL phospholipids (PC, PE, and sphingomyelin) and neutral lipids (TG and free cholesterol) secreted was enhanced from the cells supplemented with choline, but the secretion of HDL and BF lipids was not changed significantly (Fig. 4; C versus -). The concomitant increase in the amount of PC and TG of VLDL indicated that there was a constant PC/TG ratio in secreted VLDL. The addition of ethanolamine had a small inhibitory effect on the secretion of the lipoprotein lipids of VLDL compared to no addition (Fig. 4; E versus -). The secretion of VLDL and LDL lipids (both phospholipids and neutral lipids) was remarkably inhibited by the addition of N-monomethylethanolamine, and the amounts of TG and PC in secreted HDL were also slightly reduced (Fig. 4; ME versus -). A small amount of PMME was found in the lipids extracted from the whole medium as examined by two-dimensional TLC. The secretion of PMME was 0.38 nmol/mg cell protein after 15-h incubation. HPTLC of the lipids from fractionated lipoproteins indicated that PMME (co-migrated with PE in the solvent) was almost exclusively associated with the HDL fraction (Fig. 4; ME). The addition of N,N-dimethylethanolamine, on the other hand, had little effect on the secretion of lipoprotein lipids, except that marked PDME bands were observed in both the VLDL and HDL secreted (Fig. 4; DE versus -). The secretion of PDME from these cells was 0.61 nmol/mg cell protein after 15-h incubation.

The secretion of [3H]leucine-labeled lipoproteins from the hepatocytes is summarized in Table I, and the apoprotein components in each of the four lipoprotein fractions were further examined by fluorography. The total [3H]apolipoproteins in VLDL secreted from the choline-supplemented cells was stimulated by about 3-fold compared to no addition, in which the amounts of tritium in apoB100, -BL, and -E were increased by 2-, 4-, and 3-fold, respectively (Table I). Fluorography of the [3H]apolipoproteins in VLDL clearly demonstrated that the amounts of labeled apoB100, -BL, and -E were all increased (Fig. 5). Ethanolamine supplementation had no significant effect on the labeled VLDL apolipoprotein secretion. The addition of N-nonmethylethanolamine diminished the secretion of total VLDL [3H]apolipoproteins by almost 60% compared to that of no addition; the amounts of tritium in apoB100, apoB51, and apoE were decreased by 59, 56, and 67%, respectively (Table I). The secretion of labeled VLDL apolipoproteins from the cells supplemented with N,N-dimethylethanolamine was stimulated by about 50% compared to no addition, in which the amounts of tritium in apoB100 and -E were increased by 2.5- and 2-fold, respectively.

Table I

<table>
<thead>
<tr>
<th>Addition</th>
<th>VLDL Total</th>
<th>ApoB100</th>
<th>ApoB51</th>
<th>ApoE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>261</td>
<td>32 ± 8</td>
<td>16 ± 4</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Choline</td>
<td>806</td>
<td>69 ± 2</td>
<td>66 ± 3</td>
<td>142 ± 26</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>232</td>
<td>42 ± 1</td>
<td>18 ± 1</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>MME</td>
<td>110</td>
<td>13 ± 4</td>
<td>7 ± 3</td>
<td>15 ± 9</td>
</tr>
<tr>
<td>DME</td>
<td>384</td>
<td>23 ± 4</td>
<td>40 ± 6</td>
<td>93 ± 31</td>
</tr>
</tbody>
</table>

FIG. 5. Fluorography of [3H]leucine-labeled apoproteins secreted by hepatocytes supplemented with phospholipid bases. The experiment was performed as described in the legend of Fig. 4, except that the cells were incubated with [3H]leucine (15 μCi/dish) in a serum-free medium in the absence (-) or presence of various phospholipid bases for 12 h. Apoproteins were solubilized from Cab-O-Sil after fractionation, and aliquots of protein samples derived from equal amounts of culture medium were separated by PAGE as described under "Experimental Procedures." After electrophoresis, the gel was dried and subjected to fluorography using EN' HANCE as an enhancer. In this experiment, the x-ray film was exposed for 5 days for VLDL (to see apoB100) and 18 h for HDL and BF. There were no visible bands in LDL samples after a 2-day exposure. The positions of apoproteins are indicated on the left. Alb, albumin; C, E, ME, and DE are the same as defined in the legend to Fig. 4.
whereas apoB_H was slightly decreased (Table I). Fluorography of the labeled VLDL apolipoproteins confirmed that the stimulation was mainly due to the increase in VLDL apoB_L and apoE, but not apoB_H (Fig. 5). There were also small increases in the secretion of labeled LDL from choline- or N,N-dimethylethanolamine-supplemented cells and decreases from ethanolamine- or N-monomethylethanolamine-supplemented cells (Table I). Unlike VLDL and LDL, the secretion of the labeled HDL (mainly apoE and -A-I) and BF (mainly albumin) proteins from the hepatocytes was less affected by the supplementation of these phospholipid bases (Fig. 5 and Table I). Proteins separated by SDS-PAGE and stained with Coomassie Blue exhibited a pattern similar to that shown by fluorography in Fig. 5.

