Fatty acid ethyl esters and HepG2 cells: intracellular synthesis and release from the cells

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Abstract **Fatty acid ethyl esters (FAEE), esterification products of fatty acid and ethanol, have been implicated as mediators of ethanol-induced organ damage. To understand the molecular and cellular events in FAEE synthesis and secretion, we developed a system in which HepG2 cells synthesize and release FAEE into the culture medium upon incubation with ethanol. The synthesis of FAEE was observed within 5 min of the addition of ethanol, with a plateau for FAEE synthesis after 2 h of incubation. It was also observed that FAEE are synthesized by both a microsomal FAEE synthase, which preferentially uses fatty acyl-CoA as a substrate, and a cytosolic FAEE synthase, which accepts both unesterified fatty acid and fatty acyl-CoA as substrates with a slight preference for fatty acyl-CoA. Although the kinetics of cellular FAEE synthesis await further characterization, the intracellular fatty acid substrate appears to be derived principally from glycerolipids and other esters. FAEE were released into the culture medium by a mechanism independent of the vesicular transport pathway. Lipoprotein particles and albumin were found to be carriers of FAEE after FAEE secretion from the cell.—**Kabakibi, A., C. R. Morse, and M. Laposata. **Fatty acid ethyl esters and HepG2 cells: intracellular synthesis and release from the cells. *J. Lipid Res.* 1998.** 39: **1568–1582.**

Supplementary key words hepatoblastoma cells • alcohol • ethyl ester • secretion • lipoprotein • albumin

Despite the fact that millions of individuals worldwide are adversely affected by ethanol abuse, the mechanism by which ethanol induces organ damage is not well understood. Acetaldehyde, an oxidative metabolite of ethanol, has been implicated as the principal mediator of ethanol-induced organ damage, but little or no acetaldehyde production has been found in the pancreas (1), which is severely damaged by ethanol abuse. Furthermore, negligible amounts of acetaldehyde have been found in the circulation after ethanol intake (2). The possibility that fatty acid ethyl esters (FAEE), esterification products of fatty acid and ethanol (3–5), are mediators of ethanol-induced organ damage was suggested in an autopsy study (6). In this investigation of patients who died while acutely intoxicated, it was found that the organs most commonly damaged by ethanol abuse, primarily the liver and the pancreas, had the highest levels of FAEE and the enzyme responsible for FAEE synthesis. Additionally, FAEE have been shown to be toxic in several experimental systems. It has been reported that FAEE uncouple oxidative phosphorylation in isolated mitochondria (7), increase pancreatic lysosomal fragility (8), and decrease protein synthesis and DNA synthesis in intact human hepatoblastoma cells (9). Recently, FAEE injected into rats at physiologically relevant concentrations were shown to injure the pancreas (10).

Very little is known about the synthesis of FAEE by intact cells and even less is understood about FAEE liberation from cells. Grigor and Bell (11) demonstrated that a microsomal preparation from rat liver was capable of synthesizing FAEE from fatty acyl-CoA upon incubation with ethanol. Free fatty acid without prior conversion to its CoA ester could not be used for FAEE synthesis by the liver microsomes. Subsequently, Bora et al. (12) and Mogelson and Lange (13) performed a number of studies using FAEE synthase purified from the cytosol of different organs. In one study involving rabbit heart homogenates, free fatty acid, but not fatty acyl-CoA, was a substrate for FAEE synthesis (14).

We hypothesized that FAEE are released from cells because the highest levels of FAEE synthase activity have been found in the liver and the pancreas (6), which secrete products into the blood and gastrointestinal tract, respectively. In addition, we previously found FAEE in blood after ethanol ingestion (15), and because a significant percentage of the FAEE was associated with lipopro-

Abbreviations: FAEE, fatty acid ethyl ester; DMEM, Dulbecco's minimal essential medium; PBS, phosphate-buffered saline; TLC, thin-layer chromatography; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; BSA, bovine serum albumin; BFA, brefeldin A; MON, monensin; CYC, cycloheximide; apoB, apolipoprotein B; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; L-FABP, liver–fatty acid binding protein; SCP-2, sterol carrier protein-2.

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teins in that study, we speculated that the origin of these FAEE is the hepatocyte. The goal of the current study was to determine the mechanism by which FAEE are synthesized and released from HepG2 cells. The results of our studies indicate that FAEE are synthesized by both a cytosolic and a microsomal FAEE synthase. Although the kinetics of cellular FAEE synthesis await further characterization, the intracellular fatty acid substrate appears to be derived principally from glycerolipids and other esters. A portion of the synthesized FAEE is secreted into the medium by a mechanism other than the vesicular transport pathway. FAEE in the culture medium were found to be transported by lipoprotein particles as well as albumin.

MATERIALS AND METHODS

Materials

[9,10-^{3}H]palmitic acid (39.0 Ci/mmol), [9,10-^{3}H]oleic acid (14.0 Ci/mmol), [1-^{14}C]triolein (112.0 mCi/mmol), and [oleoyl-1-^{14}C]oleoyl coenzyme A (59.35 mCi/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). [1-^{14}C]tripalmitin (20 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled ethyl oleate was purchased from Nu-Chek Prep (Elysian, MN). Essentially fatty acid-free human and bovine serum albumin, brefeldin A, monensin, and the assay kit for lactate dehydrogenase (LDH) were purchased from Sigma Chemical Co. (St. Louis, MO). Cycloheximide was obtained from Janssen Chimica (Geel, Belgium). Sephacryl S-200 HR and Sephadex G-75 were products of Pharmacia (Piscataway, NJ). All tissue culture reagents were obtained from Gibco, Life Technologies (Grand Island, NY). Rabbit anti-human albumin antiserum was prepared by East Acres Biologicals (Southbridge, MA) by injecting rabbits with purified human albumin. Two different preparations of rabbit anti-rat liver fatty acid binding protein polyclonal antibody were generous gifts from Dr. Jeffrey Gordon (Washington University, St. Louis, MO) and Dr. John Lowe (University of Michigan School of Medicine). All reagents used for the polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). Agarose gel electrophoresis reagents were products of Helena Laboratories (Beaumont, TX). [^{14}C]ethyl palmitate and [^{14}C]ethyl oleate were synthesized according to the method of Turk et al. (16). Briefly, [1-^{14}C]tripalmitin (20 mCi/mmol) or [1-^{14}C]triolein (112.0 mCi/mmol) were evaporated under nitrogen and resuspended in 0.6 ml of dichloromethane. The radioactive triglyceride was incubated with 0.5 mol/L KOH in ethanol for 45 min at room temperature. The reaction was terminated by the addition of 1.0 ml of 6 mol/L HCl, and the FAEE were extracted into 1.0 ml of dichloromethane. The extracted FAEE were then isolated by solid phase extraction using an aminopropyl chromatography column as described by Bernhardt et al. (17).

Cell culture

HepG2 cells were obtained from the American Type Culture collection (Rockville, MD) and were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum, 200 U/ml penicillin, 200 μg/ml streptomycin, and 0.29 mg/ml l-glutamine (complete medium) at 37°C and 5% CO_2. An essential fatty acid-deficient cell line (HepG2-EFD) was established in our laboratory by growing HepG2 cells in medium containing delipidated serum (18). Delipidated fetal calf serum was prepared according to the method of Capriotti and Laposata (19). Serum-free medium contained all the components of the complete medium without the fetal calf serum.

FAEE synthesis and release

[^{3}H]oleic acid (14.0 Ci/mmol) or [^{3}H]palmitic acid (47.0 Ci/mmol) were dried on the wall of a 50-ml conical tube under a stream of nitrogen. The fatty acids were then resuspended in DMEM containing 0.1 mg/ml of essentially fatty acid-free human albumin (final concentration of 1.25 μm). The fatty acid suspension was then filtered through a 0.2-μm cellulose acetate filter (Nalgene, Rochester, NY) and was incubated with HepG2 cell monolayers (approximately 90% confluent) for 12 h at 37°C to allow for fatty acid uptake. At the end of the incubation period, the medium was aspirated, the cells were rinsed gently with PBS twice, and fresh serum-free medium containing 0, 50, or 100 mm absolute ethanol (final concentration) was added for an additional 9 h. In some experiments, 170 mm ethanol was used to maximize synthesis and release of FAEE in this in vitro system. The synthesis of FAEE occurred at ethanol levels <100 mm, but the amount of FAEE generated at ethanol concentrations <100 mm was relatively low in our experimental system. To increase the number of FAEE dpm and, thereby, the precision of the assay for FAEE synthesis, the ethanol concentration in the in vitro system was increased to 100 mm. This was not associated with toxicity for the HepG2 cells as only ethanol concentrations above 400 mm were found to be toxic to HepG2 cells as determined by the LDH activity in the culture medium. Dashti, Franklin, and Abrahamson (20) have reported that incubating HepG2 cells with ethanol concentrations up to 200 mm did not increase LDH activity in the medium, even after 5 days of incubation with the ethanol. Ethanol concentration was approximately maintained by the addition of ethanol (25% of the initial amount) every 3 h to compensate for ethanol metabolism and evaporation. At the end of the 9-h incubation period, the medium was aspirated, centrifuged at 3600 *g* for 15 min to remove any cells dislodged from the dish during medium changes, and then retained for analysis. Cells were washed three times with PBS at 37°C and then harvested by scraping them into 1.0 ml of ice-cold PBS with a rubber policeman. Proteins were precipitated with 2 ml of acetone after the addition of [^{14}C]ethyl palmitate or [^{14}C]ethyl oleate as internal standards. Lipids were then extracted from the cells (to measure cellular FAEE) and the medium (to measure medium FAEE) with 2 ml of acetone followed by 8 ml of hexane. The hexane layer was dried under nitrogen, resuspended in 150 μl of acetone, and FAEE were isolated from other lipid classes by thin-layer chromatography (TLC) using petroleum ether–diethyl ether–water 75:5:1 (v/v/v). The area corresponding to FAEE on the silica gel plate was identified by comparison with a known ethyl oleate standard (50 μg) in an adjacent lane visualized by iodine vapor. After sublimation of the iodine, the area corresponding to FAEE (R_f approximately 0.5) was scraped into plastic vials, and the FAEE were quantitated by liquid scintillation counting (Beckman LS 5000TD, Fullerton, CA). Palmitic acid and oleic acid were chosen for these studies because ethyl palmitate and ethyl oleate are the predominant FAEE in plasma after ethanol ingestion (15). In dose-response experiments, cells were incubated with the fatty acid suspension as described above. At the end of the incubation period, the medium was aspirated, the cells were rinsed three times with PBS, and fresh serum-free medium containing 0, 10, 20, 40, 80, 160, or 320 mm of ethanol was added for an additional 9 h. In these experiments, LDH activity in the medium did not increase above baseline, even with the highest concentration of ethanol. Time course experiments were performed as described above except that the cells were incubated with serum-free medium containing 170 mm ethanol for 0 min, 5 min, 10 min, 15 min, 30 min, or 1, 2, 4, or 9 h. In some ex-

