Mitochondrial glycerol-3-phosphate acyltransferase-1 directs the metabolic fate of exogenous fatty acids in hepatocytes

Tal M. Lewin,* Shuli Wang,* Cynthia A. Nagle, Cynthia G. Van Horn, and Rosalind A. Coleman

Department of Nutrition, University of North Carolina, Chapel Hill, North Carolina

Submitted 8 July 2004; accepted in final form 4 December 2004

Lewin, Tal M., Shuli Wang, Cynthia A. Nagle, Cynthia G. Van Horn, and Rosalind A. Coleman. Mitochondrial glycerol-3-phosphate acyltransferase-1 directs the metabolic fate of exogenous fatty acids in hepatocytes. Am J Physiol Endocrinol Metab 288: E835–E844, 2005—Because excess triacylglycerol (TAG) in nonadipose tissues is closely associated with the development of insulin resistance, interest has increased in the metabolism of long-chain acyl-CoAs toward β-oxidation or the synthesis and storage of TAG. To learn whether a mitochondrial isoform of glycerol-3-phosphate acyltransferase (mtGPAT1) competes with carnitine palmitoyltransferase I (CPT I) for acyl-CoAs and whether it contributes to the formation of TAG, we overexpressed rat mtGPAT1 13-fold in primary hepatocytes obtained from fasted rats. When 100, 250, or 750 μM oleate was present, both TAG mass and the incorporation of [14C]oleate into TAG increased more than twofold in hepatocytes overexpressing mtGPAT1 compared with vector controls. Although the incorporation of [14C]oleate into CO2 and acid-soluble metabolites increased with increasing amounts of oleate in the media, these metabolites were ~40% lower in the Ad-mtGPAT1 infected cells, consistent with competition for acyl-CoAs between CPT I and mtGPAT1. A 50–60% decrease was also observed in [14C]oleate incorporation into cholesteryl ester. With increasing amounts of exogenous oleate, [14C]TAG secretion increased appropriately in vector control-infected hepatocytes, suggesting that the machinery for VLDL-TAG biogenesis and secretion was unaffected. Despite the marked increases in TAG synthesis and storage in the Ad-mtGPAT1 cells, however, the Ad-mtGPAT1 cells secreted the same amount of [14C]TAG as the vector control cells. Thus, in isolated hepatocytes, mtGPAT1 may synthesize a cytosolic pool of TAG that cannot be secreted.

hepatic triacylglycerol; hepatic fatty acid oxidation

Previously believed to be a benign condition, the presence of excess triacylglycerol (TAG) in liver, skeletal muscle, and other nonadipose tissues is now considered a hallmark of insulin resistance. Although TAG itself may not contribute to insulin resistance, the hydrolysis of TAG to diacylglycerol (DAG) and fatty acids may trigger signaling cascades, ceramide production, and acyl-CoA accumulation. Because one or more of these downstream molecules are believed to promote insulin resistance (41), interest is growing in the metabolic pathways that lead to TAG deposition in tissues. In liver, for example, exogenous fatty acid may either be degraded via β-oxidation or enter synthetic pathways that include the esterification of cholesterol and glycerol-3-phosphate to form cholesterol esters and glycerolipids, respectively. Indirect evidence suggests that channeling of fatty acid toward a pathway of degradation or one of synthesis depends on its initial activation and esterification steps (12, 13). One of these initial steps is catalyzed by glycerol 3-phosphate acyltransferase (GPAT; EC 2.3.1.15), which uses long-chain acyl-CoAs to esterify the sn-1 position of glycerol 3-phosphate to form lysophosphatidic acid. The GPAT esterification step constitutes the committed reaction in the de novo synthesis of TAG and all the acyl-glycerophospholipids.

Three GPAT isoforms have been characterized in mammalian tissues, N-ethylmaleimide (NEM)-sensitive microsomal and mitochondrial (11, 30) isoforms that have not been purified or cloned, and an NEM-resistant mitochondrial isoform (mtGPAT1), which is a member of the gpam family of lipid acyltransferases (11, 12). Hepatic mtGPAT1 is believed to play an important role in hepatic TAG synthesis because its mRNA and protein are upregulated when fasted mice are refe2(28) and when insulin is provided to streptozotocin-diabetic mice (40). In contrast, the specific activity of the microsomal GPAT isoform does not appear to change with nutritional alterations but does increase markedly during the differentiation of 3T3-L1 adipocytes and in postnatal liver (8, 10). mtGPAT2 activity is not apparent in normal mouse liver (30).

In hepatocytes, newly synthesized TAG can be either stored in cytosolic droplets or secreted in very-low-density lipoprotein (VLDL) particles after assembly with apolipoprotein B (apoB), phosphatidylcholine, and cholesterol esters (CE). The formation and secretion of VLDL from cultured hepatocytes require ongoing TAG synthesis (17). If TAG is not cotranslationally available for association with newly forming apoB, apoB synthesis pauses, and the partially formed apoB is ubiquinated and then degraded in proteasomes or by proteasomal mechanisms (1, 17).

