Secretion of VLDL, but not HDL, by rat hepatocytes is inhibited by the ethanolamine analogue *N*-monomethylethanolamine

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Abstract The role of phospholipids in the assembly and secretion of very low density lipoproteins (VLDL) has been investigated by incubation of monolayer cultures of rat hepatocytes with monomethylethanolamine, an analogue of ethanolamine and choline. The cellular concentration of phosphatidylmonomethylethanolamine was increased 17-fold in response to treatment of hepatocytes with monomethylethanolamine. The secretion of phosphatidylcholine, triacylglycerol, and the apolipoproteins B_H, B_L, and E into VLDL was inhibited by approximately 50% in hepatocytes incubated with monomethylethanolamine, compared to untreated cells. Cell viability was unaffected by treatment with the ethanolamine analogue, as was cellular protein synthesis. The mechanism by which monomethylethanolamine reduced VLDL secretion was examined. Since monomethylethanolamine is a structural analogue of ethanolamine and choline, an obvious hypothesis for explanation of the effect on VLDL secretion was that phosphatidylcholine biosynthesis, which is required for VLDL secretion (Z. Yao and D. E. Vance. 1988. *J. Biol. Chem.* **263:** 2998–3004) was inhibited. However, the biosynthesis of phosphatidylcholine from [^{3}H]choline or from [^{3}H]glycerol was not significantly reduced in the analogue-treated, compared with the untreated, hepatocytes. Nor was the incorporation of [^{3}H]glycerol into cellular triacylglycerol altered in the monomethylethanolamine-treated cells. Furthermore, addition of monomethylethanolamine to hepatocytes did not reduce the rate of biosynthesis of phosphatidylethanolamine either from CDP-ethanolamine or from phosphatidylserine, nor was phosphatidylserine biosynthesis from [3-^{3}H]serine affected. The 50% inhibition of VLDL secretion elicited by monomethylethanolamine was apparently specific for VLDL because there was no difference in secretion of HDL (lipid or apoprotein moieties) or albumin by cells incubated with or without the ethanolamine analogue. The experiments showed that inhibition of VLDL secretion by monomethylethanolamine was not the result of decreased biosynthesis of phospholipids, triacylglycerols, or cholesteryl esters. More subtle effects of the ethanolamine/choline analogue, for example interference by the increased amount of phosphatidylmonomethylethanolamine, in the process of assembly of lipids with apoB remain a possibility.—**Vance, J. E.** Secretion of VLDL, but not HDL, by rat hepatocytes is inhibited by the ethanolamine analogue *N*-monomethylethanolamine. *J. Lipid Res.* 1991. **32:** 1971–1982.

Supplementary key words dimethylethanolamine • phosphatidylcholine • phosphatidylethanolamine • phosphatidylmonomethylethanolamine • triacylglycerol • cholesteryl ester

The mechanism and precise intracellular location of assembly of hepatic VLDL are not fully understood. Nascent VLDL particles containing apoB have been visualized by immunoelectronmicroscopy in the lumen of the endoplasmic reticulum and Golgi apparatus of rat liver (1). However, there is still disagreement about whether apoB is assembled with its full complement of lipid into a VLDL particle in the endoplasmic reticulum (2, 3) or in the Golgi (4–6). The process by which the large, hydrophobic apoB molecule is extruded into the aqueous lumen of these compartments is being actively investigated in several laboratories.

Our experiments have focused primarily on the role of phospholipids in the assembly of apoB into VLDL by cultured rat hepatocytes (7–11). Apparently, there is preference for the use of newly made, rather than pre-existing, phospholipids for assembly into VLDL (9). Phospholipids are also compartmentalized on the basis of their biosynthetic origin for assembly into VLDL (7, 8, 10). In hepatocytes prepared from choline-deficient rats, VLDL secretion was impaired (11) suggesting that the active synthesis of phosphatidylcholine (PtdCho) is required for VLDL assembly/secretion. Choline, but not its analogue monomethylethanolamine (MME), restored VLDL secretion to normal levels in the choline-deficient hepatocytes (11). Indeed, when choline-deficient hepatocytes were incubated with MME, secretion of VLDL lipids and apolipoproteins was reduced even further.

In an attempt to understand more clearly the role of phospholipids in VLDL assembly, the effect of modification of the phospholipid head-group composition of hepatocytes on the secretion of VLDL and HDL was

Abbreviations: apo, apolipoprotein; B_H and B_L, high and low molecular weight forms of apoB, respectively; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; MME, *N*-monomethylethanolamine.

examined. Monolayer cultures of rat hepatocytes were incubated with the ethanolamine analogue MME which increased the concentration of the corresponding phospholipid phosphatidylmonomethylethanolamine in cells but otherwise did not alter the phospholipid composition. Incubation of hepatocytes with MME reduced the secretion of VLDL (both lipid and apolipoprotein moieties) by approximately 50%, whereas the secretion of albumin and HDL (lipids and apoA-I) was not diminished. The hypothesis that MME inhibited phospholipid synthesis, and thereby reduced VLDL secretion, was investigated.

MATERIALS AND METHODS

Materials

The following radiochemicals were purchased from Amersham Canada, Oakville, Ont., Canada: L-[4,5-^{3}H]leucine (55 Ci/mmol), [*methyl*-^{3}H]choline chloride (15 Ci/mmol), [1-^{3}H]ethanolamine hydrochloride (29.5 Ci/mmol), [3-^{3}H]serine (32 Ci/mmol), [1(3)-^{3}H]glycerol (0.5 Ci/mmol), [9,10-^{3}H]oleic acid (2 Ci/mmol). Earle's minimum essential medium was obtained from Gibco Laboratories, Grand Island, NY, and fetal bovine serum was from BDH Chemicals, Ltd., Carle Place, NY. The culture dishes used for cell culture were Primaria (60 mm), obtained from Becton Dickson, Oxnard, CA. The standard phospholipids phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidylinositol were from Avanti Polar Lipids, Birmingham, AL. Sphingomyelin, lysophosphatidylcholine, and triacylglycerol were from Sigma, St. Louis, MO. Choline, ethanolamine, *N*-monomethylethanolamine and *N,N*-dimethylethanolamine were also obtained from Sigma, as was phospholipase C from *Clostridium welchii* and Cab-O-Sil (fumed silica). Silica gel G 60 thin-layer chromatography plates (0.25 mm thickness) and high performance thin-layer chromatography plates (silica gel 60, 10 × 20 cm) were purchased from BDH Chemicals. All reagents for polyacrylamide gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA. Soluene 350 and Hionic-Fluor scintillant were purchased from Packard Instrument Co., Downer's Grove, IL.

Preparation and incubation of hepatocytes

Primary rat hepatocytes were isolated from the livers of Sprague-Dawley rats (150–200 g body weight, fed standard laboratory diet) by the collagenase perfusion technique as described previously (12, 13). The cells were plated at a density of 3 × 10^6/60-mm dish in Earle's minimal essential medium containing 17% fetal bovine serum. The cells were allowed to adhere to the dishes overnight at 37°C in a 5% CO_2 atmosphere. The next morning the serum-containing medium was removed and replaced with fresh medium without serum but containing the appropriate additions as described for each experiment. In some experiments the cells were initially plated in medium that included the ethanolamine analogue. Cell viability was checked by Trypan blue exclusion and by leakage of lactate dehydrogenase into the medium (14). After the appropriate incubation period the culture medium was removed and centrifuged at 10,000 *g* for 15 min to remove any cell debris. The cells were washed with ice-cold phosphate-buffered saline (2 ml/dish) and scraped with a rubber policeman into 2 ml/dish of the same buffer. Centrifugation of the cell suspension for 5 min at 3,000 *g* pelleted the cells, which were subsequently lysed by being vortexed in 1 ml of ice-cold water.