periments the cells were labeled with [^{3}H]oleic acid (17.5 μCi/ml) for 12 h. The labeling medium was aspirated and the cells were rinsed three times with PBS. The cells were then incubated with serum-free medium containing 170 mm ethanol and 1.5% essentially fatty acid-free bovine serum albumin (BSA) for 9 h at 37°C. To assess the effect of lipoprotein particles in the culture medium on FAEE release from cells, the cells were labeled with [^{3}H]oleic acid for 12 h. The labeling medium was aspirated and the cells were rinsed three times with PBS. For 9 h at 37°C, the cells were incubated with serum-free medium containing 100 mm ethanol and human VLDL, LDL, or HDL (100 μg protein/ml) isolated as previously described (21). FAEE were then isolated and quantitated as described above.

Isolation of lipoprotein density classes from the cell culture medium

The medium from the cells collected after the 9-h incubation with 170 mm ethanol was subjected to discontinuous gradient ultracentrifugation according to the method of Redgrave, Roberts, and West (22). Briefly, the medium was adjusted to d 1.21 g/ml with solid KBr. Solutions of d 1.063 g/ml, d 1.019 g/ml, and d 1.006 g/ml were layered sequentially on the sample forming a discontinuous gradient. After centrifugation at 286,000 *g* at 20°C for 48 h, the secreted lipoproteins were collected in separate tubes. The lipoproteins included VLDL in the density range 1.006–1.019 g/ml, LDL in the density range of 1.019–1.063 g/ml, and HDL in the density range 1.063–1.21 g/ml, along with the d > 1.21 g/ml bottom fraction. Purity of the fractions was confirmed by agarose gel electrophoresis. The FAEE in each of these fractions were extracted, isolated, and quantitated as described above.

Analysis of the HepG2 cell culture medium by gel filtration chromatography

A separate aliquot of the medium containing radiolabeled FAEE secreted from the cells and collected after the 9-h incubation with 170 mm ethanol was fractionated using Sephacryl S-200 HR gel filtration column chromatography. The column was equilibrated with PBS at 4°C. Fractions (0.25 ml/min) were collected from the column and assayed for protein content and for radioactivity by liquid scintillation counting. The fractions containing radioactivity were pooled, extracted as described above, and their FAEE radioactivity was isolated from other lipid classes by TLC and quantitated by liquid scintillation counting. In experiments involving the determination of the molecular mass of the cytosolic protein which binds FAEE, cells were incubated with [^{3}H]oleic acid for 12 h and then with 100 mm ethanol for 9 h as described above. The cells were then harvested, homogenized, and the cytosolic fraction was isolated by ultracentrifugation as described below. The cytosol (2 ml) was applied to a Sephadex G-75 column (1.5 × 80 cm, 4°C, 0.5 ml/min), and 1-ml fractions were collected. The radioactivity and protein concentration were determined in the collected fractions. The fractions containing radioactivity were pooled (10 fractions/pool), extracted as described above, and their FAEE radioactivity was isolated from other lipid classes by TLC and quantitated by liquid scintillation counting.

Incubation of HepG2 cells with metabolic inhibitors

Stock solutions of brefeldin A, monensin, and cycloheximide were prepared in absolute ethanol. Cell monolayers were incubated with [^{3}H]oleic acid for 12 h. At the end of this incubation period, the medium was removed, the cells were rinsed with PBS twice, and serum-free medium containing 100 mm ethanol and 5 μg/ml (17.8 μm) brefeldin A, 10 μm monensin, or 10 μg/ml (35.5 μm) cycloheximide (all final concentrations) was added for 9 h. These concentrations of inhibitors were found to be nontoxic to cells as determined by an LDH enzyme assay of the medium (data not shown). FAEE were then isolated from the cells and the medium and quantitated as described above. In these studies, it was necessary to maintain the metabolic inhibitors throughout the incubation period with ethanol because the inhibitory effect of BFA (23, 24), monensin (25), and cycloheximide (26) is rapidly overcome (within 15 min) upon removal of the drugs from the culture medium.

Subcellular fractionation of HepG2 cells

Cells were seeded in 100-mm petri dishes and maintained as described above. Upon reaching confluence, the medium was aspirated, and the cells were rinsed twice with PBS and then scraped into 1.0 ml of a protease inhibitor buffer (0.32 m sucrose, 10 mm HEPES, 10 mm 2-mercaptoethanol, 20 μg/ml phenylmethylsulfonyl fluoride, 1 mm benzamidine, and 0.01% soybean trypsin inhibitor, pH = 7.4). Cells were disrupted in a Dounce homogenizer (10 strokes) and the lysate was centrifuged at 1000 *g* in a Beckman J6B centrifuge (Beckman, Fullerton, CA) for 10 min at 4°C. The supernatant was centrifuged at 10,000 *g* in a Beckman J21 centrifuge for 10 min at 4°C. The supernatant from this step was centrifuged at 100,000 *g* for 1 h in a Beckman L8-70M ultracentrifuge at 4°C. The resultant supernatant (the cytosolic fraction) was aspirated and stored at −80°C until use. The pellet from the 100,000 *g* centrifugation (microsomal fraction) was gently washed with the protease inhibitor buffer, resuspended in 500 μl of the protease inhibitor buffer, and stored in a similar manner. The cell homogenate, the cytosolic fraction, and the microsomal fraction were each diluted with the protease inhibitor buffer to an equal protein concentration (0.85 mg/ml). All fractions were dialyzed against 4 changes of 500 ml PBS buffer (pH = 7.4) for 2 h at 4°C before they were used as the enzyme source in the FAEE synthase activity assay. Dialysis of the subcellular fractions was performed to remove the high concentration of sucrose in the fractionation buffer and thereby eliminate any potential interference in the assay for FAEE synthase activity.

FAEE synthase activity assay

The assay was performed in a total reaction volume of 135 μl. In a 15-ml conical tube, 230 nmol of either [^{14}C]oleic acid (56.0 mCi/mmol) or [^{14}C]oleoyl-CoA (59.3 mCi/mmol), 0.1 m PBS (pH = 7.4), 0.1 m of absolute ethanol, 1.0 mg/ml of fatty acid-free human albumin in a total volume of 55 μl, and 80 μl of the 0.85 mg/ml enzyme source (i.e., 68 μg of the crude cell homogenate, the cytosolic fraction, or the microsomal fraction) were incubated at 37°C for 1 h. Tubes containing cytosolic or microsomal enzyme were assayed with either the [^{14}C]oleic acid or [^{14}C]oleoyl-CoA as the enzyme substrate. At the end of the incubation period, the reaction was stopped by the addition of 865 μl of ice-cold PBS. The reaction mixture was immediately extracted with 2 ml of acetone, followed by 6 ml of hexane. FAEE were isolated and quantitated as described above. FAEE synthase activity was expressed as nmol FAEE synthesized/hour per mg protein.

Immunoprecipitation and SDS-PAGE

Cells were plated in 35-mm culture dishes and maintained in complete medium as described above. After 4 days, the medium was replaced with methionine-free medium for 45 min, at which time the cells were incubated with the same medium containing [^{35}S]methionine (30 μCi/ml) for 4 h. At the end of this incubation period, the medium was aspirated, centrifuged at 15,850 *g* for 5 min to remove any cells dislodged from the dish, and used immediately for immunoprecipitation studies. The albumin from the culture medium was immunoprecipitated as described by Bonnardel and Davis (27) except that their immunoprecipita-

tion buffer (containing Triton-X 100) was replaced with PBS to eliminate the effect of the detergent on FAEE. Briefly, aliquots (200 μl) of the medium from the cells labeled with [^{35}S]methionine were combined with 800 μl PBS in 1.5-ml Eppendorf microcentrifuge tubes. The amount of rabbit anti-human albumin antiserum (or preimmune rabbit serum as a control) which produced the maximal amount of albumin in the precipitate (50–100 μl) was added. The samples were agitated overnight on a rocking platform at room temperature. Then, 100 μl of a 10% solution of protein A-Sepharose CL-4B beads (Sigma) in PBS was added, and the incubation was continued for an additional 2 h. The tubes were centrifuged in a Beckman microcentrifuge for 15 min at 15,850 *g*. The supernatant was aspirated and the pellet was washed 4 times with PBS. The final pellets were solubilized in 50 μl of sample buffer (0.5 m Tris, 13% glycerol, 4% SDS, 5% β-mercaptoethanol) and were then placed in boiling water for 3–4 min. The samples were then subjected to electrophoresis on a linear 4–20% SDS-polyacrylamide gel according to the method of Laemmli (28) under constant voltage (200 V) for 1 h. The gel was stained with Coomassie blue and then destained with 3 changes of 100 ml destaining solution (40% methanol, 10% acetic acid). The gel was finally dried for 2 h at 80°C, and was then exposed to Kodak X-Omat AR film (Rochester, NY) at −70°C for 12 h.