Although numerous studies strongly suggest that the mtGPAT1 isoform initiates the synthesis of TAG, this concept is somewhat surprising, because mtGPAT1 is an intrinsic protein of the outer mitochondrial membrane, whereas the enzymes that catalyze the final steps of TAG synthesis are located in the endoplasmic reticulum (ER) (11). Thus mitochondrial produced lysophosphatidate, the product of mtGPAT1, and/or phosphatidate produced by the subsequent 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) acylation step must be transported to the ER to complete the synthesis of TAG. We have hypothesized that mtGPAT1, which is located in the outer mitochondrial membrane, competes with CPT I, the rate-limiting step in β-oxidation, for acyl-CoAs (36). If this were true, an increase in mtGPAT1 activity should divert acyl-CoAs away from oxidation and toward TAG synthesis. To test this hypothesis directly, we used an adenovirus construct to over-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Cytoscint (ICN Biochemicals) and subjected to scintillation counting to determine the amount of $^{14}$C动生成. The acidified medium was centrifuged in a microfuge for 10 min twice to remove particulate matter. Two hundred microliters of the supernatant were added to Ecolite (ICN) and subjected to scintillation counting to determine the amount of $^{14}$C生成. The acidified medium was centrifuged in a microfuge for 10 min twice to remove particulate matter. Two hundred microliters of the supernatant were added to Ecolite (ICN) and subjected to scintillation counting to determine the amount of $^{14}$C-generated ASM. The acidified medium was centrifuged in a microfuge for 10 min twice to remove particulate matter. Two hundred microliters of the supernatant were added to Ecolite (ICN) and subjected to scintillation counting to determine the amount of $^{14}$C-generated ASM.

**Isolation of subcellular fractions.** Twelve 100-mm dishes (4 × 10⁶ hepatocytes/plate) were infected with 5 pfu/cell Ad-mtGPAT1 or Ad-GFP for 18 h. Cells were washed twice with ice-cold PBS, scrubbed into 1.5 ml/dish isolation medium (250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EGTA, 0.1% BSA), and homogenized with 10 up-and-down strokes with a Teflon-glass motor-driven homogenizer. Debris and nuclei were removed by centrifugation at 600 g for 5 min. The supernatant was centrifuged at 10,300 g for 10 min to collect the crude mitochondrial pellet. This pellet was resuspended by homogenizing in 1.2 ml of isolation medium and then layered over 30% (vol/vol) Percoll in isolation medium (20 ml). The Percoll gradient was allowed to form by centrifugation at 95,000 g for 30 min. The supernatant fraction over the crude mitochondrial pellet was centrifuged at 95,000 g for 30 min to collect microsomes. Four membrane-containing fractions (3–4 ml each) were collected from the Percoll gradient (Percoll fractions 1–3 and mitochondria), diluted in isolation medium, and centrifuged at 10,000 g for 10 min. The pellets were suspended in 10 ml of medium I (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT) and recentrifuged. The Percoll

---

**EXPERIMENTAL PROCEDURES**

**Materials.** Minimal essential medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), DMEM with high glucose (DMEM-H), nonessential amino acids (NEAA), HEPES buffer solution, and fetal bovine serum (FBS) were obtained from GIBCO-BRL Life Technologies. Collagen type I was from Collaborative Biomedical Products. Albumin (initial fractionation by heat shock, essentially fatty acid free), oleate, carnitine, and dexamethasone were purchased from Sigma. [1-14C]oleate was obtained from PerkinElmer Life Sciences. DNA restriction endonucleases and ligase for recombinant adenovirus construction were purchased from New England Biolabs. HEK-293 cells were obtained from the American Type Culture Collection. Lipid standards were from Sigma and Avanti Polar Lipids. Construction of recombinant mtGPAT1-Flag adenovirus. Shuttle vector pAdTrack-CMV, which expresses green fluorescent protein (GFP), was used to produce mtGPAT1 adenoviruses. The primers for amplification of rat mtGPAT1 cDNA were designed to include the COOH-terminal Flag epitope (DYKDDDDK) and specific restriction sites. The upper primer was 5'-AGTATGACAATACATGUGG-3', lower primer was 5'-GTCTTAGCTTGACATCGTCTGAG-3'. The PCR-derived fragment and pAdTrack-CMV were digested with XhoI, ligated, and verified to make certain that the direction of the inserted GPAT-Flag cDNA was correct. The pAdTrack/mtGPAT1-Flag construct was sequenced at the University of North Carolina (UNC) DNA sequencing facility. Expression and activity of pAdTrack/mtGPAT1-Flag were confirmed by transfection into HEK-293 cells, followed by anti-Flag immunoblot and GPAT assay. Generation of recombinant adenovirus by homologous recombination was performed by the UNC Vector Core Facility.

**Isolation of hepatocytes.** Animal protocols were approved by the UNC Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (200 g) were housed on a 12:12-h light-dark cycle. Rats were fed normally or fasted for 16 h before hepatocyte isolation. Primary hepatocytes were isolated by collagenase perfusion at the UNC Advanced Cell Technologies and Tissue Engineering Core. The cells were isolated and suspended in cold DMEM containing 10% FBS on ice.

**Hepatocyte culture and adenovirus infection.** Primary rat hepatocytes were seeded at a density of 1.5 × 10⁶ cells/60 mm collagen-coated dish and grown in DMEM-H medium supplemented with 10% (vol/vol) FBS for 5 h in 5% CO₂ before infection with adenovirus. Cells were not used if fewer than 70% attached to the dish. The medium was removed from the dish and covered with 1 ml of fresh DMEM-H containing 5 pfu/cell (total 7.5 × 10⁶ virus particles/1.5 × 10⁷ cells) of adenovirus (Ad-GFP or Ad-mtGPAT1). The hepatocytes were incubated with the infection medium for 2 h, after which it was removed, and 2 ml of incubation medium (DMEM-H, 10% FBS, 0.5% BSA, 1 mM carnitine, 10 mM HEPES, pH 7.4, 10 μM dexamethasone, and 10 mM NEAA) was added. Infection was allowed to proceed for an additional 16 h.