**Synthesis and Turnover of Apolipoproteins in Hepatocytes**—The addition of N-monomethylethanolamine and N,N-dimethylethanolamine might affect VLDL secretion by altering rates of apolipoprotein synthesis. As a test of this hypothesis, the synthetic rates of hepatic apolipoprotein B_H and B_L were examined by radiolabeling with [3H]leucine and immunoprecipitation using rabbit antiserum against apoVLDL. It was shown in the previous work (5) that the maximal labeling of the cellular apoBs with [3H]leucine was achieved after 2-h labeling. Thus, the present [3H]leucine labeling experiment was performed for a period of 2 h in order to examine the effect of different polar head groups on apolipoprotein synthesis. Addition of choline, ethanolamine, N-monomethylethanolamine, or N,N-dimethylethanolamine had no effect on the total uptake of [3H]leucine or on the incorporation of tritium into cellular trichloroacetic acid precipitable materials (data not shown). It was shown that hepatic synthesis of apolipoprotein components of VLDL was not altered by the addition of choline or methionine (5). Now it is shown that neither monomethylethanolamine or dimethylethanolamine affect apoB_H, apoB_L, apoE, and apoC synthesis (Fig. 6). These results suggest that hepatic synthesis of apolipoprotein components of VLDL is independent of VLDL secretion.

**Effect of Betaine and Homocysteine on the Secretion of Lipoproteins from Hepatocytes**—Betaine has been reported as a lipotropic agent which effectively reduces liver fat content caused by choline deficiency (18). Therefore, comparative studies were conducted to examine the effect of choline, betaine, and betaine + homocysteine on lipoprotein secretion and phospholipid synthesis in the choline-deficient hepatocytes. Lipids and [3H]leucine-labeled apoproteins from the fractionated lipoproteins were qualitatively analyzed by HPTLC and SDS-PAGE, respectively. Addition of 200 μM betaine to the culture medium stimulated the secretion of VLDL/LDL lipids (Fig. 7) and apolipoproteins (Fig. 8 and Table II) from the cells in a 12-h period. The stimulatory effect of betaine on the secretion of VLDL/LDL lipids (Fig.
The experiment was performed as described in the legend to Fig. 7. Lipoproteins secreted from the hepatocytes supplemented with betaine and/or homocysteine or choline were extracted from the culture medium by Cab-O-Sil absorption and solubilized with 2% SDS, 6 M urea solution. Aliquots of the samples were counted in 5 ml of aqueous counting scintillant. Each value from the cells supplemented with choline or none is the mean ± S.D. from three independent experiments. The values from the cells supplemented with betaine or betaine/homocysteine are the averages of two experiments, and the values in parentheses are the percentages of errors from the means. ApoB48, apoB100, and apoB of VLDL were recovered from SDS-PAGE gels, and the amounts of radioactivities were determined (5).

**TABLE II**

**Secretion of 1-[4-3H]leucine-labeled lipoproteins by hepatocytes**

7) and apolipoproteins (Fig. 8 and Table II) was further enhanced when 200 μM of homocysteine was present. There was no effect of betaine and/or homocysteine on the secretion of HDL and lipid-free proteins (d > 1.18 g/ml).

Homocysteine has been shown to be readily taken up by hepatocytes in the perfused rat liver (26). We examined the utilization of betaine by the cultured hepatocytes. In the absence of homocysteine, a nearly linear uptake of [methyl-3H]betaine (for 4 h) was observed when the cells were incubated with tritium-labeled betaine (25 μM, 100 μCi/μmol) (Fig. 9A), and about 33% of the methyl groups incorporated was integrated into cellular PC after 4 h (Fig. 9B). Thin-layer chromatography of the labeled water-soluble compounds in the cells showed that tritium exclusively co-migrated with betaine, indicating that betaine was taken up by the cells in an intact form (inset of Fig. 9A). When the cells received 100 μM homocysteine, the accumulation of [3H]betaine in the cells was about 50% less than those of cells cultured without homocysteine during the 4-h incubation period (Fig. 9A).