Determination of the binding of FAEE to immunoprecipitated albumin

Cells were incubated with [^{3}H]oleic acid (14.0 Ci/mmol, 188 μCi/ml) for 12 h. The medium was then removed, the cells were rinsed three times with PBS, and serum-free medium containing 170 mm ethanol was added for an additional 9 h. At the end of the incubation period, the medium was aspirated, centrifuged, and immunoprecipitated as described above, except that 800-μl aliquots were immunoprecipitated in these experiments. The final pellets were washed 4 times with PBS and then resuspended in 1 ml PBS. The suspension was transferred into 15-ml glass tubes, and the albumin was precipitated with 2 ml of acetone. FAEE were extracted with 8 ml of hexane in the presence of 1000 dpm of [^{14}C]ethyl oleate as a recovery marker, isolated by TLC, and quantitated by liquid scintillation counting. Most experiments were performed at least twice with similar results.

Western blot analysis

Male Sprague-Dawley rats were killed and their livers were harvested. HepG2 cells were grown and maintained as described above. The rat livers or HepG2 cells were homogenized in a cold isotonic buffer solution, consisting of 10 mm KH_2PO_4 and 154 mm KCl (pH 7.4), and their protein concentration was determined. Cytosolic proteins (100 μg/sample) were isolated by centrifugation as described above and then fractionated by SDS-PAGE (4–20%) and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Corp., Arlington Heights, IL). Blots were pretreated in blocking buffer (1% gelatin, 0.2% Tween-20 in PBS) for 1 h at room temperature and then incubated in blocking buffer with rabbit anti-rat liver fatty acid binding protein (1:1000 dilution) for 2 h at room temperature. The blots were then washed with the blocking buffer, and the antigen–antibody complexes were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) and a chemiluminescent substrate using the Western Light® kit (Tropix, Inc., Bedford, MA).

Other assays

Protein was determined by the bicinchoninic acid method (Pierce, Rockford, IL) with BSA as the standard.

RESULTS

In an effort to determine the mechanism by which FAEE are synthesized and released from cells upon exposure to ethanol, it was necessary to develop an in vitro model system. We accomplished this by incubating HepG2 cells with radiolabeled oleate for 12 h, and then with 50 or 100 mm ethanol for 9 h. In this system, the cells synthesized and liberated FAEE into the culture medium. **Figure 1** shows the relative amount of cellular and medium ethyl oleate. The dpm of cellular and medium ethyl oleate over a 9-h period was 975 ± 51 and 126 ± 20 dpm/mg cell protein, respectively (mean ± SEM) when cells were incubated with 50 mm ethanol. Corresponding dpm for the 100 mm ethanol incubation were: 2189 ± 156 (cellular) and 285 ± 10 dpm/mg cell protein (medium). Thus, approximately 12% of total ethyl oleate was released into the medium. In two other experiments, the amount of ethyl oleate liberated into the culture medium ranged from 10 to 20% (data not shown). There was essentially no FAEE synthesis or release when the cells were incubated with radiolabeled oleic acid in the absence of ethanol (Fig. 1). Similarly, there was no FAEE synthesis when the radiolabeled oleic acid was incubated with ethanol and serum-free medium in the absence of cells (data not shown). Similar results were obtained when cells were incubated

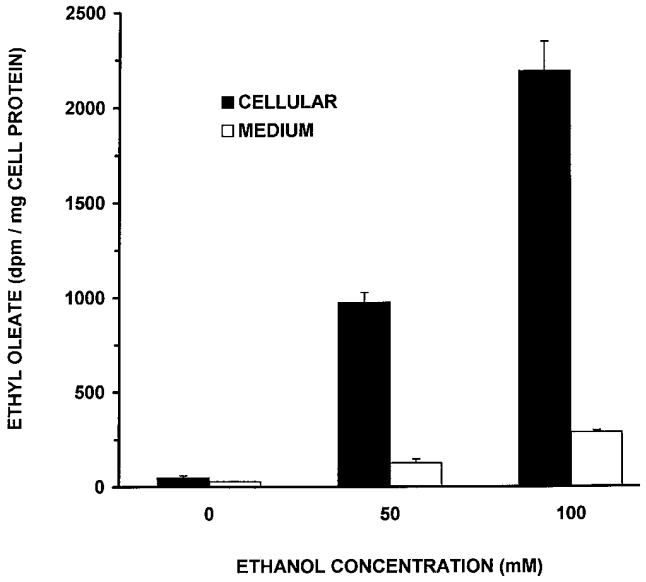

Fig. 1. Synthesis and release of ethyl oleate into the medium of HepG2 cells upon incubation with ethanol. HepG2 cells in 35-mm petri dishes (approximately 90% confluent) were incubated with 1.25 μm of [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was then removed, the cells were rinsed three times with PBS, and serum-free medium containing 0, 50, or 100 mm ethanol was added for an additional 9 h. At this time, the medium was removed, centrifuged at 3600 *g* for 15 min, and retained for analysis. The cells were rinsed twice with PBS and then harvested into 1 ml of ice-cold PBS. Ethyl oleate was isolated from the cells and quantified to measure cellular FAEE (filled bars) and isolated from the medium to measure medium FAEE (open bars). The bars represent the mean ± SEM (n = 3). The data are representative of one of three identical experiments.

with [^{3}H]palmitic acid and ethanol (data not shown). We incubated the cells with [^{3}H]palmitic acid or [^{3}H]oleic acid for 12 h to maximize the number of dpm of substrate fatty acid inside the cell and, therefore, to maximize the number of dpm FAEE synthesized and released from cells. We established this protocol on the basis of previous studies in our laboratory (29). In these studies, HepG2 cells were incubated with radiolabeled free fatty acids for different time points to assess their uptake by the cells for FAEE synthesis. Approximately 70% of the total fatty acid added to the medium was incorporated by cells when the cells were incubated with the fatty acid for 24 h, and only 22% was incorporated by cells during a 0.5-h incubation with the fatty acid. The amount of FAEE generated from fatty acids incorporated during the 24-h incubation period was approximately 3-fold more than the amount of FAEE generated during the 0.5-h incubation period. In addition, 97% of the incorporated fatty acids were esterified after 0.5 h of incubation with cells, suggesting that esterified fatty acid is the source for FAEE synthesis (29).

It was not possible to determine the mass of ethyl palmitate and ethyl oleate synthesized and released from HepG2 cells. The dilution of the exogenous radiolabeled fatty acid with an unquantifiable amount of unlabeled substrate fatty acid in the cells makes the final specific activity of the substrate fatty acid unknown. However, it was possible to measure the mass of FAEE synthesized and released using the HepG2-EFD cell line. Because there is no endogenous pool of arachidonate in HepG2-EFD, the specific activity of radioactive arachidonate does not change upon incorporation into these cells. Therefore, radioactivity data (dpm) can be converted directly to mass, and masses as low as a fraction of 1 pmol can be accurately measured. Upon incubating HepG2-EFD cells with arachidonate for 12 h followed by 100 mm ethanol for 9 h, we calculated the mass of intracellular ethyl arachidonate to be 0.30 pmol/mg cell protein (data not shown). The mass of medium ethyl arachidonate was 0.04 pmol/mg cell protein. Therefore, approximately 12% of the total amount of ethyl arachidonate was released into the medium. Ethyl arachidonate represents between 2% and 6% of total FAEE synthesis in HepG2 cells (29). Therefore, the total FAEE production within 9 h by HepG2-EFD exposed to 100 mm ethanol was calculated to be 15–45 pmol/mg cell protein. **Figure 2** shows the time course for FAEE synthesis and appearance in the medium. The top panel (Fig. 2A) shows that ethyl oleate is synthesized rapidly after the addition of ethanol with a plateau value achieved by 2 h of incubation. The FAEE appearance in the medium, on the other hand, as shown in the lower panel (Fig. 2B), follows a lag phase of approximately 1 h during which time there is no FAEE release. After 1 h, the FAEE release into the medium occurs at a linear rate. **Figure 3** shows the concentration dependence curves for synthesis and release of ethyl oleate with increasing ethanol concentration in the medium. There was a positive correlation between ethanol concentration in the culture medium and the amount of cellular ethyl oleate (Fig. 3A). A similar correlation was observed between ethanol concentration in the culture

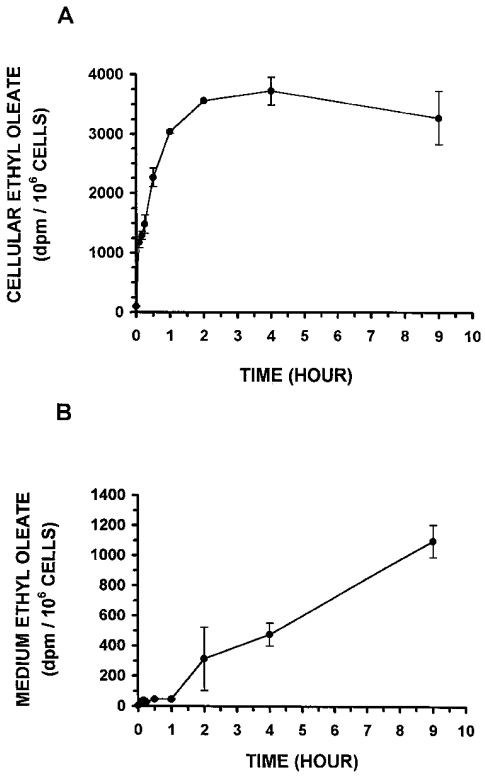

Fig. 2. Time course for the synthesis (A) and the release (B) of ethyl oleate into the medium of HepG2 cells. HepG2 cells in 35-mm petri dishes (approximately 90% confluent) were incubated with 1.25 μm of [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was then removed and the cells were rinsed three times with PBS. Serum-free medium containing 170 mm ethanol was added for 0, 5, 10, 15, 30 min, or 1, 2, 4, or 9 h. At each time point, the medium and the cells were harvested and ethyl oleate was isolated and quantified as described under Materials and Methods. The bars represent the mean ± SEM (n = 2). When not showing, the error bars are within the symbols. The data are representative of one of two identical experiments.

medium and the amount of ethyl oleate liberated into the culture medium (Fig. 3B).