**Cell labeling and lipid analysis.** Bovine serum albumin (BSA)-conjugated oleate was prepared by dissolving Na oleate (C18:1, Sigma Chemical) in 2.5% BSA (essentially fatty acid free) at a concentration of 3.75 mM and heating at 65°C until the Na oleate dissolved completely. Then [1-14C]oleic acid (5 μCi) dissolved in ethanol (PerkinElmer Life and Analytical Sciences) was dried with a stream of N₂ gas, resuspended in DMEM-H culture medium, combined with the unlabeled Na oleate solution, and added to DMEM medium to give a final concentration of 100, 250, or 750 μM. Hepatocytes were infected for 2 h as described above, and then 100, 250, or 750 μM [14C]oleic acid (500,000–750,000 dpm in 2 ml) was added together with the incubation medium. Sixteen hours later, the labeling medium was removed and centrifuged to remove floating cells. Media lipids were extracted (6) and concentrated in a SpeedVac concentrator. To obtain cell lipids, hepatocyte cultures were washed with 1 ml of cold 0.9% NaCl containing 10 mg BSA and then with 2 ml of cold phosphate-buffered saline and scraped in 2 ml of cold methanol and 0.5 ml of H₂O. Lipids were extracted and concentrated as described above. Neutral lipids were resolved on LK6 silica plates (Whatman) by thin-layer chromatography using a solvent system consisting of hexane-ethyl ether-acetic acid (80:20:2, vol/vol). All samples were chromatographed in parallel with pure lipid standards. The 14C-labeled lipids were detected and quantified with a Bioscan 200 Image System.

**Quantification of lipids.** Unlabeled oleate was conjugated with BSA as described above. Hepatocytes were infected for 2 h as described above, and then 100, 250, or 750 μM of unlabeled oleate was added together with incubation medium. Medium and cell lipids were extracted as described above. TAG mass was determined using an enzymatic colorimetric method (Staibio Laboratory, Boerne, TX). Following the manufacturer’s instructions except for the sample preparation, in which 100 μl of total cell lipids or 200 μl of media lipids were dried in a SpeedVac concentrator and dissolved in 30 μl of isopropyl alcohol containing 1% Triton X-100. Phosphatidylcholine and sphingomyelin mass were determined by HPLC (27). For cholesterol content, lipids were extracted (18) and subjected to alkaline hydrolysis in 10% KOH in methanol for 48 h at room temperature. Free and total cholesterol were determined using enzymatic colorimetric assays (Free Cholesterol E and Cholesterol CII, Wako Chemical). CE was determined by subtracting free cholesterol from total cholesterol.

**β-Oxidation.** Hepatocytes were infected and labeled with 100, 250, or 750 μM [14C]oleic acid (500,000–750,000 dpm in 2 ml) as described above. The labeling medium was removed 16 h after label was added and centrifuged to remove floating cells. [14C]oleate oxidized to CO₂ and acid-soluble metabolites (ASM) was measured (36). Although we did not use closed flasks for our CO₂ measurements, the percentage of [14C]CO₂ that we measured per total [14C]oleate oxidized was similar to that in other studies that used closed flasks (3, 16). Briefly, CO₂ was driven from 1 ml of medium by adding 200 μl of 70% perchloric acid and trapped on a suspended filter wick (Kontes) saturated with NaOH. Wicks were placed in Cytoscient (ICN Biochemicals) and subjected to scintillation counting to determine the amount of [14C]CO₂ generated. The acidified medium was centrifuged in a microfuge for 10 min twice to remove particulate matter. Two hundred microliters of the supernatant were added to Ecolite (ICN) and subjected to scintillation counting to determine the amount of 14C-labeled ASM generated. The acidified medium was centrifuged in a microfuge for 10 min twice to remove particulate matter. Two hundred microliters of the supernatant were added to Ecolite (ICN) and subjected to scintillation counting to determine the amount of 14C-labeled ASM generated.

---

**E836** *mtGPAT1 REGULATES HEPATIC FATTY ACID METABOLISM*
fractions 1–3, mitochondrial, and microsomal pellets were resuspended by homogenization in medium I and stored in 100-μl aliquots at −80°C. The supernatant fraction over the microsomal pellet was saved as the cytosolic fraction. Purity of the subcellular fractions was established by measuring the activity of marker enzymes, NADH cytochrome c reductase (14) and cytochrome c oxidase (Cytochrome C Oxidase Kit, Sigma), for ER and mitochondria, respectively.

Isolation of total membrane fractions. Uninfected, Ad-GFP infected, or Ad-mtGPAT1 infected hepatocytes were washed with cold PBS, scraped into medium I, and homogenized with 10 up-and-down strokes in a Teflon-glass motor-driven homogenizer. The total membrane fraction was obtained by centrifuging at 100,000 g for 1 h. The total membrane pellet was rehomogenized in medium I and stored in 100-μl aliquots at −80°C for enzyme assay.

Immuno blotting. Proteins (60 μg) from the total membrane fraction were separated by electrophoresis on an 8% polyacrylamide gel containing 1% SDS, transferred to a PVDF membrane (Bio-Rad), and incubated with antibody against the Flag epitope (M2 anti-flag monoclonal antibody, Sigma). For chemiluminescent detection, the immuno reactive bands were visualized by incubating the membrane with horseradish peroxidase-conjugated goat anti-mouse IgG and PicoWest reagents (Pierce). For subcellular fractions, 20 μg of subcellular and Percoll fractions were immuno blotted for the Flag epitope or voltage-dependent ion channel (Anti-VDAC1, Abcam), as described above.