Isolation of lipoproteins and apolipoproteins

The culture medium from eight dishes of cells combined (16 ml) was fractionated by single-spin ultracentrifugation as described previously (7, 13). Briefly, the medium was applied to a salt gradient and centrifuged for 42 h at 100,000 *g* in a Beckman Ti70 rotor. The following density fractions were collected from the gradient: VLDL (including intermediate density lipoproteins), density < 1.02 g/ml; LDL, density between 1.02 and 1.06 g/ml; HDL, density between 1.06 and 1.18 g/ml; bottom fraction density > 1.18 g/ml. The addition of 0.2 ml of an aqueous slurry (50 mg/ml) of Cab-O-Sil (fumed silica) to each of the four density fractions concentrated the lipoproteins (7, 13). The Cab-O-Sil, which contained the bound lipoproteins, was pelleted by centrifugation for 15 min at 12,000 *g*. The apolipoproteins were solubilized from the Cab-O-Sil pellet by addition of 1 ml of an extraction buffer that contained 2% sodium dodecyl sulfate and 6 M urea (13). The pellet was heated for 5 min at 95°C in the extraction buffer and the extract was electrophoresed on a 3–15% gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate. The apolipoproteins and albumin were identified by comparison with standard lipoproteins obtained from rat serum (15). The gels were stained with Coomassie blue and the bands corresponding to apoB_H, B_L, E, A-I, and albumin were cut from the gels. The gel slices were solubilized by incubation in 1 ml of Soluene 350 overnight at room temperature and the radioactivity in each protein band was counted after incubation of the samples with 10 ml Hionic-Fluor scintillant for 3 days.

Isolation and analysis of lipids from hepatocytes and lipoproteins

Lipids were extracted from the hepatocytes and from the secreted lipoproteins in the medium, or bound to Cab-O-Sil, with chloroform–methanol 2:1 (v/v) as described previously (7). The individual phospholipids—phosphatidylcholine, phosphatidylethanolamine (including phosphatidylmonomethylethanolamine), phosphatidylserine, and phosphatidylinositol—were isolated by thin-layer

chromatography of the lipid extract on silica gel G plates in the solvent system chloroform–methanol–acetic acid–formic acid–water 70:30:12:4:2 (v/v). In experiments in which triacylglycerol was analyzed, in conjunction with the phospholipids, the solvent was run 12 cm from the origin in the above solvent to separate the phospholipids. The plate was then dried and run to the top in the solvent system hexane–diethyl ether–acetic acid 65:35:2 (v/v). The plate was sprayed with Primulin (16) and glycerolipids were identified by comparison with authentic standards. The spots containing glycerolipids were scraped from the plate and either counted for radioactivity directly or analyzed for phosphorus (17) or triacylglycerol (18).

Phosphatidylethanolamine and phosphatidyl-*N*-methylethanolamine were separated by two-dimensional thin-layer chromatography in the following solvent system: first dimension, chloroform–methanol–acetic acid–formic acid–water 70:30:12:4:2 (v/v); second dimension, butanol–acetic acid–water 90:20:20 (v/v). The phospholipids were visualized by exposure to iodine vapor and identified by comparison with authentic standards.

High performance thin-layer chromatography of total lipids of lipoprotein fractions was performed as described previously (19). For visualization of the lipids, the high performance thin-layer plate was dipped in a solution of 3% (w/v) cupric acetate and phosphoric acid (8% v/v) and heated at 180°C for 15 min.

In labeling experiments using [3-^{3}H]serine the radioactivity present in the ethanolamine and choline moieties of phosphatidylethanolamine and phosphatidylcholine, respectively, was analyzed. The phospholipids were scraped from the thin-layer plate and extracted from the silica gel using chloroform–methanol–acetic acid–water 50:39:1:10 (v/v). The solvent was evaporated and the sample was hydrolyzed with phospholipase C (7, 20). The ethanolamine and choline products of the hydrolysis were present in the aqueous phase after hydrolysis. The completeness of hydrolysis was verified by thin-layer chromatography (7). The extent of hydrolysis of phosphatidylcholine and phosphatidylethanolamine by this method was 100% and 85%, respectively.

Other methods

Protein concentration was measured according to the method of Lowry et al. (21) using bovine serum albumin as the standard. For measurement of cellular protein synthesis, [^{3}H]leucine-labeled cells were harvested and pelleted by centrifugation, then disrupted by vigorous vortexing in water. The proteins were precipitated with ice-cold trichloroacetic acid at a final concentration of 10%. The precipitated proteins were pelleted by centrifugation for 5 min at 2,000 *g*. The pellet was washed 3 times with 1 ml of ice-cold 5% trichloroacetic acid and the proteins were solubilized by addition of 0.8 ml of 0.1 M sodium hydroxide. The mixture was neutralized with 0.5 M HCl in 0.12 M Tris/HCl and the samples were counted for radioactivity.

For assay of acyl-CoA:cholesterol acyltransferase, ten dishes of hepatocytes were homogenized by hand in a buffer containing 0.5 M sucrose, 1% dextran, 5 mM $MgCl_2$, 37.5 mM Tris/maleate, pH 6.5, using 75 strokes in a Dounce homogenizer. Microsomes were prepared by centrifugation of the homogenate for 10 min at 10,000 *g*, then recentrifugation of the supernatant at 100,000 *g* for 1 h from which a pellet of microsomes was obtained. The microsomes were resuspended in buffer containing 0.25 M sucrose, 10 mM Tris (pH 7.4), and 0.1 mM phenylmethylsulfonyl fluoride. The enzyme was assayed according to a published procedure (22) using [^{3}H]oleoyl-CoA as substrate. The ^{3}H-labeled substrate was synthesized according to the method of Hajra and Bishop (23).

Analysis of statistical significance of all data was performed using the unpaired Student's *t*-test.

RESULTS

Effect of MME on glycerolipid composition of hepatocytes and culture medium

Monolayer cultures of rat hepatocytes that had been plated overnight in the absence or presence of 200 μM MME were incubated in fresh medium containing either 0 or 200 μM MME for 6 h. The glycerolipid composition of cells and culture medium is shown in **Fig. 1.** In the presence of MME the only significant change observed in the cellular lipid composition (Fig. 1A) was an approximately 17-fold increase in the amount of the phospholipid phosphatidylmonomethylethanolamine compared to that in cells grown in the absence of MME (15.0 ± 2.4 and 0.9 ± 0.1 nmol/mg cell protein, respectively). The cellular content of PtdCho, phosphatidylethanolamine (PtdEtn), and triacylglycerols was unaffected by incubation with MME.

The glycerolipid content of medium from hepatocytes maintained in the presence or absence of MME is shown in Fig. 1B. The amount of triacylglycerols and PtdCho secreted by cells treated with MME was reduced by 46% and 53%, respectively. Although the amount of PtdEtn secreted from MME-treated cells was slightly reduced compared with untreated cells, the reduction was not statistically significant. In contrast, the amount of phosphatidylmonomethylethanolamine secreted into the culture medium was increased by 3-fold, from 0.06 ± 0.01 nmol/mg cell protein to 0.19 ± 0.01 nmol/mg cell protein.