To determine whether FAEE synthase is present in the cytosol and/or the microsomes of HepG2 cells, and whether this enzyme uses free fatty acid or fatty acyl-CoA as a substrate, we performed an experiment in which the HepG2 cells were lysed and the microsomes and cytosol were tested for FAEE synthase activity with either oleic acid or oleoyl-CoA as a substrate. As shown in **Fig. 4**, FAEE synthase activity was detectable in both the cytosolic and the microsomal preparations. Free fatty acid and fatty acyl-CoA were acceptable substrates for FAEE synthesis by the cytosolic and the microsomal enzymes. However, the microsomal FAEE synthase preferred oleoyl-CoA as a substrate approximately 4-fold over oleic acid, with the cytosolic enzyme showing only a slight preference for oleoyl-CoA. The homogenate reflects primarily the FAEE synthase activity in microsomes which showed a distinct preference for oleoyl-CoA. To exclude non-enzymatic formation of FAEE, [^{3}H]oleic acid was incubated with 100 mm ethanol

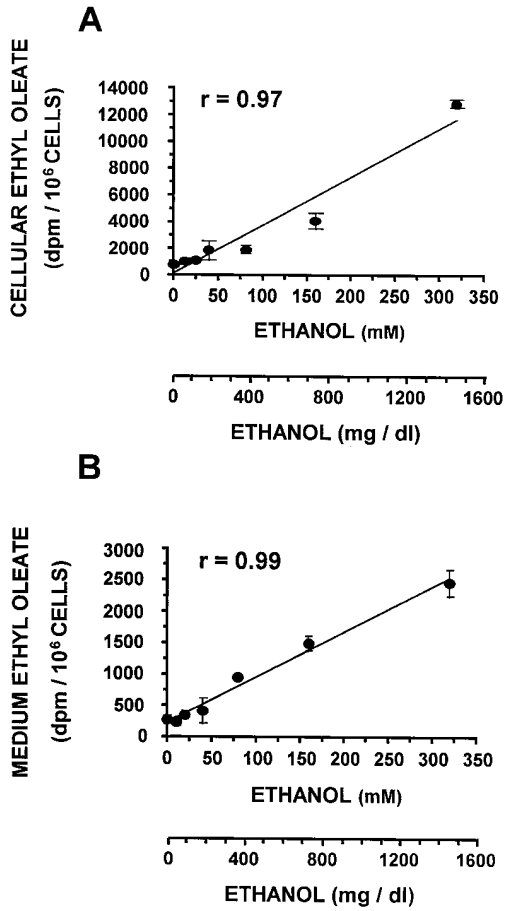

Fig. 3. Synthesis (A) and the release (B) of ethyl oleate into the medium relative to ethanol concentration in the medium. HepG2 cells in 35-mm petri dishes were incubated with 1.25 μm of [^{3}H] oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was then removed and the cells were rinsed three times with PBS, and serum-free medium containing 0, 10, 20, 40, 80, 160, or 320 mm ethanol was added for an additional 9 h. The medium and the cells were harvested and ethyl oleate was isolated and quantified as described under Materials and Methods. The results are expressed as mean ± SEM (n = 2). When not showing, the error bars are within the symbols. The data are representative of one of two identical experiments.

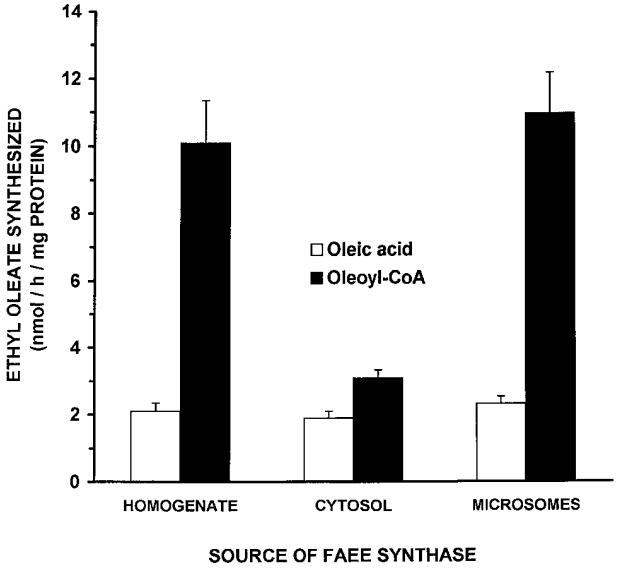

Fig. 4. Substrate specificity of FAEE synthase in subcellular fractions of HepG2 cells. HepG2 cells were fractionated to separate the microsomal fraction from the cytosolic fraction. In a conical tube, 230 nmol of either [^{14}C]oleic acid (56.0 mCi/mmol) (open bars) or [^{14}C]oleoyl-CoA (59.3 mCi/mmol) (filled bars), 0.1 m PBS (pH = 7.4), 0.1 m of absolute ethanol, 1.0 mg/ml of fatty acid-free human albumin in a total volume of 55 μl, and 80 μl of the 0.85 mg/ml enzyme source (i.e., 68 μg of the crude cell homogenate, the cytosolic fraction, or the microsomal fraction) were incubated at 37°C for 1 h. Ethyl oleate was immediately extracted from the reaction mixture, isolated, and quantified as described under Materials and Methods. The results are shown as mean ± SEM (n = 2). The data are representative of one of two identical experiments.

in the presence of a HepG2 cell homogenate, a boiled homogenate (100°C, 5 min), or saline for 1 h at 37°C. The amount of ethyl oleate synthesized in each of these three preparations was 1.35 ± 0.01, 0.034 ± 0.004, and 0.036 ± 0.001 nmol/h per mg cell protein, respectively (data not shown). These results confirm that the synthesis of FAEE by HepG2 cells is an enzyme catalyzed process.

In our in vivo studies (15), FAEE were present in blood after ethanol ingestion and were found to be associated with lipoprotein particles and with a plasma protein having a density much greater than any lipoprotein (d > 1.21 g/ml). Therefore, we performed an ultracentrifugation experiment to determine whether the FAEE released from HepG2 cells into the medium were associated with lipoproteins or a non-lipoprotein particle from the d > 1.21 g/ml fraction. In these studies, the medium containing radiolabeled ethyl oleate released from cells was fractionated by discontinuous ultracentrifugation. The results of the experiment are shown in **Fig. 5**. Ethyl oleate was found to be predominantly associated with lipoproteins after liberation from the HepG2 cells, primarily the HDL fraction. However, approximately 18% of the ethyl oleate was bound to a protein in the d > 1.21 g/ml fraction. Similar results were obtained when the cells were incubated with palmitic acid and ethanol to synthesize and release ethyl palmitate into the culture medium (data not shown).

Earlier evidence suggested that the protein in the d > 1.21 g/ml fraction to which FAEE was bound was albumin (15, 30). The data in **Fig. 6A** show that when HepG2 cells were incubated with 1.5% BSA, the intracellular level of FAEE decreased below the amount with serum-free medium. However, FAEE release from cells was markedly enhanced when compared to release in serum-free medium (Fig. 6B). The sum of cellular and medium FAEE indicates that BSA in the culture medium enhanced the overall production of FAEE by cells.

To further investigate the possibility that FAEE released into the medium are bound to albumin, cells were incubated with [^{3}H]oleic acid and then with ethanol for 9 h, and the medium was fractionated by gel filtration chromatography. As shown in **Fig. 7**, the bulk of the radiolabeled ethyl oleate eluted with a protein of a molecular mass of 66 kDa, which is consistent with the molecular mass of albumin. Additionally, to obtain direct evidence that FAEE in the cell culture medium bind to albumin, cells were in-

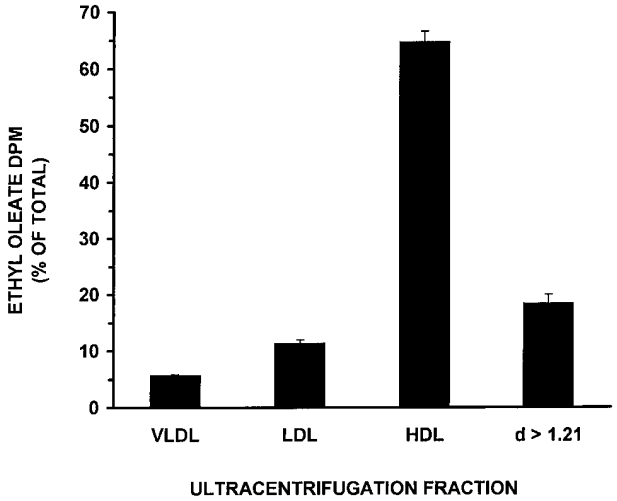

Fig. 5. Fractionation of the ethyl oleate-containing medium by discontinuous ultracentrifugation. HepG2 cells in 35-mm petri dishes (approximately 90% confluent) were incubated with 1.25 μm [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was removed, the cells were rinsed three times with PBS, and serum-free medium containing 170 mm ethanol was added for an additional 9 h. At this time, the medium was removed, centrifuged at 3600 *g* for 15 min, and then fractionated by discontinuous ultracentrifugation as described under Materials and Methods. The secreted lipoproteins, including VLDL in the density range 1.006–1.019 g/ml, LDL in the density range 1.019–1.063 g/ml, and HDL in the density range 1.063–1.21 g/ml, along with the d > 1.21 g/ml bottom fraction were collected in separate tubes. Ethyl oleate in the fractions was isolated and quantified as described under Materials and Methods. The results are shown as mean ± SEM (n = 2). The data are representative of one of two identical experiments.

