

# Glycerol-3-phosphate Acyltransferase (GPAT)-1, but Not GPAT4, Incorporates Newly Synthesized Fatty Acids into Triacylglycerol and Diminishes Fatty Acid Oxidation\*

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**Background:** The independent functions of the glycerol-3-phosphate acyltransferase (GPAT) isoforms are unknown.

**Results:** When compared with *Gpat4*<sup>-/-</sup>, *Gpat1*<sup>-/-</sup> hepatocytes oxidized more fatty acids (FA) and were unable to incorporate *de novo* synthesized FA into triacylglycerol.

**Conclusion:** GPAT1, but not GPAT4, metabolizes FA synthesized *de novo* from [<sup>14</sup>C]acetate and diverts FA away from mitochondrial oxidation.

**Significance:** GPAT1 and GPAT4 use different cellular pools of FA.

Four glycerol-3-phosphate acyltransferase (GPAT) isoforms, each encoded by a separate gene, catalyze the initial step in glycerolipid synthesis; in liver, the major isoforms are GPAT1 and GPAT4. To determine whether each of these hepatic isoforms performs a unique function in the metabolism of fatty acid, we measured the incorporation of *de novo* synthesized fatty acid or exogenous fatty acid into complex lipids in primary mouse hepatocytes from control, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> mice. Although hepatocytes from each genotype incorporated a similar amount of exogenous fatty acid into triacylglycerol (TAG), only control and *Gpat4*<sup>-/-</sup> hepatocytes were able to incorporate *de novo* synthesized fatty acid into TAG. When compared with controls, *Gpat1*<sup>-/-</sup> hepatocytes oxidized twice as much exogenous fatty acid. To confirm these findings and to assess hepatic  $\beta$ -oxidation metabolites, we measured acylcarnitines in liver from mice after a 24-h fast and after a 24-h fast followed by 48 h of refeeding with a high sucrose diet to promote lipogenesis. Confirming the *in vitro* findings, the hepatic content of long-chain acylcarnitine in fasted *Gpat1*<sup>-/-</sup> mice was 3-fold higher than in controls. When compared with control and *Gpat4*<sup>-/-</sup> mice, after the fasting-refeeding protocol, *Gpat1*<sup>-/-</sup> hepatic TAG was depleted, and long-chain acylcarnitine content was 3.5-fold higher. Taken together, these data demonstrate that GPAT1, but not GPAT4, is required to incorporate *de novo* synthesized fatty acids into TAG and to divert them away from oxidation.

Glycerol-3-phosphate acyltransferase (GPAT<sup>3</sup>; EC 2.3.1.15) initiates the pathway of TAG synthesis by esterifying a long-

chain fatty acyl-CoA to a glycerol-3-phosphate backbone at the *sn*-1 position to form *sn*-1-acylglycerol-3-phosphate (lysophosphatidic acid) (1). A second esterification at the *sn*-2 position is catalyzed by acylglycerol-3-phosphate acyltransferase. The phosphate is then hydrolyzed by phosphatidic acid phosphohydrolase (lipin), and then a final acylation is catalyzed by diacylglycerol acyltransferase to form TAG. In addition to their roles in TAG synthesis, the phosphatidic acid and diacylglycerol intermediates in this pathway are precursors for all the glycerophospholipids, and they initiate signaling pathways.

The pathway of TAG biosynthesis is remarkable for the number of isoenzymes that catalyze each step. For example, four independent GPAT isoforms, each encoded by a separate gene, catalyze the formation of lysophosphatidic acid, but it is unclear why GPAT enzyme redundancy is required. Studies of mice with deleted GPATs show that the isoforms present in liver include primarily GPAT1 (2) and GPAT4 (3, 4), with lower expression of GPAT2 (5) and GPAT3 (6). Each of the GPAT isoforms is an intrinsic membrane protein whose active site faces the cytosol (7). GPAT1, located on the outer mitochondrial membrane and at contact sites with the endoplasmic reticulum (8), has a strong preference for saturated fatty acids (1). GPAT1 comprises 30–50% of total activity in liver (1), and *Gpat1* mRNA expression is highly responsive to SREBP1c and is up-regulated when dietary carbohydrate intake and circulating insulin concentrations are high (9). Absent GPAT1 protects against the development of hepatic steatosis caused by a high fat diet (2) or by the up-regulation of SREBP1c in *ob/ob* mice (10). Conversely, GPAT1 overexpression causes hepatic steatosis (11) and insulin resistance (12). GPAT4, which is located on the endoplasmic reticulum and on lipid droplets (13), contributes 40–50% of total hepatic GPAT activity (3). Like GPAT1, GPAT4 also appears to be important for hepatic TAG synthesis

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<sup>§</sup> The abbreviations used are: GPAT, glycerol-3-phosphate acyltransferase; ASM, acid-soluble metabolite(s); C75, 4-methylene-2-octyl-5-oxotetrahy-

drolfuran-3-carboxylic acid; CPT, carnitine palmitoyltransferase-1; DGAT, diacylglycerol acyltransferase; NEM, *N*-ethylmaleimide; PL, phospholipid; TAG, triacylglycerol; PPAR, peroxisome proliferator-activated receptor.

## Unique Functions of GPAT1 versus GPAT4 Isoforms

because the TAG content in *Gpat4*<sup>-/-</sup> liver is 42% lower than in control mice (3).