The concentration dependence of MME on lipoprotein secretion was determined in an experiment in which hepatocytes were plated overnight in various concentrations of MME (0–1 mM). The next morning the medium was replaced with fresh medium containing [^{3}H]choline and the same concentration of MME. Cells were incu-

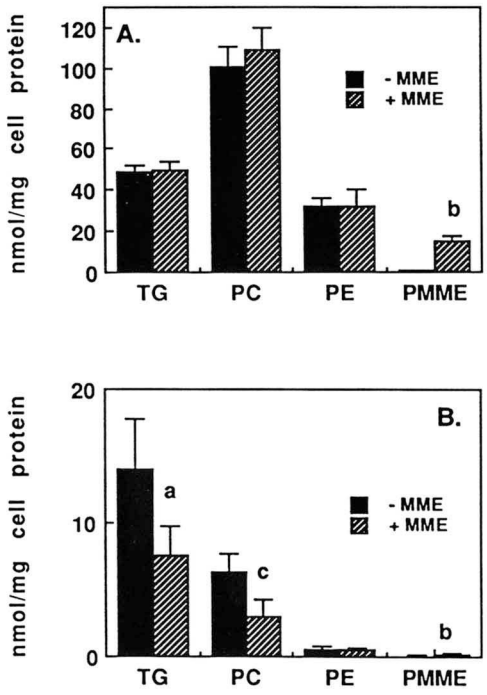

Fig. 1. Effect of MME on glycerolipid composition of cells and culture medium. Monolayer cultures of rat hepatocytes were plated overnight in medium containing 0 or 200 μM MME. The next morning the medium was removed and replaced with fresh medium containing the same concentration of MME. After 6 h lipids were extracted from cells (panel A) and medium (panel B) and individual lipids were isolated by thin-layer chromatography. The content of triacylglycerols (TG), PtdCho (PC), PtdEtn (PE), and phosphatidyl-*N*-monomethylethanolamine (PMME) was measured. Solid bars, no MME; hatched bars, 200 μM MME. Data are averages ± SD of four independent experiments. Statistical significance of differences between samples from cells grown in the presence and absence of MME was determined from the unpaired Student's *t* test. $a = 0.0005 < P < 0.005$; $b = P < 0.0005$; $c = 0.01 < P < 0.025$. All other differences were not statistically significant.

bated for 6 h, at which time PtdCho was isolated from cells and medium. Although MME treatment slightly reduced the incorporation of [^{3}H]choline into cellular PtdCho (by approximately 20% with 0.5 mM MME), MME caused a marked dose-dependent decrease in PtdCho secretion (**Fig. 2**). Cell viability, as assessed by leakage of lactate dehydrogenase into the medium was unaffected by all concentrations of MME used. Since a large effect of MME on VLDL secretion was elicited with an MME concentration of 200 μM and the viability of the cells was unaffected, this concentration was used for all subsequent experiments.

The speed at which MME inhibited VLDL secretion was investigated in an experiment in which hepatocytes were incubated with 200 μM MME for different periods of time, up to 24 h. The incorporation of [^{3}H]choline into secreted PtdCho was measured as an indicator of lipoprotein secretion. As shown in **Fig. 3A,** MME only slightly inhibited the incorporation of [^{3}H]choline into cellular PtdCho. In contrast, the secretion of PtdCho (Fig. 3B) was markedly reduced by MME, the effect being evident within 4 h.

Monolayer cultures of rat hepatocytes secrete both VLDL and HDL into the medium. Thus, four lipoprotein fractions of densities corresponding to VLDL (including intermediate density lipoproteins), LDL, HDL, and a fraction of density > 1.18 g/ml, from cells incubated for 18 h in the presence or absence of MME, were separated by ultracentrifugation of the medium on a single-spin salt gradient (13). The mass of triacylglycerols in the VLDL fraction was reduced from 40.9 ± 5.4 nmol/mg cell protein in the control cells to 13.2 ± 2.4 nmol/mg protein in MME-treated cells. In the LDL and HDL fractions from treated and untreated cells, smaller amounts of triacylglycerols (approximately 5–10 nmol/mg cell protein in LDL and less than 4 nmol/mg cell protein in HDL) were detected. Since the reduction in secreted triacylglycerols was in the VLDL fraction, and there was no increase in any other density fraction, triacylglycerols had not been redistributed to particles of density higher than that of the VLDL fraction.

The lipid profile of VLDL, LDL, HDL, and the bottom fraction (density > 1.18 g/ml) from the medium of cells incubated in the presence or absence of MME was examined by high performance thin-layer chromatography. A general reduction in the content of many lipids (phospholipids, triacylglycerols, cholesterol, and cholesteryl esters) of the VLDL fraction resulted from MME treatment, as shown in **Fig. 4.** MME did not significantly affect secretion of lipids into the HDL fraction or into the fraction of density > 1.18 g/ml. Thus, the effect of MME was specifically on VLDL.

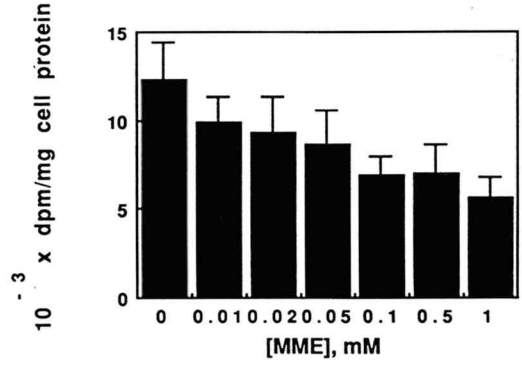

Fig. 2. Concentration dependence of MME for inhibition of lipoprotein secretion. Hepatocytes were plated overnight in various concentrations of MME (0–1 mM). The next morning the medium was replaced with fresh medium containing [*methyl*-^{3}H]choline (10 μCi/dish) and the same concentrations of MME. Cells were incubated for 6 h, then PtdCho was isolated from cells and medium. Data are averages ± SD of three determinations from one experiment that was repeated with a similar results.

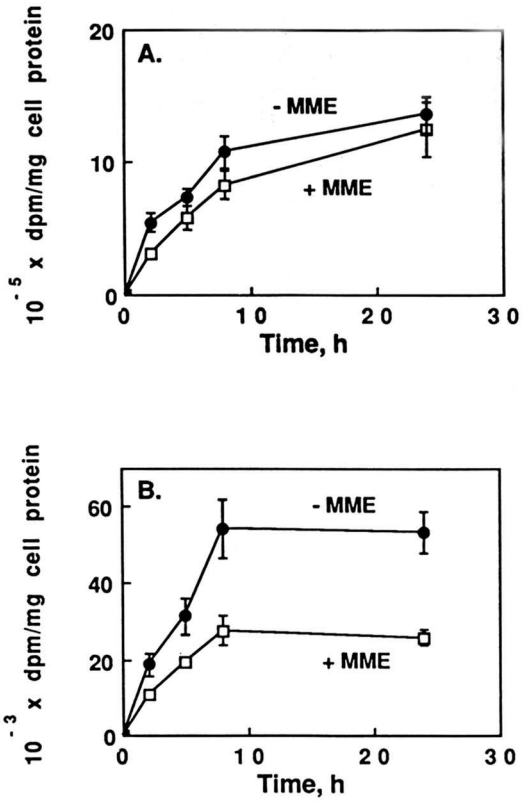

Fig. 3. Effect of time of incubation with MME on PtdCho secretion. Monolayer cultures of rat hepatocytes were plated overnight in medium without MME. The next morning, the medium was removed and replaced with medium containing 10 μCi/dish of [*methyl*-^{3}H]choline and either 0 (closed symbols) or 200 (open symbols) μM MME. At the indicated times lipids were extracted from cells (panel A) and medium (panel B) and PtdCho was isolated by thin-layer chromatography. The data are averages ± SD of three experiments, each with duplicate determinations. In some cases the error bars are too small to be visible.