Enzyme assays. sn-2-[3H]glycerol-3-phosphate was synthesized enzymatically from [2-3H]glycerol (1 mM/ml) and purified and assayed as described previously (7). GPAT specific activity was assayed at room temperature in a 200-μl mixture containing 75 mM Tris-HCl, pH 7.5, 4 mM MgCl2, 1 mg/ml BSA (essentially fatty acid free), 1 mM DTT, 8 mM NaF, 800 μM [3H]glycerol-3-phosphate, and 80 μM palmityl-CoA (10). The reaction was initiated by adding 5–20 μg of total membrane protein to the assay mix after incubation for 15 min on ice in the absence or presence of 1 mM NEM. mtGPAT1 activity is calculated as the activity that is uninhibited by NEM.

[14C]oleoyl-CoA was synthesized enzymatically from [1-14C]oleate (0.1 mCi/ml) (45). ACAT specific activity was assayed in a 200-μl mixture containing 50 μg of total membrane protein, medium I, and 1.67 μg/ml BSA, as described previously (42). The protein mixture was preincubated at 37°C for 5 min in the absence, or 50 min in the presence, of 8 μg of cholesterol (phosphatidylcholine-cholesterol, 8:1 by weight). The assay was started by adding 25 μM [14C]oleoyl-CoA (38,000 dpm/nmol). After incubation at 37°C for 6 min, the reaction was stopped by adding 3 ml of chloroform-methanol (2:1), followed by 1 ml of H2O for Folch lipid extraction (18). The organic phase (2 ml) was dried down in a SpeedVac concentrator, and the lipids were resuspended in 50 μl of 0.5 mg/ml cholesteryl oleate in chloroform. The entire sample was spotted onto an LK6D silica gel plate (Whatman, and neutral lipids were resolved as described above. The 14C-labeled CE was detected and quantified using a Bioscan Image System.

Other methods. Protein concentrations were determined by the bicinchoninic acid method (Pierce) using BSA as the standard. Data are presented as means ± SD. Significant differences between the Ad-mtGPAT1 infected hepatocytes and Ad-GFP-infected controls were analyzed by two-tailed Student’s t-test.

RESULTS

Adenoviral mediated overexpression of mtGPAT1 in hepatocytes increases NEM-resistant GPAT activity. Mitochondrial GPAT activity comprises 30–50% of the total GPAT activity measured in rat liver (11), but its contribution to the synthesis of cellular phospholipid and TAG and to VLDL-TAG is not known. We infected primary rat hepatocytes with adenovirus containing GFP (Ad-GFP) or with adenovirus containing both GFP and mtGPAT1-Flag (Ad-mtGPAT1). Uninfected hepatocytes provided an additional control. NEM-resistant GPAT specific activity (mtGPAT1) began to increase 12 h after infection with Ad-mtGPAT1 and was maximal by 18 h (Fig. 1A). At 18 h, infection with Ad-mtGPAT1 increased NEM-resistant specific activity 13-fold compared with uninfected or Ad-GFP controls (Fig. 1B). No change was observed in microsomal (NEM-sensitive) GPAT activity. To verify that overexpressed mtGPAT1-Flag was targeted to mitochondria, we obtained microsomal and mitochondrial fractions from Ad-GFP infected hepatocytes (Table 1). Immunoblotting of these fractions with antibody against the Flag epitope revealed that mtGPAT1 was present primarily in mitochondria but was also present in an ER fraction (Fig. 1C). Although little of the mitochondrial marker enzyme cytochrome oxidase was present in the microsomal fraction (Table 1), the presence of VDAC, an outer mitochondrial membrane marker, in the microsomal fraction (Fig. 1C) clearly indicates that the microsomal fraction was contaminated with broken mitochondrial outer membrane. VDAC appears to be equally distributed in the microsomal and mitochondrial fractions, consistent with the distribution of NEM-resistant GPAT activity (total activity: microsomal, 3.47 nmol/min; mitochondria, 5.00 nmol/min). Although not conclusive, these data suggest that overexpressed mtGPAT1-Flag is properly localized to the mitochondrial outer membrane.

Overexpression of mtGPAT1 decreases fatty acid oxidation. Because both mtGPAT1 and CPT I are located on the outer mitochondrial membrane, it seemed that mtGPAT1 might play a significant role in liver in directing the fate of exogenous fatty acids toward glycerolipid synthesis and away from fatty acid oxidation. To test this hypothesis, we used hepatocytes from fasted rats to determine whether increasing mtGPAT1 activity would affect their high oxidation rates. When primary hepatocytes from fasted rats were either uninfected or infected with Ad-GFP or Ad-mtGPAT1 and incubated with [14C]oleate, a 13-fold overexpression of mtGPAT1 resulted in decreased oxidation of [14C]oleate to ASM and CO2. With 100, 250, or 750 μM [14C]oleate, incorporation of label into ASM decreased 95, 50, and 53%, respectively, compared with controls (Fig. 2B). Although ASM is considered a more accurate measure of β-oxidation (47), we also measured [14C]oleate oxidation to CO2. At 100, 250, or 750 μM [14C]oleate, labeled CO2 decreased 72, 60, and 40%, respectively (Fig. 2A). Similar results were obtained for hepatocytes from fed rats incubated with either 250 or 750 μM oleate; at each oleate concentration, ASM and CO2 each decreased ~60% (data not shown). As previously reported (34), hepatocytes obtained from fasting rats incorporate less [14C]oleate into TAG and more into ASM than do hepatocytes from fed rats. When hepatocytes from fasted rats were incubated with 250 μM oleate, the ratio of [14C]oleate incorporation into TAG:ASM was 1.0, whereas the ratio in hepatocytes from fed rats was 2.9. These data show that, although we incubated the fasted hepatocytes in 10% serum and 25 mM glucose, the resulting metabolic profile was similar to that reported for fasted hepatocytes that had been incubated without serum and in low glucose (34).