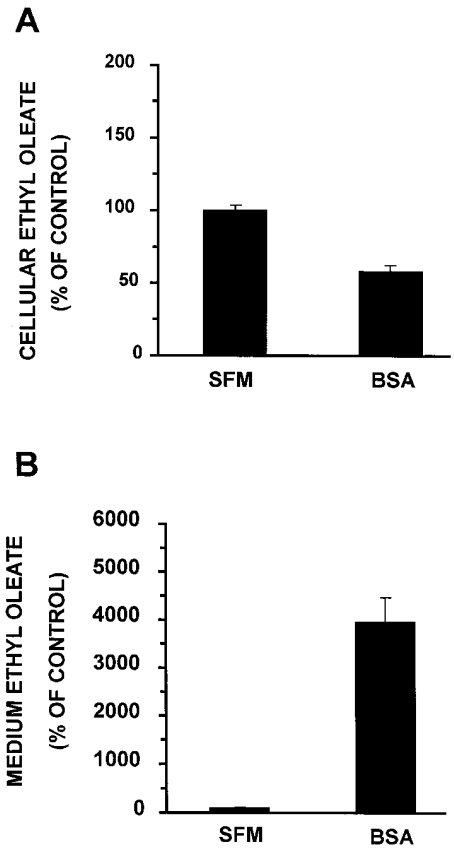

Fig. 6. Effect of BSA in the culture medium on the synthesis and release of FAEE from HepG2 cells. HepG2 cells in 35-mm petri dishes were incubated with 1.25 μm of [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The labeling medium was aspirated, the cells were rinsed three times with PBS, and serum-free medium containing 170 mm ethanol and 1.5% bovine serum albumin (BSA) (essentially fatty acid free) was added for 9 h at 37°C. Control dishes were incubated with serum-free medium containing 170 mm ethanol only (SFM). At the end of the incubation period, the medium was aspirated, centrifuged, and retained for analysis. The cells were rinsed twice with PBS and then harvested into 1 ml of ice-cold PBS. Ethyl oleate was extracted from the cells (A) and from the medium (B), and quantified as described under Materials and Methods. The results are shown as mean ± SEM (n = 3). The data are representative of one of two identical experiments.

cubated with [^{3}H]oleic acid for 12 h and then with 170 mm of ethanol for 9 h. Albumin was immunoprecipitated from the culture medium by rabbit anti-human albumin antiserum. The pellets were washed with PBS and their [^{3}H]ethyl oleate was extracted, isolated by TLC, and quantitated by scintillation counting. SDS-PAGE analysis of [^{35}S]albumin showed that the rabbit anti-human albumin antiserum, but not the preimmune rabbit serum, precipitated albumin released from the cells into the culture medium (data not shown). Furthermore, ethyl oleate was recovered from the immunoprecipitate produced by incubating the medium with the antiserum, but not from the immunoprecipitate with the preimmune serum (**Fig. 8**). There was essentially no ethyl oleate in the pellet produced by immunoprecipitating albumin from the medium in the absence of ethanol. Thus, these results indicate that ethyl oleate liberated into the cell culture medium binds to albumin.

Because FAEE in the culture medium were bound to lipoproteins and albumin, one possibility for FAEE exiting from cells is that FAEE are secreted via the vesicular transport pathway as an integral part of lipoproteins or as albumin–FAEE complexes. Another possibility is that FAEE exit the cell by a mechanism independent of the vesicular transport pathway, and that the association with lipoproteins and albumin occurs in the culture medium. To assess the involvement of the vesicular transport pathway in FAEE exiting from cells, we performed experiments in which the HepG2 cells were incubated with inhibitors that interfere with the vesicular transport pathway. Brefeldin A (BFA), a lipophilic fungal metabolite, interferes with protein secretion at the level of *cis*-Golgi by the disassembly of the Golgi complex and redistribution of Golgi components to the endoplasmic reticulum (31–33). Monensin is a carboxylic ionophore that inhibits protein secretion at the level of *trans*-Golgi (34). The results of the experiments showed that brefeldin A and monensin have a profound inhibitory effect on the release of ethyl oleate into the serum-free medium (**Fig. 9**). Notably, brefeldin A markedly increased the cellular level of FAEE. This is in agreement with the studies in which brefeldin A has been found to increase cholesteryl ester synthesis in HepG2

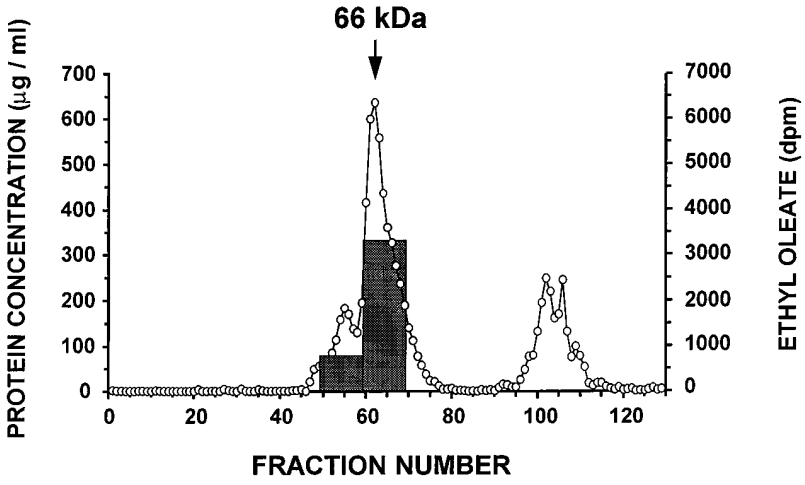

Fig. 7. Fractionation of the FAEE-containing medium by gel filtration chromatography. HepG2 cells in 35-mm petri dishes (approximately 90% confluent) were incubated with [^{3}H]oleic acid (100 μCi/ml) for 12 h at 37°C. The medium was removed, the cells were rinsed three times with PBS, and serum-free medium containing 170 mm ethanol was added for an additional 9 h. At this time point, the medium was removed, centrifuged to remove cells, and an aliquot (3 ml) was applied to a Sephacryl S-200 HR column which was equilibrated with PBS at 4°C. Fractions (0.25 ml/min) were collected from the column, assayed for protein content (open circles), and radioactivity in the fractions was measured by liquid scintillation counting. The fractions containing radioactivity were pooled (10 fractions/pool), extracted, and their ethyl oleate content was isolated by TLC and quantified by liquid scintillation counting (bars). The data are representative of one of two identical experiments.

cells (35), and sphingomyelin synthesis in CHO cells (36). Cycloheximide also impaired FAEE release into the medium (Fig. 9), suggesting that FAEE release is linked to the synthesis of a protein required for its transport out of the cell.

Although the results shown in Fig. 9 suggest that FAEE exit the cell via the vesicular transport pathway, it was still possible that FAEE are transported to the plasma membrane by a lipid carrier protein in the cytoplasm, and then delivered onto lipoprotein and albumin particles which are secreted via the vesicular transport pathway without the FAEE. In this case, the inhibitory effect of BFA and monensin on FAEE liberation from cells could be explained by inhibiting the secretion of lipoproteins and albumin which serve as acceptors for FAEE in the medium. To test this hypothesis, it was necessary to determine whether the addition of acceptor molecules for FAEE in the medium enhance FAEE release into the medium. As shown in **Fig. 10**, the addition of VLDL, LDL, or HDL to the culture medium markedly increased FAEE release when compared to release in serum-free medium. Lipoprotein particles in the culture medium did not significantly alter the cellular level of FAEE. However, the sum of the cellular and the medium FAEE indicates that for each lipoprotein, the addition of lipoprotein particles to the culture medium stimulated the overall production of FAEE by HepG2 cells (Fig. 10). When the same concentration of lipoprotein particles was incubated with [^{3}H]oleic acid and ethanol in a cell-free medium, no FAEE synthesis occurred, eliminating the possibility that FAEE in the culture are a result of the synthesis by the lipoproteins in the medium in the presence of ethanol (data not shown). Increasing HDL concentration in the medium was associated with an increase in the amount of medium FAEE, with no apparent change in the cellular level of FAEE (**Fig. 11A**). Additionally, when the release of FAEE into the HDL-containing medium was assessed as a function of time, FAEE were detectable in the medium approximately 15 min after the addition of ethanol (Fig. 11B), suggesting that FAEE exiting from cells occurred faster than apolipoprotein and albumin secretion. It has been reported that approximately 30–60 min is required for apolipoprotein and albumin molecules to be secreted from HepG2 cells after the synthesis is complete (37, 38). The level of FAEE in the medium increased over 9 h. Similarly, the cellular level of FAEE increased with time, although it decreased after 9 h of incubation with ethanol (Fig. 11B).