Viability of the hepatocytes was not compromised by treatment with 200 μM MME for at least 44 h. The appearance of the cells by phase contrast microscopy and the amount of protein per dish of cells were identical whether the cells were incubated in the presence or absence of MME. Moreover, cell viability, as assessed by Trypan blue exclusion or by leakage of lactate dehydrogenase into the medium, was >90% and was unchanged by MME treatment. In addition, cellular protein synthesis (see Table 1) and secretion of albumin (see Fig. 5) were unaffected by MME.

Effect of MME on secretion of [^{3}H]leucine-labeled apolipoproteins

Inasmuch as MME inhibited secretion of lipid moieties of VLDL, the effect of MME on secretion of the apolipoprotein components of lipoproteins was examined. Hepatocytes were plated overnight in medium containing either 0 or 200 μM MME. The medium was removed and replaced with fresh medium containing [4,5-^{3}H]leucine and the same concentration of MME. After the cells had been incubated for an additional 16 h, cellular protein synthesis was unaffected by MME (**Table 1**). However, MME reduced secretion of the total proteins in the VLDL fraction but not in LDL, HDL, or the fraction of density > 1.18 g/ml (Table 1). Apolipoproteins were separated by electrophoresis on a 3–15% gradient polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Secretion of [^{3}H]leucine-labeled apolipoproteins B_H, B_L, and E in VLDL (**Fig. 5A**), was markedly inhibited (by 58%, 61%, and 72%, respectively) by incubation with MME. Even though little lipoprotein material was present in the LDL fraction from rat hepatocytes, the data for this fraction are included (Fig. 5B and Table 1) as confirmation that there had not been redistribution of VLDL apolipoproteins into the LDL density range. MME did not significantly affect the incorporation of [^{3}H]leucine into any proteins in the HDL fraction, for example apoA-I, apoE, or albumin (Fig. 5C). Thus, MME selectively inhibited secretion of VLDL.

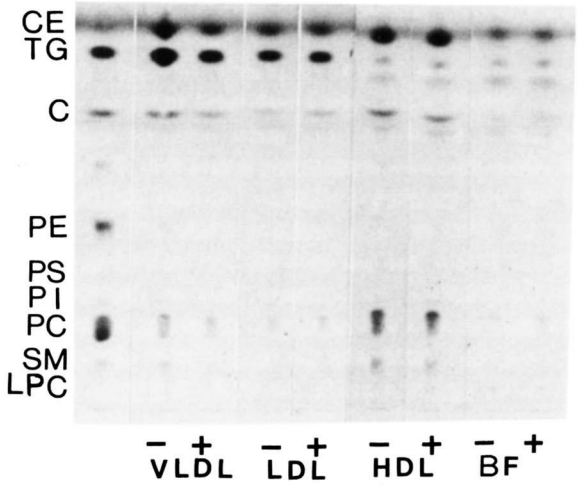

Fig. 4. High performance thin-layer chromatography of lipids of lipoproteins in the culture medium. Hepatocytes were plated overnight in the presence or absence of 200 μM MME. The next morning the medium was replaced with fresh medium containing the same concentration of MME and the incubation was continued for 6 h. Medium was harvested and lipoprotein classes were separated on the basis of density into four classes—VLDL, LDL, HDL, and a fraction of density >1.18 g/ml (BF). Lipids were extracted and subjected to high performance thin-layer chromatography. The VLDL sample contained lipid from two dishes whereas the LDL, HDL, and BF (density >1.18 g/ml) were from four dishes. The lanes marked + and – contain lipids from cells incubated in the presence and absence of MME, respectively. Data are from one experiment that was repeated 3 times with similar results. The left-hand lane contains standard lipids. CE, cholesteryl esters; TG, triacylglycerol; C, cholesterol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; LPC, lysophosphatidylcholine.

TABLE 1. Effect of MME on incorporation of [^{3}H]leucine into cellular and secreted proteins

Sample	+ MME	− MME	Statistical Significance of Difference
	10^{-3} × dpm/mg cell protein		
VLDL	10.2 ± 1.9	37.7 ± 0.3	$P < 0.0005$
LDL	4.2 ± 0.6	4.6 ± 2.2	NS
HDL	19.1 ± 2.4	14.1 ± 2.3	NS
BF	330.1 ± 6.2	357.0 ± 17.5	NS
Cells	986.4 ± 107.5	947.4 ± 95.1	NS

Monolayer cultures of rat hepatocytes were incubated as described in the legend to Fig. 5. Proteins secreted into the medium in the lipoprotein density ranges of VLDL, LDL, HDL, and the fraction of density > 1.18 g/ml (BF) were concentrated by addition of Cab-O-Sil. One-tenth of the total protein from eight dishes combined was counted for radioactivity. Cellular proteins were precipitated by addition of 10% trichloroacetic acid. The data are averages ± SD of quadruplicate analyses from one experiment that was repeated twice with similar results. Qualitatively similar results were obtained from two additional experiments in which cells were incubated for 7 h. Statistical significance of differences between cells grown in the presence and absence of MME was determined by the unpaired Student's *t* test; NS, not significant.

Effect of MME on PtdCho synthesis from [^{3}H]choline

Since MME is a structural analogue of choline, the initial hypothesis was that MME reduced VLDL secretion by competitive inhibition of cellular PtdCho synthesis. This hypothesis was tested in a pulse-chase experiment. Hepatocytes that had been plated overnight in medium without MME were pulsed with fresh medium containing [^{3}H]choline for 0.5 h. The pulse medium was replaced with chase medium containing either 0 or 200 μM MME and 28 μM unlabeled choline. At the indicated times (**Fig. 6**), PtdCho was isolated from cells and culture medium. MME did not alter the rate of PtdCho synthesis from [^{3}H]choline. In contrast, but in agreement with other experiments (for example, those depicted in Figs. 2 and 3), MME inhibited the secretion of choline-labeled PtdCho by approximately 40–50%. A similar result was obtained when [1(3)-^{3}H]glycerol was used as the label for PtdCho biosynthesis, i.e., MME did not affect the labeling of PtdCho from [^{3}H]glycerol. These data strongly suggest that the effect of MME on VLDL secretion was not mediated by reduction in PtdCho synthesis from [^{3}H]choline.