The present studies show that, as a percentage of total oleate incorporation, CO2 plus ASM increased from 7% at 100 μM oleate to 21% at 250 μM oleate in control Ad-GFP-infected cells (Fig. 3). Although with increasing exogenous fatty acid one expects an increase in fatty acid oxidation because of decreased malonyl-CoA inhibition of CPT I, the percentage of
[14C]oleate oxidized to CO2 plus ASM is the same with both 250 and 750 μM oleate. This result is similar to our previous observation that conversion of [14C]oleate to [14C]oxidation products is linear from 0 to 250 μM oleate but reaches a plateau at higher concentrations (34). Consistent with these findings is our result that overexpressing mtGPAT-1 inhibits 96% of the incorporation of [14C]oleate into CO2 plus ASM at 100 μM oleate but inhibits only 66% at 250 or 750 μM oleate (Fig. 3).

This reduction in mtGPAT-1-mediated inhibition of [14C]oxidation would be expected because of the increased competition from CPT I at higher fatty acid concentrations. Despite the increase in labeled oxidation products that depends on exogenous fatty acid, the relative percentage of oxidation at all oleate concentrations decreased with the addition of mtGPAT1 (Figs. 2 and 3). These results suggest that mtGPAT1 competes with CPT I to direct exogenous fatty acids away from [14C]oxidation.

Overexpression of mtGPAT1 increases cell, but not media, TAG. We previously showed in Chinese hamster ovary (CHO) cells that a 3.8-fold increase in mtGPAT1 activity increases TAG content 2.7-fold and increases [14C]oleate incorporation 3.4-fold (25). In the Ad-GPAT1-infected hepatocytes, a 13-fold increase in GPAT activity resulted in 2.4-, 3-, and 2.4-fold increases in TAG mass at 100, 250, and 750 μM oleate, respectively (Fig. 4A). When hepatocytes prepared from fasted rats were incubated with [14C]oleate, [14C]TAG in uninfected and vector control cells increased sixfold with 750 μM oleate compared with 100 μM oleate, and infection with Ad-mtGPAT1 further increased [14C]oleate incorporation into cellular TAG 2.4- to 2.7-fold compared with the Ad-GFP controls at each fatty acid concentration (Fig. 4B). As expected, the pattern for [14C]oleate incorporation into cellular DAG paralleled that for TAG, although DAG values were always ~10% of TAG values (Fig. 4C). As a percentage of total [14C]oleate incorporation, labeled TAG plus DAG increased from 56% in Ad-GFP cells to 69% in Ad-GPAt cells with 250 μM oleate (Fig. 3). Similar increases were observed with the 750 μM oleate incubation; labeled TAG plus DAG increased from 66% in Ad-GFP cells to 82% in Ad-GPAT cells.
These data support the hypothesis that mtGPAT1 enhances the incorporation of exogenous fatty acid into TAG, even in hepatocytes from fasted rats.

In hepatocytes, newly synthesized TAG can be stored in cytosolic droplets or assembled together with apoB, phosphatidylcholine, and CEs to form VLDL particles. Because it remains unclear whether newly synthesized TAG or TAG previously stored in cytosolic droplets is preferentially secreted from liver (20), we determined whether an increase in TAG synthesis would be reflected by an increase in secreted TAG. The effect of increased availability of exogenous oleate was apparent in the 7.9- and 37.6-fold increases in labeled media TAG secreted from cells incubated with 250 or 750 μM oleate compared with 100 μM 14C-oleate (Fig. 4D). However, although overexpression of Ad-mtGPAT1 increased 14C-oleate incorporation into cell TAG 2.4- to 2.7-fold, incorporation into media TAG was identical for Ad-mtGPAT1 and control cells. In hepatocytes from fed rats, overexpression of GPAT did not increase media TAG more than the vector control, although the total incorporation of oleate into cell and media TAG increased two- to fourfold compared with hepatocytes from fasted rats (data not shown), reflecting greater synthesis of TAG after a meal. Because exogenous fatty acids stimulate the formation and secretion of apoB by primary mouse hepatocytes (31), we considered the possibility that either the oleate concentration or the cellular machinery required to assemble and secrete VLDL was limiting. It was clear, however, in the two controls that sufficient oleate was present: both cell and media 14C-TAG increased in direct proportion to the exogenous oleate concentration, whereas overexpression of mtGPAT increased label incorporation only into cellular 14C-TAG. Furthermore, VLDL assembly and secretion appeared sufficient: 750 μM oleate led to fivefold more media 14C-TAG than 250 μM oleate (Fig. 4D), demonstrating that, when additional exogenous fatty acid was available, Ad-mtGPAT1 infected cells could secrete more TAG. In fact, at 250 μM oleate, the amount of 14C-TAG present in control cell and medium were each ~50 nmol/1.5 × 10⁶ cells and at 750 μM oleate, each was ~120 nmol/1.5 × 10⁶ cells. In contrast, at 250 and 750 μM oleate, the Ad-mtGPAT1 cell TAG was ~80 and 400 nmol, respectively, and should have resulted in more secreted 14C-TAG. It appears that, despite an unimpaired ability to assemble and secrete VLDL, the excess synthesis of TAG initiated by mtGPAT1 could not be secreted.