However, the incorporation of the methyl groups into PC was increased by 4.5-fold, about 25% of the incorporated methyl groups was integrated into cellular PC at the end of the 4-h incubation (Fig. 9B). PC was the major labeled phospholipid in the cells, accounting for about 75% of the total tritium in the cellular chloroform-soluble materials after 4 h, whereas 3, 2, and 3.5% of the chloroform-soluble tritium was associated with lyso-PC, PS, and PE, respectively (inset of Fig. 9B). The rest (unidentified materials, about 12.5% of total tritium in the chloroform-soluble materials) migrated with the solvent front. In parallel experiments, exposure of the hepatocytes to betaine (200 μM) for 12 h did not significantly change cellular PC concentrations compared to no addition (70.9 ± 5.0 versus 66.0 ± 4.0 nmol/mg cell protein; p > 0.1; n = 4). However, the PC level was remarkably increased in the cells when both betaine (200 μM) and homocysteine (200 μM) were added to the medium (80.5 ± 3.8 versus 66.0 ± 4.0; p < 0.01; n = 4).

These data suggest that the lipotropic effect of betaine (18) resulted from a stimulated VLDL secretion due to the active synthesis of PC via PE methylation. This, in turn, is most likely a consequence of increased synthesis of methionine by betaine-homocysteine methyltransferase.

**DISCUSSION**

Previous studies showed that VLDL secretion from cultured hepatocytes required the active biosynthesis of PC (5), suggesting the integration of PC into TG-rich lipoproteins is essential for the intracellular assembly and/or transport of VLDL. The failure of hepatocytes to use pre-existing PE for VLDL secretion led us to examine the head group specificity in the requirement of phospholipid synthesis for VLDL secretion.

The present work clearly demonstrates that the impaired hepatic VLDL secretion by the choline- and methionine-deficient hepatocytes can be corrected only by PC synthesis. The requirement for PC synthesis was not replaced by the synthesis of PE or PMME and only to a limited extent by PDME synthesis. There are two lines of evidence indicating that the impaired hepatic VLDL secretion was due to inhibited PC synthesis in choline deficiency, but not due to inhibited synthesis of apolipoprotein components or core lipids of VLDL. First of all, we have demonstrated that the synthetic rates of hepatic B apoproteins (evaluated by [3H]leucine incorporation) were not altered significantly by any of the polar head groups (Fig. 6). The present data are consistent with our previous observations that the deficiency in choline and/or methionine had no effect on hepatic VLDL apoprotein synthesis (5). These results suggest that the synthesis of VLDL apoproteins is not affected by the supplementation of the
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head groups. Second, the rate of hepatic TG synthesis, determined by \(^{[3]H}\)oleate incorporation (Fig. 2) or \(^{[3]}H\)glycerol incorporation was not decreased but increased in the presence of monomethylethanolamine or no addition (Fig. 2). Thus, the reduced VLDL secretion in hepatocytes supplemented with monomethylethanolamine or no addition was not reflecting reduced TG synthesis. Interestingly, although dimethylethanolamine and choline appeared to correct equally well the increased hepatic TG synthesis in choline deficiency (Fig. 2), they were not equally effective in promoting TG secretion (Fig. 3). The reason for this PC specificity in promoting VLDL secretion is presently unknown. However, two explanations might be suggested. First, the less effective utilization of PE, PMME, and PDME for VLDL secretion might be explained by lipid topogenesis. The lipids synthesized on the cytosolic leaflet of the endoplasmic reticulum bilayer probably undergo a slow transbilayer movement, whereas the transbilayer movement of PC can be facilitated by a special protein called “flipase” (30–32). It has been proposed that during the assembly of VLDL, the monolayer of phospholipid on the surface of the particles is acquired from the luminal leaflet of the endoplasmic reticulum bilayer (27). The highly hydrophobic nature of apoB and its association with endoplasmic reticulum membrane in human Hep G2 cells (28) and rat hepatocytes (29) are in agreement with this hypothesis. Thus, active PC synthesis and the facilitated PC transbilayer movement might be required to drive apoB into the lumen of the endoplasmic reticulum during VLDL assembly. At present, however, little is known about the rates of transbilayer movement of PE and the partially methylated intermediates on endoplasmic reticulum, nor has the asymmetric distribution of phospholipids across the bilayer of microsomal membranes been established.

An alternative explanation for the failure of PE (probably PMME as well) to replace PC in the VLDL assembly process might be an inability of PE to transfer from endoplasmic reticulum membranes to lipoprotein particles in the lumen. It is well known that PE is less effective as a lipid component for lipoproteins and accounts for only 2–3% of the total phospholipids in the plasma (33). In addition, there is very little secretion of \(^{[3]H}\)ethanolamine-derived PE from cultured rat hepatocytes, although the cellular PE pool is heavily labeled (34). Analysis of lipid compositions present in the membranes and contents of isolated microsomes (35), as well as Golgi (35, 36), has shown striking differences between these two compartments. Although PE contributes about 20% of total phospholipids in the membranes of microsomes and Golgi, there was barely detectable PE in the content of these organelles (35). Howell and Palade (36) have obtained a similar result in Golgi using \(^{32}P\) to label the phospholipids. PC and sphingomyelin, on the other hand, are the predominant phospholipids present in the contents of microsomes and Golgi. Thus, the exchange or transport of PE between microsomal membrane and its content might be slow. Recently, a slow rate of intravesicular transfer of PE (5- to 7-fold slower than the rates for PC) has been reported with model membranes (37).