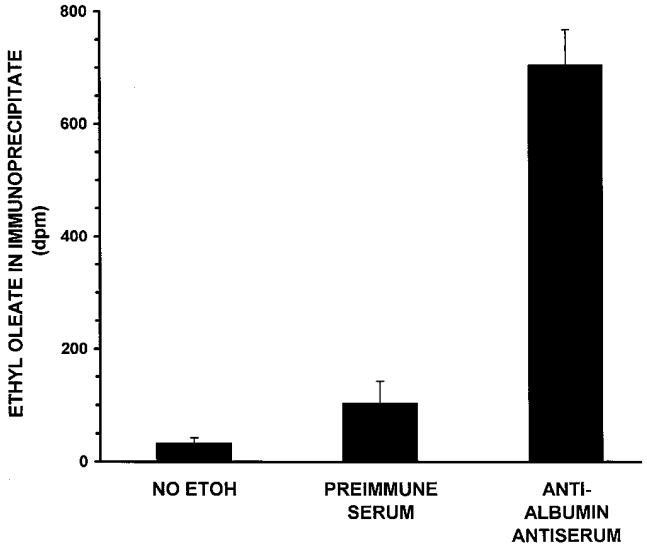

Fig. 8. Immunoprecipitation of albumin from the medium of HepG2 cells. Cells were incubated with [^{3}H]oleic acid (100 μCi/ml) for 12 h at 37°C. The medium was then removed, the cells were rinsed three times with PBS, and serum-free medium containing 170 mm ethanol was added for an additional 9 h. At the end of the incubation period, the medium was aspirated, centrifuged, and immunoprecipitated using a polyclonal rabbit anti-human albumin antiserum or preimmune rabbit serum as described under Materials and Methods. The final pellets were washed and resuspended in 1 ml PBS. The suspension was transferred into 15-ml glass tubes and the albumin was precipitated with 2 ml of acetone. Ethyl oleate was extracted, isolated by TLC, and quantified by liquid scintillation counting. The results are expressed as mean ± SEM (n = 2). The data are representative of one of two identical experiments.

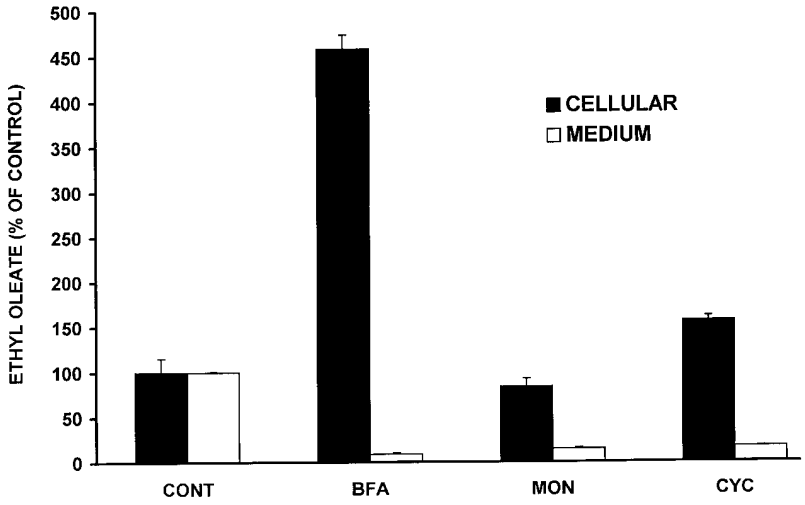

Fig. 9. Effect of metabolic inhibitors on ethyl oleate synthesis and release from HepG2 cells. Cell monolayers were incubated with 1.25 μm [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. At the end of this incubation period, the medium was removed, the cells were rinsed with PBS twice, and serum-free medium containing 100 mm ethanol and 5 μg/ml (17.8 μm) brefeldin A (BFA), 10 μm monensin (MON), or 10 μg/ml (35.5 μm) cycloheximide (CYC) was added for 9 h. Control dishes (CONT) contained 100 mm ethanol without any of the inhibitors. The medium and the cells were harvested and ethyl oleate was isolated from the cells (filled bars) and from the medium (open bars) and quantified as described under Materials and Methods. The synthesis and release of ethyl oleate by control dishes without any of the test compounds was designated as 100%. The results are shown as mean ± SEM (n = 2). The data are representative of one of four identical experiments.

If FAEE are transported to the plasma membrane by a mechanism independent of the vesicular transport pathway, and if they reside at the plasma membrane awaiting the secretion of lipoprotein and albumin particles to serve as carriers in the extracellular medium, disrupting the vesicular transport pathway is likely to decrease the release of FAEE into the serum-free medium, as it will interrupt the secretion of lipoproteins and albumin. However, the release of FAEE into medium containing exogenously added lipoproteins should not be affected when the vesicular transport pathway is disrupted, as the liberation of FAEE is no longer dependent on the secretion of lipoprotein and albumin particles from the cells. As shown in **Fig. 12A**, BFA at a concentration as low as 0.5 μg/ml completely abolished the release of FAEE into the serum-free medium. However, the addition of HDL (100 μg protein/ml) to the culture medium resulted in an increase in the amount of FAEE in the medium. FAEE release into the medium was not affected by BFA at concentrations as high as 10 μg/ml (Fig. 12A). Thus, FAEE are secreted from cells by a route other than the vesicular transport pathway. As expected, incubating the cells with BFA increased the intracellular level of FAEE by approximately 5-fold (Fig. 12B). There was no difference in the cellular level of FAEE whether or not HDL was added to the culture medium (Fig. 12B). This suggested that HDL, like BFA, can enhance the synthesis of FAEE by cells, and thereby maintain cellular FAEE levels despite increased FAEE release into the medium. Indeed, HDL added to the medium was found to increase the overall production of FAEE by cells (Figs. 10, 11A). Therefore, although FAEE release increases with the presence of HDL in the medium, the cells

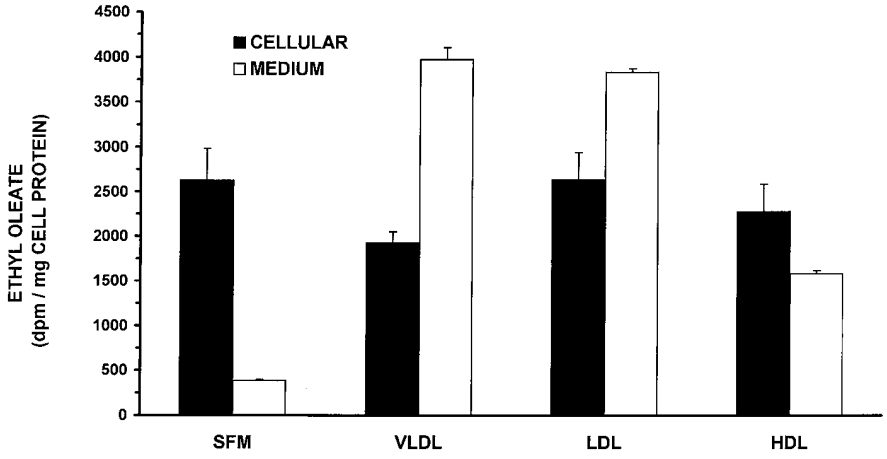

Fig. 10. Effect of human lipoproteins in the culture medium on FAEE synthesis and release from HepG2 cells. HepG2 cells in 35-mm petri dishes were incubated with 1.25 μm of [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was then removed, the cells were rinsed three times with PBS, and serum-free medium containing 100 mm ethanol and VLDL, LDL, or HDL (100 μg protein/ml) was added for an additional 9 h. Ethyl oleate was isolated from the cells (filled bars) and the medium (open bars) and quantified as described under Materials and Methods. The results are shown as mean ± SEM (n = 3).

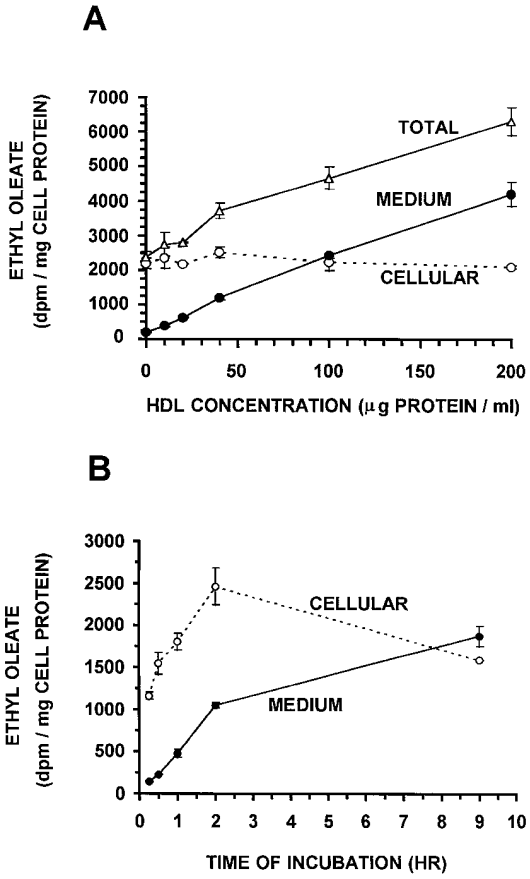

Fig. 11. Effect of HDL in the culture medium on the synthesis and release of FAEE from HepG2 cells as a function of concentration (A) and the time of incubation (B). (A): HepG2 cells were incubated with 1.25 μm of [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was then removed, the cells were rinsed three times with PBS, and serum-free medium containing 100 mm ethanol and 0, 10, 20, 40, 100, or 200 μg protein/ml of human HDL was added for an additional 9 h. (B): The cells were incubated with 1.25 μm of [^{3}H]oleic acid for 12 h at 37°C. The medium was then removed, the cells were rinsed three times with PBS, and serum-free medium containing 100 mm ethanol and human HDL (100 μg protein/ml) was added for 15 min, 30 min, 1, 2, or 9 h at 37°C. Ethyl oleate was isolated from the cells (dashed lines) and the medium (solid lines) and quantified as described under Materials and Methods. When not showing, the error bars are within the symbols. The results are shown as mean ± SEM (n = 3).