In Ad-GPAT-infected hepatocytes 14C-oleate incorporation into cellular phospholipids increased 30–60% compared with vector control cells (Fig. 4E) but did not change as a percentage of total label incorporation (Fig. 3). Phosphatidylcholine and sphingomyelin mass did not change (Table 2), suggesting that overexpression of mtGPAT1 did not alter the relative amount of phospholipid in cells and that impaired synthesis of phosphatidylcholine did not contribute to the diminished secretion of VLDL-TAG by the mtGPAT1 cells (50).

Overexpression of mtGPAT1 decreases CE synthesis. When the amount of exogenous 14C-oleate increased from 250 to 750 μM, incorporation into CE increased 2.5-fold in vector control cells. A 13-fold overexpression of mtGPAT1, however, resulted in a marked decrease in the incorporation of 14C-oleate into cellular CE. At 100, 250, and 750 μM oleate, the amount of 14C-CE in the cells decreased 84, 60, and 30%, respectively, compared with controls (Fig. 5A). Similarly, although increasing exogenous oleate raised the amount of secreted 14C-CE proportionally, the presence of Ad-mtGPAT1 severely blocked 14C-oleate incorporation into media CE at 100 μM oleate and diminished it 50–60% at concentrations of 250 and 750 μM oleate (Fig. 5B). With hepatocytes obtained from fed rats, similar decreases in 14C-oleate incorporation into cell and media CE were observed at 250 and 750 μM (data not shown). Because the decrease in label incorporation into
cell CE diminished as the exogenous olate concentration increased, it appeared that olate might be limiting for CE secretion, possibly because mtGPAT1 diverted fatty acyl-CoA away from ACAT. Arguing against limiting olate, however, is the fact that media $[^{14}C]$CE as a percentage of total $[^{14}C]$olate incorporation were essentially identical (13 and 19%) at both 250 and 750 M olate (Fig. 3).

To understand why CE synthesis was diminished, we measured ACAT activity in total membrane fractions. ACAT activity depends on the availability of both the amount of labeled olate and the amount of cholesterol substrate present in the ER. In either the absence or the presence of cholesterol added to the assay mixture, ACAT activity in Ad-mtGPAT1-infected hepatocytes was unchanged compared with the two controls (Fig. 6), and the amount of free cholesterol in the cell was identical in uninfected (data not shown), Ad-GFP, and Ad-mtGPAT1 infected cells (Table 2). As would be expected, hydroxymethylglutaryl (HMG)-CoA reductase specific activity was similar (62–67 pmol·min$^{-1}$·mg$^{-1}$·protein) in membranes from all treatments. If the cholesterol content in the ER had decreased, HMG-CoA reductase specific activity should have increased due to increased nuclear content of sterol regulatory element-binding protein-2 (SREBP-2) and the resulting upregulation of genes involved in the pathway of cholesterol synthesis.

Fig. 4. Ad-mtGPAT1 increases incorporation of $[^{14}C]$olate into cell but not media triacylglycerol (TAG). Hepatocytes were treated as described in the legend for Fig. 2 and incubated with 100, 250, or 750 M $[^{14}C]$olate as indicated. A: cell TAG mass was measured by colorimetric assay. $[^{14}C]$olate incorporated into cell TAG (B), cell diacylglycerol (DAG; C), media TAG (D), and cell PL (E) was determined as described in EXPERIMENTAL PROCEDURES. Data for B–D are means of experiments performed 3 times in triplicate. Data for A at 250 and 750 M olate and for E at 100 and 250 M olate are means ± SD of experiments performed 3 times in triplicate. Data for A at 100 M olate and for E at 750 M olate are averages of experiments performed 2 times in triplicate. *$P \leq 0.05$, **$P < 0.01$, ***$P = 0.005$. 

**AJP-Endocrinol Metab • VOL 288 • MAY 2005 • www.ajpendo.org**
study strongly suggested that mtGPAT1 directs fatty acid \([^{14}C]\)oleate incorporation into TAG 3.4-fold. Although that activity in CHO cells increases TAG content 2.7-fold and change minimally, and mtGPAT2, the third isoform, is not.

**DISCUSSION**

GPAT activity in cells is comprised of at least three isoforms encoded by different genes (11, 30). The independent functions of these isoforms have remained unclear, but studies of mtGPAT1 mRNA under different physiological conditions suggest that mtGPAT1 contributes specifically to TAG synthesis. For example, liver mtGPAT1 mRNA abundance is downregulated under conditions where TAG synthesis declines, as in fasting and streptozotocin-diabetes, whereas mRNA expression increases 19-fold 6 h after streptozotocin-diabetic mice are injected with insulin (40). Furthermore, when mice are fasted for 24 h and then refed a high-carbohydrate, fat-free diet for 8 h, mtGPAT1 mRNA increases 20-fold, and this increase is blocked 70% by injecting the animals with dibutyryl-cAMP (40). mtGPAT1 is also upregulated by SREBP-1c, a powerful inducer of lipogenic genes (15). Livers from mtGPAT1 null mice contain 37% less TAG than livers from wild-type mice, consistent with the observation that the mitochondrial isoform comprises 30–50% of total hepatic GPAT activity (22).