The rate of secretion of VLDL is reduced in choline deficiency, and there is no effect on apoprotein synthesis or turnover. In addition, immunoblot studies have shown no accumulation of apoproteins in choline-deficient livers. What happens to the apoproteins that are not secreted? Apparently, the apoproteins not assembled into VLDL particles in choline-deficient cells are degraded. Where this degradation occurs is not known. There is evidence for lysosomal and nonlysosomal degradation of incompletely assembled subunits of T cell receptors (38). The liver cell might handle the incompletely assembled VLDL proteins in a similar manner.

A striking difference in the effect of synthesis of the two partially methylated phospholipids (PMME and PDME) on hepatic VLDL secretion was observed. Although the synthesis of PDME did not correct the impaired secretion of TG, the secretion of VLDL apoproteins, mainly apoE and apoB, (but not apoAI), was stimulated to a limited degree (Table 1). Significant amounts of PDME, associated with VLDL and HDL in the medium, were observed. On the other hand, VLDL secretion from N-monomethylethanolamine-supplemented cells was markedly inhibited when synthesis of PMME was initiated. Why N-monomethylethanolamine would inhibit the basal secretion of VLDL from the cells is not understood. One explanation is the altered structure and function of membranes due to accumulation of PMME. However, studies conducted in mouse fibroblast cells showed the accumulation of PMME on microsomes, mitochondria, and plasma membranes (up to 37% of total membrane phospholipid) did not affect the activities of some membrane-bound enzymes (10) or the characteristic temperatures (transition temperatures) of plasma membranes and the intracellular membranes (11). In addition, our data have shown that the secretion of HDL and lipid-free proteins (such as albumin) is not altered by the elevation of cellular PMME (20% of four major cellular phospholipids), indicating that intracellular transport of secretory vesicles (one of the major functions of intracellular membranes) is maintained. An alternative explanation for the inhibitory effect of N-monomethylethanolamine on VLDL secretion could be due to the reduced cellular PC levels (Fig. 1). However, a similar decrease in cellular PC levels was also observed in N,N-diethylthanolamine-supplemented cells (Fig. 1), but the basal rate of VLDL secretion was not altered.

Although synthesis of PC, PE, PMME, and PDME in the presence of free bases is well known in cultured hepatocytes (7), the synthetic pathways through which PMME and PDME are formed from the corresponding free bases are still unknown. We have found that formation of PMME and PDME is accompanied by significant decreases in cellular PC and PE levels. Results from \(^{[3]H}\)oleate labeling experiments (Fig. 2) indicated that N-monomethylethanolamine or N,N-diethylthanolamine did not alter the rate of PC and PE synthesis. Thus, the decreased cellular PC and PE levels might result from an enhanced degradation. The lack of an effect of ethanolamine on cellular PC levels observed in the present work might be due to the pre-existing elevated concentration of PE in these choline-deficient cells (5, 39, 40). It has also been shown in a human tumor cell line that ethanolamine supplementation has no effect on cellular PC levels or on PE N-methyltransferase activity (41). The other explanation for the lack of an effect of ethanolamine might be that the synthesis of PE in hepatocytes is mainly via phosphatidylethanolamine decarboxylation rather than the CDP-ethanolamine pathway, as observed in cultured baby hamster kidney cells (42).

The lipotropic effect of betaine reported by Best and his co-workers (18) in the 1950s led us to investigate the effect of betaine supplementation on lipoprotein secretion from choline-deficient cells. In the absence of homocysteine, the addition of betaine (100 or 200 \(\mu\)M) to the culture medium for 12 h had essentially no effect on lipoprotein secretion, nor the cellular PC concentration. This result suggests that betaine alone is not an effective substitute for choline to stimulate PC synthesis and correct the impaired VLDL secretion in the choline-deficient cells. In the liver, conservation of methionine is achieved by remethylation of homocysteine...
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catalyzed by either betaine-homocysteine methyltransferase or N\(^-\)methyltetrahydrofolate-homocysteine methyltransferase (43). Although which reaction is more important for the formation of methionine in rat liver remains to be defined (44), homocysteine is an indispensable substrate for either reaction. Supplementation of high concentrations of homocysteine (200 \(\mu\)M) together with betaine effectively corrects impaired VLDL secretion and also increases cellular PC levels. Hence, the utilization of methyl groups from betaine depends on the availability of the methyl group acceptor, homocysteine, in the hepatocytes. The elevated cellular PC level upon addition of betaine and homocysteine probably results from increased synthesis of methionine which in turn promotes PE methylation to form PC.

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