Effect of MME on PtdEtn and PtdCho synthesis from [1-^{3}H]ethanolamine

In liver PtdCho is synthesized both from choline via the CDP-choline pathway and from PtdEtn by three successive methylation reactions (24). Since MME did not affect the biosynthesis of PtdCho from the CDP-choline pathway (Fig. 6), the effect of MME on the synthesis of PtdEtn and PtdCho from [^{3}H]ethanolamine was investigated. The rationale for this experiment was that MME, which is a

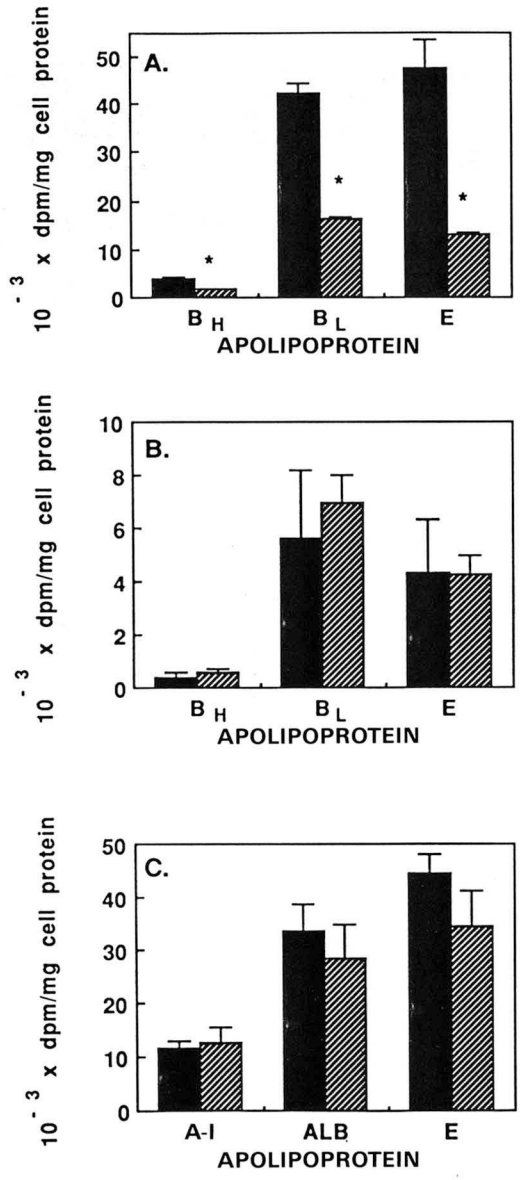

Fig. 5. Effect of MME on incorporation of [4,5-^{3}H]leucine into secreted apoproteins. Monolayer cultures of rat hepatocytes were plated overnight in the absence (solid bars) or presence (hatched bars) of 200 μM MME. The next morning the medium was replaced with fresh medium containing 15 μCi/dish of [4,5-^{3}H]leucine in the absence (solid bars) or presence (hatched bars) of 200 μM MME. After 16 h the medium was harvested and lipoproteins from eight dishes combined were separated by ultracentrifugation into VLDL (panel A), LDL (panel B), and HDL (Panel C). Lipoproteins were concentrated by adsorption on to Cab-O-Sil and proteins were extracted and separated by electrophoresis on gradient (3–15%) polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Unlabeled carrier lipoproteins from rat serum were added for visualization by Coomassie blue staining of the gels and apolipoproteins were identified by comparison with standard VLDL, HDL, and albumin (ALB). The bands were cut from the gels and counted for radioactivity. The data are averages ± SD from quadruplicate samples from one experiment that was repeated twice with similar results. Qualitatively similar results were obtained in three additional experiments in which cells were incubated with radiolabel for 7 h. In some cases error bars are too small to be visible. Statistical significance of differences between samples from cells growth in the presence and absence of MME was determined by the Student's unpaired *t* test; * is $P < 0.0005$; for all other samples the control and MME-treated cells were not significantly different.

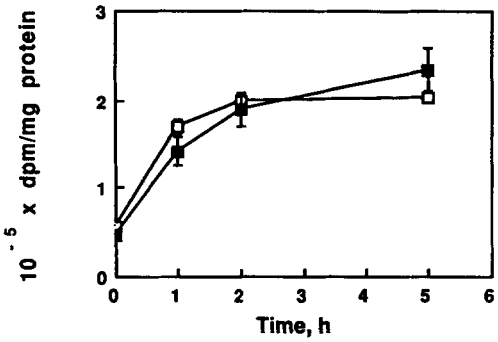

Fig. 6. Pulse-chase labeling of hepatocytes with [*methyl*-^{3}H]choline. Hepatocytes were pre-incubated overnight without MME then pulse-labeled for 0.5 h with 10 μCi/dish of [*methyl*-^{3}H]choline. The medium was removed and replaced with fresh medium containing 28 μM unlabeled choline and either 0 (closed symbols) or 200 μM MME (open symbols). At the indicated times cellular PtdCho was isolated and radioactivity was counted. Data are averages ± SD of triplicate determinations from one experiment which was repeated.

structural analogue of ethanolamine, might compete with ethanolamine for the enzymes of PtdEtn biosynthesis (25–27), which consequently might limit VLDL secretion. As shown in pulse-chase experiments, MME did not significantly affect incorporation of [1-^{3}H]ethanolamine into cellular PtdEtn (**Fig. 7A**) or PtdCho (Fig. 7B). MME did, however, reduce the amount of [^{3}H]ethanolamine-labeled PtdEtn and PtdCho secreted into the medium. For example, 4 h after the end of the pulse, radioactivity in secreted PtdCho was 884 and 337 dpm/mg cell protein from MME-treated and control cells, respectively. Similarly, MME reduced the amount of secreted [^{3}H]ethanolamine-labeled PtdEtn from 680 to 447 dpm/mg cell protein after 4 h.

Thus, apparently, the reduction of VLDL secretion by MME is not the result of inhibition of the biosynthesis of PtdEtn or PtdCho from [^{3}H]ethanolamine.

Effect of MME on incorporation of [3-^{3}H]serine into phospholipids

PtdEtn is made by two biosynthetic routes in mammalian cells: *a*) from the CDP-ethanolamine pathway (28); and *b*) via the decarboxylation of PtdSer (29). PtdEtn from both biosynthetic origins is secreted by rat hepatocytes, as is PtdCho derived from the methylation of PtdEtn produced from either source (7). The possibility that MME inhibited VLDL secretion by interfering with the biosynthesis of PtdSer or PtdSer-derived PtdEtn or PtdCho was considered. For example, MME might have competed with serine for synthesis of PtdSer by the so-called "base-exchange" enzyme (PtdSer synthase) (30). Hepatocytes were plated overnight in the presence or absence of MME (200 μM). The next morning 20 μCi/dish of [3-^{3}H]serine was added. The uptake of serine into cells was not affected by MME for 16 h of preincubation and 24 h incubation. After various time intervals PtdSer, PtdEtn, and PtdCho were isolated from cells and culture medium. Since [3-^{3}H]serine labels the fatty acid, glycerol and polar head-group moieties of phospholipids (7) PtdEtn and PtdCho were digested with phospholipase C so that radioactivity in the ethanolamine and choline moieties, respectively, was measured. PtdSer was not digested with phospholipase C since > 95% of the radioactivity in PtdSer from [3-^{3}H]serine resides in the serine moiety (7). The radioactivity in PtdSer was 15–30% lower in MME-treated cells than in untreated cells (**Fig. 8A**) and the amount of [^{3}H]-labeled PtdEtn was very similar in both types of cells (Fig. 8B). However, the radioactivity in [^{3}H]serine-derived PtdCho from the MME-treated cells was at all times significantly higher than from cells incubated without MME (Fig. 8C). Consequently, when the radioactivity in PtdSer + PtdEtn + PtdCho was combined, as a measure of total radiolabel incorporated into phospholipids derived from [^{3}H]serine, there was no difference in total radioactivity in the presence or absence