Acutely, mtGPAT1 specific activity is inhibited 40% by AMP-activated protein kinase (36), a kinase that is active under conditions when cellular energy stores are low and synthetic pathways such as cholesterol and TAG synthesis are downregulated (26). In contrast to the responsiveness of mtGPAT1 to lipogenic stimuli, the microsomal GPAT activity appears to change minimally, and mtGPAT2, the third isoform, is not normally expressed in liver (30).

We (25) have shown that a 3.8-fold increase in mtGPAT1 activity in CHO cells increases TAG content 2.7-fold and \([^{14}C]\)oleate incorporation into TAG 3.4-fold. Although that study strongly suggested that mtGPAT1 directs fatty acid toward TAG synthesis, CHO cells provide limited information because their oxidation rate is low and exogenous fatty acids are primarily metabolized to glycerolipids. Therefore, to determine whether mtGPAT1 would alter pathways of fatty acid oxidation and TAG secretion, we overexpressed mtGPAT in primary hepatocytes obtained from fasted rats to maximize the cells’ oxidative capacity.

Liver from mtGPAT1 null mice contains 40% less TAG mass than that from wild-type mice (22), whereas TAG mass and label incorporation into TAG increase in CHO cells when mtGPAT1 is stably overexpressed (25). Thus we expected that overexpression of mtGPAT1 in rat hepatocytes would similarly result in an increase in TAG mass and in the incorporation of labeled fatty acid into TAG. These increases occurred as predicted. In addition, we wanted to determine whether high mtGPAT1 activity would decrease β-oxidation. We found that label incorporation into CO₂ and ASM decreased by at least 40%. When mtGPAT1 was overexpressed, decreases occurred at each fatty acid concentration provided to hepatocytes obtained from both fasted and fed animals. Thus neither the availability of fatty acid nor the physiological attributes of the cells played a determining role, and the percent decrease in CO₂ plus ASM was identical at 250 and 750 μM oleate, consistent with the hypothesis that the presence of mtGPAT1 activity on the outer mitochondrial membrane competes with CPT I for acyl-CoAs and diverts long-chain acyl-CoAs toward TAG synthesis and away from β-oxidation.

Although a decrease in ASM production in rat hepatocytes that overexpress mtGPAT1 has been recently reported (32), our detailed study reveals additional novel findings. Measuring the effect of overexpressed mtGPAT1 at three different physiological concentrations of exogenous fatty acids, we found that mtGPAT1 overexpression also markedly increased the incorporation of \([^{14}C]\)oleate into TAG. The incorporation of \([^{14}C]\)oleate into TAG was always more than sixfold higher than the incorporation into DAG. Surprisingly, the other study, in which murine mtGPAT1 was overexpressed, showed an increase in \([^{3}H]\)palmitate incorporation into DAG but not TAG unless the cells were preloaded with 300 μM 18:1–16:0 (1:1) (32). It is likely that, in the absence of 18:1, either dipalmitoylglycerol was formed and was a poor substrate for diacylglycerol acyltransferase (9) or that the exogenously provided 16:0 caused the hepatocytes to undergo apoptosis (33, 38).

**Table 2. Hepatocyte metabolites**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Ad-GFP</th>
<th>Ad-mtGPAT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine, nmol/1.5 × 10⁶ cells</td>
<td>131.3 ± 4.5</td>
<td>125.9 ± 6.1</td>
</tr>
<tr>
<td>Sphingomyelin, nmol/1.5 × 10⁶ cells</td>
<td>12.0 ± 0.3</td>
<td>10.1 ± 0.7</td>
</tr>
<tr>
<td>Cholesterol, μg/1.5 × 10⁶ cells</td>
<td>12.2 ± 1.3</td>
<td>12.2 ± 1.9</td>
</tr>
<tr>
<td>Cholesterol ester, μg/1.5 × 10⁶ cells</td>
<td>15.1 ± 0.6</td>
<td>11.6 ± 2.7</td>
</tr>
</tbody>
</table>

Results indicate means ± SD for triplicates. Hepatocytes were plated, infected with adenovirus-infected green fluorescent protein (Ad-GFP) or adenovirus-infected mitochondrial glycerol-3-phosphate acyltransferase-1 (Ad-mtGPAT1) at 5 pfu/cell, and incubated with 250 μM oleate. Lipids were extracted and analyzed for phosphatidylcholine, sphingomyelin, and cholesterol as described in EXPERIMENTAL PROCEDURES. *P = 0.04.
Our study also documents and examines, for the first time, the lack of effect of mtGPAT1 overexpression on TAG secretion from hepatocytes. Despite greater than 2.4-fold increases in TAG mass and [14C]oleate incorporation into TAG, the amount of [14C]TAG secreted was unaffected by mtGPAT overexpression. Studies in cultured hepatoma cells show that exogenous fatty acid promotes the amount of VLDL-TAG secreted (17) and that inhibiting the de novo synthesis of TAG with triacin (48), an inhibitor of acyl-CoA synthetase, long-chain (ACSL)1 and ACSL4 (29), or with troglitazone (19), an inhibitor of ACSL4 (29), decreases both TAG synthesis and apoB secretion. Although the initiation of apoB synthesis is constitutive, apoB undergoes both proteasomal and nonproteasomal degradation if TAG is not synthesized concomitantly. Because the rate of TAG synthesis critically affects the production of apoB-containing lipoproteins, the increase in TAG synthesis in the Ad-mtGPAT1 hepatocytes should have increased their secretion of VLDL-TAG. The discrepancy cannot be explained by a defect in the machinery for VLDL assembly and secretion, because both control and Ad-mtGPAT1 hepatocytes incubated with 750 μM oleate increased TAG secretion appropriately (Fig. 4D). Nor can the lack of increased TAG secretion be attributed to a deficiency of phosphatidylcholine (Table 2) (50).