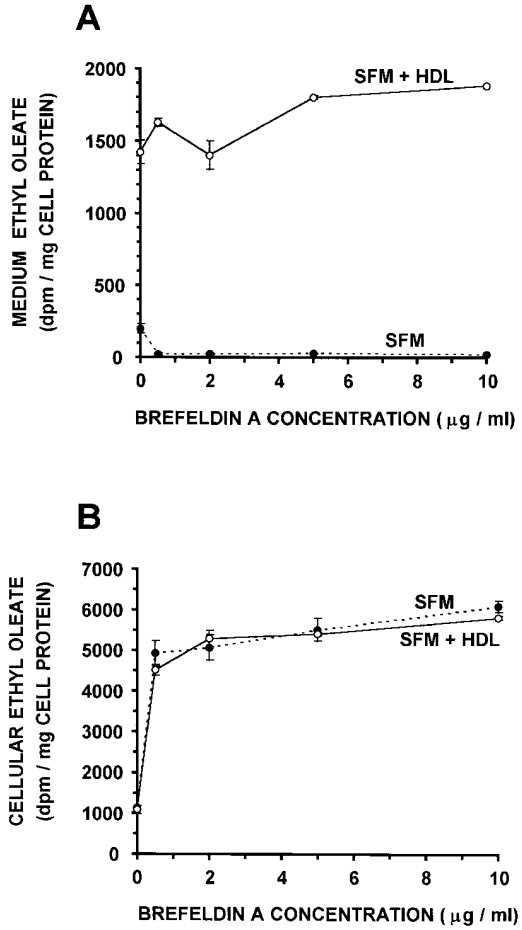

Fig. 12. Effect of brefeldin A on FAEE synthesis and release into the medium in the presence and absence of human HDL in the medium. Cell monolayers were incubated with 1.25 μm [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. At the end of this incubation period, the medium was removed, the cells were rinsed with PBS twice, and serum-free medium containing 100 mm ethanol and 0, 0.5, 2, 5, or 10 μg/ml brefeldin A in the presence (solid lines) or the absence (dashed lines) of human HDL (100 μg protein/ml) was added for 9 h. Ethyl oleate was isolated from the medium (A) and the cells (B) and quantified as described under Materials and Methods. When not showing, the error bars are within the symbols. The results are expressed as mean ± SEM (n = 3).

appear to maintain a certain amount of FAEE regardless of the amount of FAEE liberated into the medium. The results in Figs. 10 and 11A support this hypothesis.

The results in Fig. 12A support the hypothesis that FAEE are transported intracellularly by a mechanism independent of the vesicular transport pathway. Such transport may occur via fatty acid transfer proteins (FABP) which have been reported to transfer long chain fatty acids from either the plasma membrane or the site of de novo synthesis to other organelles (39, 40). To obtain direct evidence that FAEE are transported intracellularly by a cytosolic protein, the cells were labeled with [^{3}H]oleic acid for 12 h, the labeling medium was removed and the cells were rinsed, and serum-free medium containing 100 mm ethanol was added for 9 h. The cells were immediately harvested, lysed, and fractionated into cytosol and non-cytosol fractions by centrifugation. The cytosol was then subjected to gel filtration chromatography to determine the molecular mass of the protein which binds FAEE in the cytosol. The column eluates were extracted and their ethyl oleate content was isolated by TLC and the FAEE radioactivity was determined. As shown in **Fig. 13**, FAEE were predominantly associated with a protein with a molecular mass of 13–15 kDa, a mass similar to that of fatty acid binding proteins (41, 42). To demonstrate that FABP is expressed by HepG2 cells, Western blot analyses were performed using rabbit anti-rat L-FABP polyclonal antibody which is known to crossreact with human L-FABP. As shown in **Fig. 14**, the anti-L-FABP antibody reacted with a protein with a molecular mass of approximately 14 kDa in the cytosol of HepG2 cells, suggesting that L-FABP is present in the cytosol of HepG2 cells.

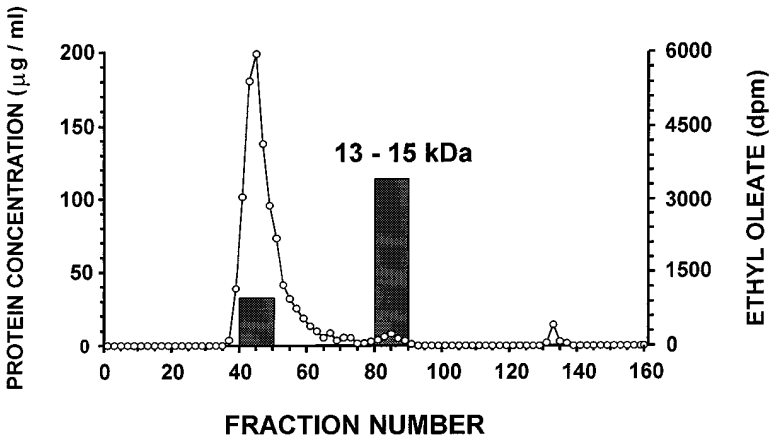

Fig. 13. Fractionation of the FAEE-containing cytosol of HepG2 cells by gel filtration chromatography. HepG2 cells in 35-mm petri dishes were incubated with 1.25 μm [^{3}H]oleic acid (17.5 μCi/ml) for 12 h at 37°C. The medium was removed, the cells were rinsed three times with PBS, and serum-free medium containing 100 mm ethanol was added for an additional 9 h. At this time point, the cells were harvested, homogenized, and the cytosol was isolated by ultracentrifugation. The cytosol (2 ml) was applied to a Sephadex G-75 column (1.5 × 80 cm, 4°C, 0.5 ml/min), and 1-ml fractions were collected. Protein concentration (open circles) was determined in the collected fractions. The fractions were then pooled (10 fractions/pool), extracted, and their ethyl oleate content was determined as described under Materials and Methods (bars).

DISCUSSION

HepG2 cells possess a number of normal liver cell functions such as the ability to synthesize and secrete apolipoproteins and major plasma proteins (43–45). Ethanol metabolism in HepG2 cells occurs primarily through a non-alcohol dehydrogenase (ADH) pathway because HepG2 cells possess low ADH activity (46). Therefore, this cell line represents a unique model of a liver cell for the study of the nonoxidative metabolism of ethanol to form FAEE because it does not present the confounding variable of significant ethanol oxidation to acetaldehyde via alcohol dehydrogenase. The results of the studies demonstrate that FAEE are synthesized and released from HepG2 cells exposed to ethanol. FAEE synthase was present in the cytosol and the microsomes of HepG2 cells. Both free fatty acid and fatty acyl-CoA were substrates for FAEE synthesis, although fatty acyl-CoA was a preferred substrate by the microsomal enzyme. FAEE were transported intracellularly by a mechanism independent of the vesicular transport pathway. FAEE released into the medium were associated with lipoproteins and albumin.

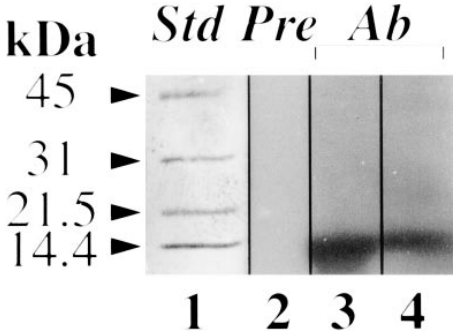

Fig. 14. Western blot analysis of L-FABP in the cytosol of HepG2 cells. Lane 1 represents the Coomassie blue-stained molecular weight standards. Cytosolic proteins (100 μg/lane) from rat liver (lanes 2, 3) and HepG2 cells (lane 4) were isolated by centrifugation, fractionated by SDS-PAGE (4–20%), and electrophoretically transferred to polyvinylidene difluoride membranes. Blots were probed with rabbit anti-rat L-FABP (1:1000 dilution) (lanes 3, 4) or preimmune rabbit serum (lane 2) for 2 h at room temperature. The blots were then washed, treated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) and a chemiluminescent substrate, and finally exposed to X-ray film for 10 sec.

Grigor and Bell (11) demonstrated that rat liver microsomes were able to synthesize FAEE upon exposure to ethanol using fatty acyl-CoA, but not free fatty acid, as a substrate. Treloar et al. (47) performed experiments assessing the synthesis of ethyl oleate by human liver microsomes using oleic acid or oleoyl-CoA as a substrate. A much higher rate (7328-fold) of ethyl oleate synthesis was found with oleoyl-CoA as a substrate. We also found (Fig. 4) that microsomal FAEE synthase prefers fatty acyl-CoA over free fatty acid for FAEE synthesis. Thus, there is significant evidence that the microsomal FAEE synthase (also called acyl-CoA:ethanol acyltransferase) greatly prefers fatty acyl-CoA as a substrate. It is important to note that in studies involving cell homogenates, the free fatty acid may be converted to fatty acyl-CoA, and vice versa, due to the presence of fatty acyl-CoA synthetase and fatty acyl-CoA hydrolase in the homogenate. In such experiments, therefore, it is impossible to conclusively identify the actual substrate for the microsomal and the cytosolic FAEE synthase. However, because the interconversion between fatty acid and fatty acyl-CoA usually involves only a minor fraction of the fatty acid or fatty acyl-CoA added to the homogenate and because there has been much greater synthesis of FAEE by the microsomal fraction using fatty acyl-CoA as a substrate in several studies (11, 47) (Fig. 4), there is now substantial evidence that FAEE are preferentially formed in microsomes using fatty acyl-CoA as a substrate. It should be noted that an FAEE synthase, which may represent a unique enzyme, has been purified to homogeneity from rabbit and human myocardium (13, 48). However, FAEE synthesis can be catalyzed by a variety of well-characterized enzymes such as cholesterol esterase (49), carboxylesterase (50), and lipoprotein lipase (51, 52). Additionally, FAEE synthase has been found to possess other catalytic activities (53). Furthermore, FAEE have been shown to rapidly hydrolyze into free fatty acids and ethanol when incubated with isolated rat mitochondria (7) or injected within the core of LDL particles into the circulation of rats (54). Therefore, one proposed mechanism for FAEE

toxicity has been that FAEE deliver free fatty acids to critical locations in the cell, such as the mitochondrial membrane, in an abundance not otherwise attainable. These free fatty acids have been reported to uncouple oxidative phosphorylation in mitochondria (7).