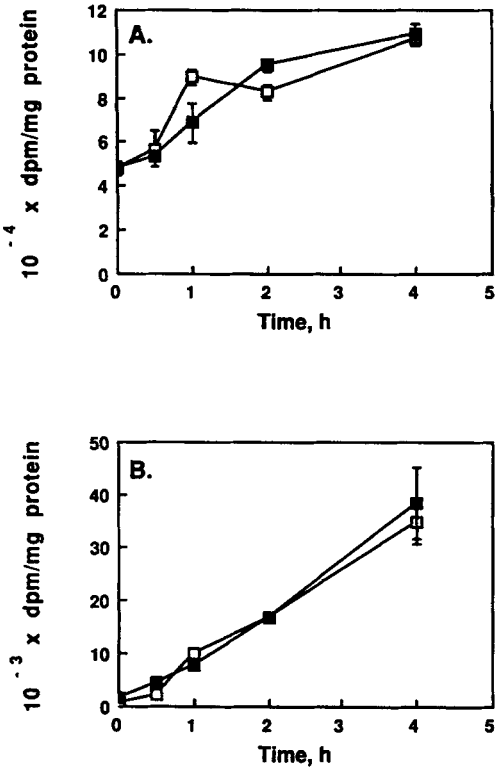

Fig. 7. Effect of MME on incorporation of [1-^{3}H]ethanolamine into cellular phospholipids. Hepatocytes were plated overnight in the absence of MME. The following morning the medium was replaced for 15 min with pulse medium containing 10 μCi/dish of [1-^{3}H]ethanolamine. The pulse medium was removed, the cells were washed, and chase medium with (open symbols) or without (closed symbols) 200 μM MME was added. Incorporation of ^{3}H into cellular PtdEtn (panel A) and PtdCho (panel B) was determined. Data are averages of triplicate analyses from one experiment that was repeated with a similar result. In some instances error bars are too small to be visible.

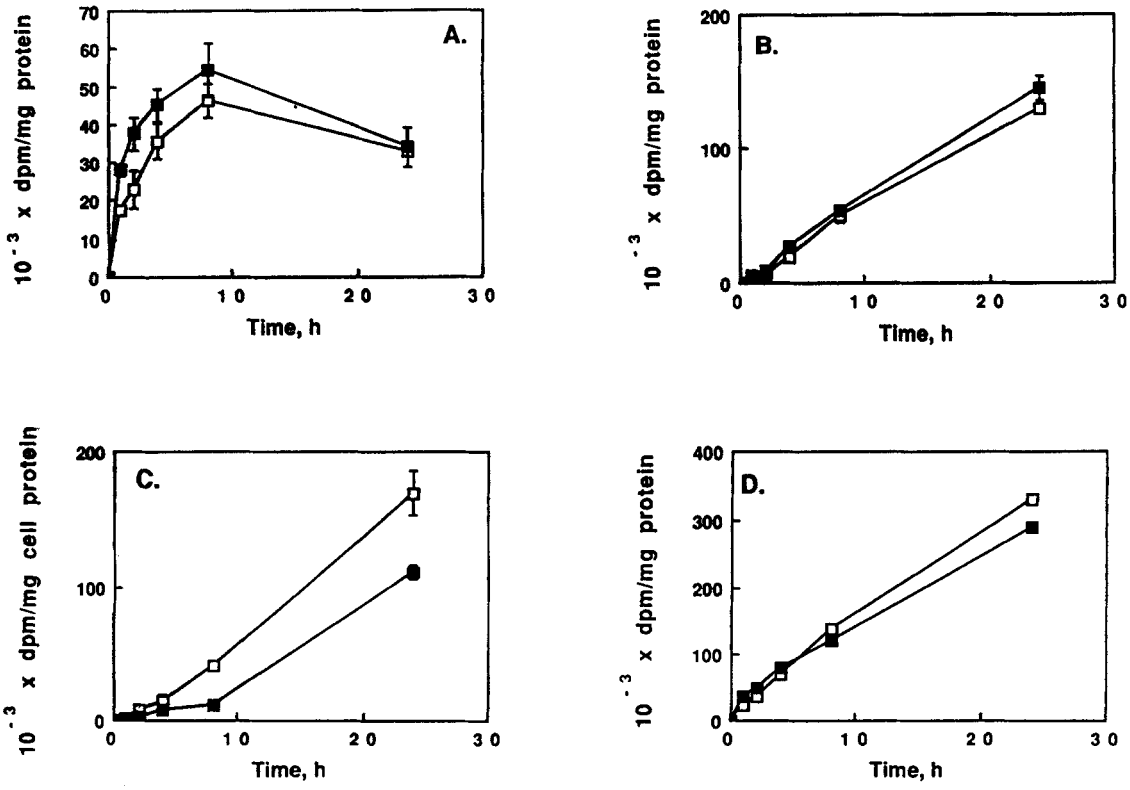

Fig. 8. Effect of MME on incorporation of [3-^{3}H]serine into cellular phospholipids. Hepatocytes were plated overnight in the presence (open symbols) or absence (closed symbols) of 200 μM MME. The next morning the medium was replaced with fresh medium containing 20 μCi/dish of [3-^{3}H]serine in the presence or absence of 200 μM MME. At the indicated times the cells were washed, and cells and medium were harvested (six dishes combined). Incorporation of [^{3}H]serine into cellular PtdSer (panel A) and the ethanolamine and choline moieties, respectively, of PtdEtn (panel B) and PtdCho (panel C) was determined. In panel D radioactivity in PtdSer + PtdEtn + PtdCho has been plotted. Data are averages ± SD of triplicate samples from one experiment that was repeated with a similar result. In some cases error bars are too small to be visible.

of MME (Fig. 8D); the label had, however, been redistributed, with a significant increase in radioactivity in PtdCho in MME-treated cells (Fig. 8C). The explanation for this increase is at present not clear.

[3-^{3}H]Serine is also incorporated into phospholipids of secreted lipoproteins (7–9). MME reduced incorporation of [^{3}H]serine into secreted PtdEtn; additional data from the experiments depicted in Fig. 8 showed that after 6 h the radioactivity values in PtdEtn secreted from MME-treated and control cells were 19,100 and 9,700 dpm/mg cell protein, respectively. In contrast, incorporation of [^{3}H]serine into secreted PtdCho was slightly higher in MME-treated cells than in those maintained in the absence of MME (900 vs. 460 dpm/mg cell protein). The latter observation most likely reflects increased incorporation of [^{3}H]serine into cellular PtdCho in the presence of MME (Fig. 8C).

Effect of MME on incorporation of [1(3)-^{3}H]glycerol into glycerolipids

Another possible explanation for why MME inhibited VLDL secretion was that the biosynthesis of triacylglycerols might have been diminished by MME treatment. This hypothesis was tested by incubation of cells overnight in the presence or absence of MME. The medium was replaced with fresh medium containing [1(3)-^{3}H]glycerol and either 0 or 200 μM MME. Cellular triacylglycerol synthesis from [^{3}H]glycerol was unaltered by incubation with MME whereas decreased amounts of ^{3}H-labeled triacylglycerols were secreted by MME-treated cells. For example, after 24 h the dpm in triacylglycerols secreted from cells incubated in the presence and absence of MME were 26,300 ± 1,900 and 44,000 ± 3,300 dpm/mg cell protein, respectively. From the same experiments, no significant reduction in incorporation of [^{3}H]glycerol into cellular PtdCho or PtdEtn over the 24-h time period was observed. These data are in agreement with the experiments depicted in Figs. 1 and 4.