Contradictory data exist regarding whether a decrease in CE interferes with VLDL secretion. In HepG2 cells, inhibition of CE synthesis and VLDL-CE content variably inhibit VLDL-stimulated or oleate-stimulated apoB secretion (5, 21, 48). Conversely, increasing CE synthesis and mass by overexpression of HMG-CoA reductase (48) or treatment with sphingomyelinase (48) does not affect apoB secretion. Thus neither diminishing nor overproducing cellular CE consistently alters lipoprotein secretion from HepG2 cells. Studies that do show a relationship between CE production and lipoprotein secretion (37, 43, 46) suggest that Ce content or production must be higher than basal for subsequent inhibition to show an effect.

mtGPAT1 overexpression diminished the incorporation of [14C]oleate into CE, but when the amount of exogenous oleate was increased, the decrease in CE was partially ameliorated (Fig. 5). These data suggest that overexpressed mtGPAT1 might be diverting oleate away from cholesterol esterification but that the diversion can be overcome by increasing the amount of acyl-CoA available. In our experiments, it is unlikely that the decrease observed in CE synthesis in the Ad-mtGPAT1 hepatocytes diminished the ability of the cells to secrete more TAG. In all treatment groups, TAG secretion was proportional to the amount of exogenous oleate provided, despite the fact that incorporation of [14C]oleate into CE decreased only in the Ad-mtGPAT1 cells.

Because VLDL assembly was adequate, the rate of TAG synthesis was high, cellular phosphatidylcholine content was sufficient, and CE was not limiting, how can we explain the lack of mtGPAT1 effect on secretion of labeled TAG? A likely possibility is that the glycerolipid synthesis initiated by mtGPAT1 produces TAG that cannot be secreted and, instead, becomes sequestered in lipid droplets. Studies in Hep G2 cells suggest that two TAG pools exist, a microsomal pool that is coupled to secretion and a cytosolic pool that is not (49). Several other studies suggest the presence of separate and functionally different intracellular lipid pools. In Neutral Lipid Storage Disease, for example, hydrolyzed DAG intermediates are available for TAG, but not phospholipid, biosynthesis (24). Furthermore, the human deficiency of AGPAT2 leads to a severe congenital lipodystrophy (2), and apparently none of the other five putative AGPAT isoforms can alter the excess of the lysophosphatidic acid substrate or the deficiency of the phosphatic acid product that contributes to the pathophysiology. Separate pools of cardiac TAG synthesized by distinct diacylglycerol acyltransferase (DGAT) activities have also been proposed (44). A final example of separate phospholipid pools is that of a temperature-sensitive mutation in the cytidine diphosphocholine pathway in CHO cells that cannot be complemented by overexpression of an enzyme that contributes to an alternate phosphatidylcholine-synthetic pathway (23).

Overexpression of mtGPAT1 leads to both an increase in cell TAG content and an increase in the entry of fatty acid as measured by the total amount of [14C]label in cellular metabolites. With a 13-fold overexpression of mtGPAT1, at each concentration of oleate, the total [14C]-labeled metabolites (cell plus medium) increased 40–50% compared with the vector control. Smaller changes were observed with stable mtGPAT1 overexpression in CHO cells, where a 3.8-fold increase in mtGPAT specific activity resulted in a 16% increase in total labeled glycerolipids (25). Fatty acid entry into cellular lipids
also increases with overexpression of other acyltransferases, such as AGPAT1 activity in 3T3-L1 adipocytes and C2C12 myotubes (39), and DGAT-1 in 3T3-L1 adipocytes (51). The only exception to this pattern of increased fatty acid uptake is a study in which stable overexpression of DGAT-1 activity in SV40-transformed human lung fibroblasts caused a marked decrease in cell proliferation and thus phospholipid synthesis (4). Taken as a whole, observations on the overexpression of three different acyltransferases in the Kennedy pathway suggest that metabolism, rather than transport, is the major factor limiting fatty acid entry into cells.

Altered fatty acid metabolism plays a significant role in the development of obesity and type 2 diabetes. We have shown that overexpressing mtGPAT1 decreases fatty acid oxidation and increases storage of fatty acid as TAG. A similar hepatic diversion of fatty acid toward TAG storage is observed in obesity, and our data suggest that dysregulation at the mitochondrial outer membrane may be significant in controlling fatty acid partitioning. Nonadipose TAG accumulation is also a hallmark of insulin resistance. Although beyond the scope of these studies, our model of hepatic TAG accumulation may be useful in studying TAG-associated hepatic insulin resistance.

ACKNOWLEDGMENTS

We thank Dr. S. K. Krisans (San Diego State University) for the measurements of HMG-CoA reductase, and Mei-Heng Mar for the choline metabolite measurements.

Current address of C. G. Van Horn: Department of Biochemistry, Rm. 239 Nutrition Research Center, Wake Forest University Health Sciences Medical Center Blvd, Winston-Salem, NC 27157

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-59935 (R. A. Coleman), DK-59931 (T. M. Lewin), and F32-DK-61190 (C. G. Van Horn) from the National Institutes of Health, and by Grant 230323N from the American Heart Association (T. M. Lewin), the Center for Gastrointestinal Biology and Disease, Vector and ACT Cores (F30-DK-34987).

REFERENCES