FAEE were detectable in the plasma after ethanol ingestion (15). These FAEE were found to be associated with lipoprotein particles and albumin. One source of FAEE in the circulation may be synthesis in the vascular compartment. Indeed, FAEE have been reported to be synthesized by white blood cells, particularly the lymphocyte-monocyte fraction (55), and by red blood cells (56). Alternatively, FAEE in the circulation may be a result of FAEE release from the liver because it has been found to have a high FAEE synthetic activity (6). Our results clearly indicate that FAEE are released from hepatocytes when incubated with ethanol. However, the contribution of FAEE liberated from the liver to the total amount present in the circulation is currently unknown. The data in Fig. 1 show that approximately 12% of the total FAEE were released into the medium after 9 h of incubation with ethanol. It should be noted that this amount represents the net result of continuous synthesis, release, and hydrolysis of FAEE during the incubation period. Further studies are required to determine the fate of the de novo synthesized FAEE that are not liberated into the medium.

FAEE have a hydrophobicity similar to triglycerides (57). For this reason, it was expected that FAEE would be bound to a lipid carrier in aqueous medium. We have previously reported that, after ethanol ingestion, FAEE in human serum are bound to lipoproteins and to a protein in the d $>$ 1.21 g/ml fraction, most likely albumin. In the current study, approximately 70% of FAEE in the medium were associated with a lipoprotein in the d 1.063–1.21 g/ml range which corresponds to the density of HDL. Thrift et al. (58) reported that HepG2 cells secrete LDL and HDL and very little VLDL. However, the lipid composition of these lipoproteins is somewhat different than that of human plasma lipoproteins. The particles in the d $<$ 1.063 g/ml range (this includes primarily LDL as the cells secrete very little VLDL) have an elevated unesterified cholesterol, minimal amounts of cholesteryl ester, and triglyceride as the major core lipid, unlike LDL in human plasma. The HDL particles (d 1.063–1.21 g/ml) produced by HepG2 cells differed significantly in composition from their plasma counterparts in that they possess, by comparison to human HDL, an elevated unesterified cholesterol and phospholipid, and an extremely low cholesteryl ester content. In addition, for HepG2 cells, apoA-I was the major apolipoprotein; apoE was the next most abundant apolipoprotein; and small quantities of apoA-II and apoC were also present. McCall, Forte, and Shore (59) also analyzed HDL particles (d 1.063–1.235 g/ml) isolated from HepG2 cells. Four subclasses were identified with different diameters and enrichment levels with apoA-I, apoA-II, and apoE. These subclasses of HDL appear to share many similarities with those isolated from patients with lecithin:cholesterol acyltransferase deficiency, in that they were rich in free cholesterol and extremely poor in cholesteryl ester (59). The unique structure of HDL secreted by HepG2 cells might account for the higher association of FAEE with the HDL versus LDL in the medium. FAEE have been found in the neutral lipid core of the lipoprotein particle (30). The core of HDL particle secreted by HepG2 cells is relatively unoccupied by cholesteryl ester and would, therefore, be expected to accommodate a considerable amount of FAEE. Very little FAEE were associated with VLDL, most likely because HepG2 cells secrete a low amount of VLDL particles (58).

It has been somewhat more problematic to determine whether the FAEE, once released into the culture medium, are bound specifically to albumin in the d $>$ 1.21 g/ml fraction or to a different protein. The results in Fig. 6B indicate that FAEE are able to bind to albumin, because in those experiments there were no other proteins added to the medium. Thus, although there may be other non-lipoprotein carriers of FAEE, albumin has been shown to be a carrier for FAEE. To further support the finding that albumin is a carrier for FAEE, we demonstrated that a protein of the molecular mass of albumin (66 kDa), in the medium of the HepG2 cells, bound FAEE which were released from cells (Fig. 7). The immunoprecipitation experiments provide direct evidence that FAEE bind to albumin in the cell culture medium. Ethyl oleate was detected when albumin was immunoprecipitated from the medium by rabbit anti-human albumin antibody (Fig. 8).

There are at least two likely mechanisms by which FAEE can move from their site of synthesis in the microsomes and/or the cytosol to the plasma membrane to be released to the extracellular environment. These are: *1*) vesicular transport and *2*) liver fatty acid binding protein (L-FABP) or related cytoplasmic protein-assisted transport. Although at the present time the relative contribution of each of these pathways toward the total amount of FAEE transport within cells could not be conclusively determined, three pieces of evidence suggest that L-FABP is involved in the intracellular movement of FAEE.

First, the association of FAEE with lipoprotein and albumin particles in the culture medium (Figs. 5, 7, and 8) initially suggested that FAEE may exit from cells incorporated in newly synthesized lipoprotein particles, or bound to albumin molecules. The results of the experiment with the inhibitors of the vesicular transport pathway (Fig. 9) supported this suggestion because protein and lipoprotein particles are known to be secreted by way of endoplasmic reticulum/Golgi secretory vesicles. However, the release of FAEE in these studies was into serum-free medium. Therefore, it was possible that the inhibition of FAEE liberation, when the cells were treated with compounds that disrupt the vesicular transport pathway, could have been a result of impaired secretion of lipoproteins and albumin particles required as acceptors for FAEE in the aqueous medium. Indeed, when HDL particles were exogenously provided as acceptors for FAEE in the culture medium, FAEE release was not interrupted by BFA even at high concentrations (Fig. 12A). Thus, it is likely that the association between FAEE and lipoproteins and albumin occurs in the culture medium. It should be noted

as a caveat that these findings do not discern whether FAEE, after exiting the cell, associate first with lipoproteins and then transfer to albumin molecules or vice versa. However, studies in our laboratory have shown that the transfer of FAEE between LDL particles and albumin is a rapid process (30). Additionally, we have demonstrated that the association of FAEE with lipoproteins versus albumin in the $d > 1.21$ g/ml is a concentration-dependent process. At low concentration of FAEE, these FAEE preferentially bind to albumin. However, with increasing FAEE concentration, the amount of FAEE bound to albumin decreases and that associated with lipoprotein particles increases (60). Thus, the association of FAEE with lipoproteins and albumin under our experimental conditions (after 9 h of incubation with ethanol) represents the equilibrium partitioning of FAEE among their carriers in the medium.

Second, while there was no FAEE liberation into the serum-free medium for the first hour of incubation with ethanol (Fig. 3B), FAEE were detectable in medium spiked with HDL within 15 min of incubation with ethanol (15 min was the first time point studied) (Fig. 11B). These findings suggest that the intracellular transport of FAEE, similar to the intracellular transport of free fatty acids (61), is a rapid process.

The transport of newly synthesized cholesterol from the sites of synthesis to the plasma membrane has been reported to be rapid ($t_{1/2}$ of approximately 10 min), independent of the Golgi apparatus, and ATP-dependent (62). If FAEE were primarily released from cells via this proposed mechanism, ATP depletion from cells should interrupt FAEE liberation to the medium. Depleting HepG2 cells from ATP by potassium cyanide did not significantly decrease the amount of FAEE in the medium when the medium was spiked with human HDL (data not shown). ATP depletion significantly decreased the efflux of FAEE into the serum-free medium, presumably because the vesicular transport of lipoprotein and albumin particles by way of Golgi requires ATP (63). Thus, it is unlikely that this proposed pathway is responsible for the bulk transport of FAEE to the plasma membrane. These results are consistent with our hypothesis that FAEE are transported to the plasma membrane by a cytoplasmic lipid carrier protein, as the transport of free fatty acids by fatty acid binding proteins to the plasma membrane is found to be ATP-independent (61).

Third, the association of FAEE in the cytosol of HepG2 cells with a 13–15 kDa protein (Fig. 13), combined with the detection of L-FABP in the same cytosol by Western blot analysis (Fig. 14) strongly suggests that L-FABP is involved in the intracellular transport of FAEE. L-FABP has been reported to play a role in the intracellular transport of long chain fatty acids (40), and it is possible that it also binds FAEE. Another 13–15 kDa protein which might participate in the cellular transport of FAEE is sterol carrier protein-2 (SCP-2). SCP-2 has been found to be involved in the intracellular transport of free fatty acids (64), fatty acyl-CoA (65), and cholesterol (66). Therefore, it is possible that SCP-2 is involved in the intracellular transport of FAEE. Although SCP-2 is a peroxisomal protein, it is possible that upon cell homogenization and disruption of peroxisomal membranes, SCP-2 is released into the cytosolic fraction of HepG2 cells. Thus, these findings suggest that FAEE are transported to the plasma membrane by a cytosolic lipid carrier protein, and then delivered onto lipoprotein and albumin molecules secreted via the vesicular transport pathway without the FAEE. It should be noted that these findings do not conclusively demonstrate that FAEE reside at the plasma membrane during the exiting process. Additional studies involving the isolation of the plasma membrane and the determination of the membrane diffusion constant for FAEE are necessary to address this question and provide useful information regarding the kinetics of FAEE partitioning between cellular membranes and different carriers.

Taken together, we have demonstrated that FAEE are formed by two pools of FAEE synthase in the cell, that the microsomal enzyme preferentially uses fatty acyl-CoA as a substrate with the cytosolic enzyme showing no such preference, that the intracellular transport of FAEE occurs by a mechanism independent of that used for the transport of newly synthesized proteins, and that the primary lipid carrier of FAEE upon release from the cell is lipoprotein, with albumin also serving as a significant carrier of FAEE.

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