Effect of MME on cholesteryl ester synthesis

Although cholesteryl esters are quantitatively minor components of nascent VLDL secreted by rat hepatocytes, a recent study has reported that in fibroblasts incubated with MME the biosynthesis of cholesteryl esters

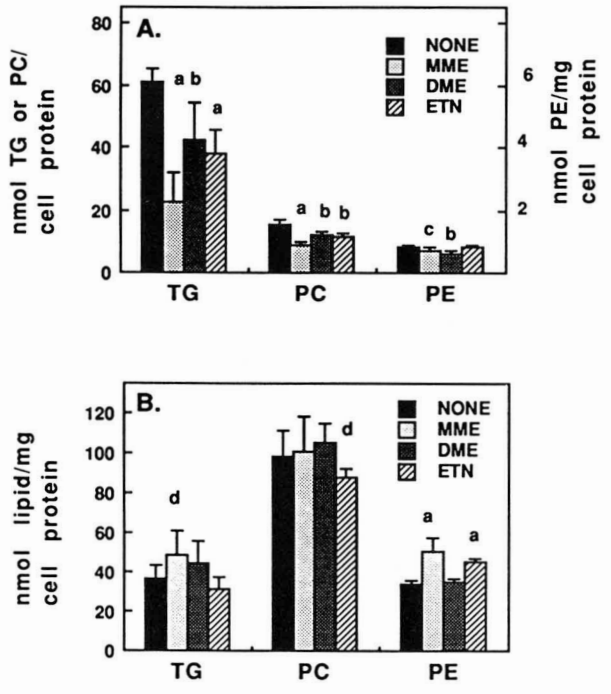

Fig. 9. Comparison of the effect of MME, *N,N*-dimethylethanolamine and ethanolamine on secretion of glycerolipids. Hepatocytes were plated in medium containing no addition (NONE), 200 μM MME, 200 μM dimethylethanolamine (DME), or 200 μM ethanolamine (ETN). After the cells had adhered to the dishes, the serum-containing medium was removed and replenished for 18 h with fresh medium containing the same concentrations of ethanolamine analogues. The medium (panel A) and cells (panel B) were harvested and the mass of triacylglycerols (TG), PtdCho (PC), and PtdEtn (PE) was measured. Data are averages ± SD from triplicate determinations of three experiments. Statistical significance of data from the treated cells compared with untreated cells was calculated using the Student's *t* test: a = $P < 0.0005$; b = $0.0005 < P < 0.005$; c = $0.01 < P < 0.025$; d = $0.025 < P < 0.05$. All other values were not statistically different from control values.

was reduced by 40–50% (31). Moreover, in HepG2 cells there are parallel changes in cholesteryl ester synthesis and apoB secretion (32). The activity of acyl-CoA:cholesterol acyltransferase, a key enzyme in cholesteryl ester biosynthesis, was therefore assayed in microsomes from rat hepatocytes that had been incubated overnight in the presence or absence of 200 μM MME. The specific activity of the enzyme was not significantly different in MME-treated (0.023 ± 0.008 nmol/min per mg protein) and control (0.020 ± 0.005 nmol/min per mg protein) cells.

Comparison of the effect of MME, ethanolamine, and *N,N*-dimethylethanolamine on lipoprotein secretion

The specificity of inhibition of VLDL secretion by MME was investigated by incubation of hepatocytes with *N,N*-dimethylethanolamine and ethanolamine, as well as MME. First, the effects of MME, dimethylethanolamine, and ethanolamine on the appearance of [^{3}H]choline-labeled PtdCho in the medium were compared. Cells were plated overnight and incubated in medium containing one of MME, dimethylethanolamine, ethanolamine (200 μM of each), or no addition. The next morning fresh medium containing [^{3}H]choline and the same ethanolamine analog was added, and the incubation was continued for 6 h. [^{3}H]Choline-labeled PtdCho was analyzed from cells and medium. The only ethanolamine analog that decreased secretion of labeled PtdCho was MME (by 56%). In agreement with previous experiments (Fig. 6), MME did not affect the incorporation of [^{3}H]choline into cellular PtdCho. Dimethylethanolamine increased ^{3}H-labeled PtdCho secretion slightly (by approximately 30%) but decreased the amount of [^{3}H]choline in cellular PtdCho by 28%, the latter being most likely the result of competition between dimethylethanolamine and choline for the enzymes of PtdCho biosynthesis. Although ethanolamine did not affect the secretion of [^{3}H]PtdCho, the incorporation of [^{3}H]choline into cellular PtdCho was stimulated by approximately 40%; the mechanism and significance of this stimulation are not known. Cell viability was unaffected by 24 h of incubation of cells with 200 μM MME, dimethylethanolamine, or ethanolamine, as measured by leakage of lactate dehydrogenase into the medium.

In a second series of experiments the mass of secreted and cellular lipids was measured after incubation of cells for 24 h with MME, dimethylethanolamine, or ethanolamine, all at a concentration of 200 μM. MME reduced secretion of triacylglycerols and PtdCho by 63% and 43%, respectively, whereas PtdEtn secretion was inhibited by only 14% (**Fig. 9A**). However, Ptd Etn analyzed in this experiment also included phosphatidylmonomethylethanolamine, the secretion of which was substantially increased by MME (Fig. 1). Dimethylethanolamine and ethanolamine each produced smaller, but statistically significant, decreases in secretion of triacylglycerols and PtdCho (Fig. 9A).

No major differences were observed in the levels of cellular triacylglycerols, PtdCho, and PtdEtn of cells treated with MME, dimethylethanolamine, or ethanolamine compared with untreated cells (Fig. 9B). The small increase in mass of cellular PtdEtn after treatment of cells with ethanolamine or MME was not unexpected (26). The apparent increase in PtdEtn content of cells treated with MME was most likely due to increased content of phosphatidylmonomethylethanolamine, since the PtdEtn-containing band scraped from the thin-layer chromatography plate also contained this lipid, the cellular content of which was greatly increased by MME treatment (Fig. 1).

The conclusion from the studies in which ethanolamine, MME, and dimethylethanolamine are compared is that MME is the most potent of the three analogues tested for inhibition of VLDL secretion.

DISCUSSION

In an attempt to determine the effect of alteration of phospholipid composition of hepatocytes on lipoprotein

secretion, monolayer cultures of rat hepatocytes were incubated with MME which caused a rapid, dose-dependent decrease in secretion of lipid and apolipoprotein constituents of VLDL. At a concentration of 200 μM MME, secretion of apolipoproteins B_H, B_L, and E, and lipids (triacylglycerols and phospholipids) was reduced by approximately 50% compared with that in cells incubated without MME. Neither cell viability nor overall protein synthesis was compromised by incubation with MME. Moreover, albumin was secreted at the same rate in MME-treated and control cells. Interestingly, secretion of apoA-I and lipids in the HDL fraction was not affected by MME. Therefore, MME appears to reduce specifically the secretion of VLDL, but not HDL, components.

Phosphatidylmonomethylethanolamine, the phospholipid containing the MME head-group, is almost undetectable in phospholipid mixtures obtained from normal mammalian cells. However, the level of phosphatidylmonomethylethanolamine in several cell types treated with MME can be increased dramatically. For example, treatment of mouse LM fibroblasts (33, 34) and human fibroblasts (31) with MME increased the cellular concentration of phosphatidylmonomethylethanolamine to 45% of total phospholipids. Similarly, when rat hepatocytes were incubated with 0.4 mM MME, the cellular content of phosphatidylmonomethylethanolamine increased to 20% of total phospholipids (25, 26). Moreover, when rats were fed a diet containing 1% MME in a single meal there was a large increase in the amount of phosphatidylmonomethylethanolamine in their liver and lungs (35). In agreement with these reports, in the present study phosphatidylmonomethylethanolamine was increased 17-fold upon MME treatment.

Effects of MME on phospholipid synthesis

The first plausible mechanism considered for reduced secretion of VLDL was that MME, which is a methylated analogue of ethanolamine and a demethylated analogue of choline, inhibited the biosynthesis of either PtdEtn or PtdCho by competition with the normal substrates of the enzymes involved. It has been suggested that formation of phosphatidylmonomethylethanolamine occurs via the same enzymes used for PtdEtn biosynthesis via CDP-ethanolamine, probably at the level of CDP-base formation (25–27). In contrast, formation of phosphatidyl-*N,N*-dimethylethanolamine from dimethylethanolamine probably occurs via enzymes of the CDP-choline pathway (25–27). In the present study no effect by MME on PtdCho biosynthesis from choline was observed. These data agree with the recent study of Jamil and Vance (36) who demonstrated that incubaton of choline-deficient hepatocytes with MME caused a large increase in phosphatidylmonomethylethanolamine content but did not significantly affect the activity of the enzyme CTP:phosphocholine cytidylyltransferase. (In many instances this enzyme is rate-limiting for the CDP-choline biosynthetic pathway.)

The effect of MME on VLDL secretion was compared with that of the same concentrations of ethanolamine and dimethylethanolamine. MME reduced (by approximately 60%) secretion of [^{3}H]choline-labeled PtdCho, whereas ethanolamine and dimethylethanolamine each reduced slightly the chemical amounts of triacylglycerols and PtdCho secreted, but the effect was smaller than that produced by MME.

The active biosynthesis of PtdCho, by either the CDP-choline pathway or by methylation of PtdEtn, is apparently required for VLDL assembly and/or secretion (11). In choline-deficient hepatocytes, in which PtdCho biosynthesis was restricted, the secretion of VLDL, but not HDL, was diminished by approximately 70% compared to that in control cells (11), although the exact mechanism of inhibition is not understood. Addition of MME to choline-deficient hepatocytes did not restore normal levels of VLDL secretion but reduced VLDL secretion even further (37). *N,N*-dimethylethanolamine partially reversed the inhibition of VLDL secretion caused by choline deficiency (37). In light of these studies, it seemed feasible that MME might exert its effect on VLDL secretion by inhibition of PtdCho biosynthesis. This hypothesis was tested in pulse-chase experiments in which hepatocytes were incubated with [^{3}H]choline in the presence or absence of MME. MME did not decrease PtdCho biosynthesis from either [^{3}H]choline or [^{3}H]glycerol.

Another possible explanation for inhibition of VLDL secretion by MME was that formation of PtdEtn (from [^{3}H]ethanolamine) was inhibited by competition between MME and ethanolamine for ethanolamine kinase or another enzyme of the CDP-ethanolamine pathway (25–27). Also feasible was the possibility that methylation of PtdEtn to PtdCho might have been diminished by MME treatment. Both of these possibilities were eliminated because neither the biosynthesis of PtdEtn from ethanolamine nor the methylation of ethanolamine- or PtdSer-derived PtdEtn to PtdCho was inhibited by incubation with MME.

We have previously demonstrated a preferential selection of PtdCho and PtdEtn derived from PtdSer for assembly and secretion into rat hepatocyte VLDL (7). The possibility that MME interfered with production of phospholipids derived from the biosynthetic route, serine to PtdSer to PtdEtn to PtdCho, was investigated. MME and serine might be expected to compete in the base-exchange reaction (30), thus inhibition of PtdSer biosynthesis by MME might occur. However, MME did not inhibit PtdSer biosynthesis from [^{3}H]serine. Nor was incorporation of [^{3}H]serine into cellular PtdCho or PtdEtn decreased in MME-treated cells.

Effects of MME on neutral lipid synthesis

Triacylglycerols are the major neutral lipids of VLDL secreted by rat hepatocytes. Recently, Maziere et al. (31) reported that when cultured human fibroblasts were grown in MME, so that phosphatidylmonomethylethanolamine comprised approximately 45% of total phospholipids, the incorporation of oleic acid into cholesteryl esters and triacylglycerols was reduced by 40–50%; the activities of the enzymes that synthesize these neutral lipids, i.e., acyl-CoA:Cholesterol acyltransferase and diacylglycerol acyltransferase, were also decreased. In the present study, however, in which phosphatidylmonomethylethanolamine levels were more modestly increased and the cell type was different, MME inhibited neither cellular triacylglycerol synthesis as measured by incorporation of [^{3}H]glycerol into triacylglycerols, nor the activity of a key enzyme in cholesteryl ester biosynthesis, acyl-CoA:cholesterol acyltransferase.

In conclusion, no mechanism for inhibition of VLDL secretion by MME has been deduced. The effect may be due to increased levels of either phosphatidylmonomethylethanolamine or MME. The secretory process per se is not affected since albumin, apoA-I and other proteins are secreted normally in MME-treated cells. Reduction of VLDL secretion as a result of MME treatment cannot be ascribed to inhibition of: *a*) PtdCho biosynthesis from the CDP-choline or methylation pathways; *b*) PtdEtn biosynthesis from either CDP-ethanolamine or PtdSer decarboxylation; *c*) PtdSer biosynthesis; *d*) triacylglycerol or cholesteryl ester biosynthesis; or *e*) protein synthesis.

A possible explanation, not yet examined, for the effect of MME on VLDL assembly and/or secretion is that the rate of apoB synthesis might have been decreased by MME treatment. On the basis of current information, however, this possibility does not seem likely, since in no instance so far examined has alteration in the rate of apoB synthesis been responsible for alteration of the rate of apoB secretion (38). Apparently, apoB is synthesized constitutively and excess apoB is degraded (39). Most likely, the limiting factor in assembly and secretion of VLDL is the supply of lipid (11, 32, 40–42). The possibility that intracellular apoB degradation might be increased by MME treatment also deserves consideration.

An intriguing alternative explanation for inhibition of VLDL secretion by MME may be that the increased concentration of phosphatidylmonomethylethanolamine might disrupt the transbilayer movement of newly made glycerolipids. Glycerolipids are synthesized on the cytosolic surface of endoplasmic reticulum (and Golgi) membranes (43) and must cross the membrane bilayer to the luminal surface from which the lipids are presumably assembled into nascent VLDL. Any compound that interfered with this transbilayer movement might inhibit VLDL assembly. Alternatively, the abnormally large amounts of phosphatidylmonomethylethanolamine in MME-treated cells might interfere with the normal process by which lipids and apoB aggregate in formation of VLDL particles.

The work was supported by a grant from the Heart and Stroke Foundation of Alberta. I thank Penney Bandura, Grazyna Wierzbicka and Alexis Chen for excellent technical assistance.

Manuscript received 13 June 1991 and in revised form 13 September 1991.

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