Hepatic triacylglycerol synthesis is enhanced by the increased availability of long-chain fatty acids synthesized within hepatocytes from dietary glucose or from fatty acid that enters hepatocytes exogenously from dietary chylomicron remnants or as free fatty acids lipolyzed from adipose tissue. Because SREBP1c concomitantly up-regulates both GPAT1 and the enzymes that catalyze the *de novo* synthesis of fatty acids, including acetyl-CoA carboxylase and fatty acid synthase (14), we hypothesized that the function of GPAT1 and *de novo* lipogenesis might be intrinsically linked. To test this hypothesis, we compared the ability of GPAT1 and GPAT4 to use exogenous *versus de novo* synthesized fatty acids in control and knock-out mice and in their hepatocytes.

### EXPERIMENTAL PROCEDURES

**Materials**—Type I collagenase was from Worthington Biochemical Corp. [1-<sup>14</sup>C]Acetic acid and [1-<sup>14</sup>C]palmitic acid were purchased from PerkinElmer Life Sciences. Silica G gel plates were from Whatman. Tissue culture plates were from BD Biosciences, and media were obtained from Invitrogen. Sigma was the source of 4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid (C75) and all other chemicals, unless otherwise indicated.

**Animal Care and Hepatocyte Isolation**—Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Mice were housed in a pathogen-free barrier facility on a 12-h light/dark cycle with free access to water and food (Prolab 5P76 Isopro 3000; 5.4% fat by weight). *Gpat1*<sup>-/-</sup> (2) and *Gpat4*<sup>-/-</sup> (3, 15) mice had been backcrossed at least eight times onto a C57BL6/J background. At 8 weeks of age, littermate controls and *Gpat1*<sup>-/-</sup> and *Gpat4*<sup>-/-</sup> mice were either fasted for 24 h or fasted for 24 h and then refed a high sucrose diet for 48 h before sacrifice. The high sucrose diet was modified from Research Diets D12450B (10% kcal of fat, 20% kcal of protein, and 70% kcal of carbohydrate (525 g/kg of sucrose, 175 g/kg of corn starch)).

To isolate hepatocytes, after mice were anesthetized with 250 mg/kg of Avertin, a 22-gauge catheter was inserted into the vena cava, and the liver was perfused with wash buffer (10 mM Hepes, 132 mM NaCl, 6.7 mM KCl, 20 mM glucose, 0.5 M adenosine, 100 mM EGTA, 140 nM insulin, pH 7.4) and then with digestion buffer (100 mM HEPES, 57 mM NaCl, 6.7 mM KCl, 5 mM CaCl<sub>2</sub>, 20 mM glucose, 0.45 mg/ml type 1 collagenase, pH 7.4). The digested liver was removed and placed in a 100-mm dish on ice with Hanks'/BSA buffer (10 mM HEPES, 137 mM NaCl, 5.4 mM KCl, 1.26 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM glucose, pH 7.4). The hepatocytes were dissociated and filtered through a cell strainer, centrifuged at 4 °C at 50 × *g* for 3 min, and then washed once in Williams' medium E. The cells were resuspended in Williams' medium E:Percoll (16 ml:14 ml), centrifuged at 4 °C at 400 × *g* for 8 min, and washed with Williams' medium E. Cells were plated onto 60-mm collagen-coated dishes and incubated for 90 min in Williams' medium E + 10% FBS. After the cells attached, the medium was changed to Wil-

liams' medium E, 100 units of penicillin/streptomycin, 50 nM insulin, 0.1 μM dexamethasone, and 0.1% BSA.

**GPAT Activity**—Hepatocytes were cultured overnight in 60-mm plates and then scraped and homogenized in cold Medium I (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) with 10 up-and-down strokes with a Teflon glass motor-driven homogenizer. Liver was minced and then homogenized similarly. Total membranes were isolated by centrifuging the homogenate at 100,000 × *g* for 1 h. GPAT specific activity was assayed for 10 min at room temperature in a 200-μl reaction mixture containing 75 mM Tris-HCl, pH 7.5, 4 mM MgCl<sub>2</sub>, 1 mg/ml BSA (essentially fatty acid-free), 1 mM dithiothreitol, 8 mM NaF, 800 μM [<sup>3</sup>H]glycerol 3-phosphate, and 80 μM palmitoyl-CoA (20). The reaction was initiated by adding 10–30 μg of membrane protein after incubating the membrane protein on ice for 15 min in the presence or absence of 2 mM *N*-ethylmaleimide (NEM), which inactivates GPAT isoforms 2, 3, and 4. The reaction products were extracted into CHCl<sub>3</sub>, dried under N<sub>2</sub>, resuspended in 4 ml of Cytosint, and counted on a scintillation counter. NEM-resistant activity (GPAT1) was calculated by subtracting NEM-sensitive activity from total activity.

**RNA Extraction and RT-PCR**—Total RNA was extracted from overnight cultures of hepatocytes with TRIzol (Invitrogen). cDNA synthesis and real time RT-PCR were performed as described (3). Primer sequences for each GPAT isoform and data normalization used were described (3, 32).

**Labeling, Oxidation, and Lipid Extraction**—Sixteen to 20 h after plating, hepatocytes were labeled with 0.5 ml of labeling buffer (Krebs Ringer buffer, 10 mM HEPES, 5.5 mM glucose, 0.25% fatty acid-free bovine serum albumin (BSA), and 1 mM carnitine) containing 500 μM [1-<sup>14</sup>C]acetic acid or [1-<sup>14</sup>C]palmitic acid for 2 h. Media were removed, and CO<sub>2</sub> was liberated by adding 100 μl of 70% perchloric acid and trapped in 1 M NaOH. An aliquot of the NaOH was counted to measure radiolabeled CO<sub>2</sub>. To quantify intracellular oxidation metabolites, fresh Krebs Ringer buffer and 100 μl of 70% perchloric acid were added to each plate, and acidified supernatants were collected. The acidified media and cell supernatants were incubated overnight with BSA and then centrifuged at 14,000 × *g* for 20 min. Aliquots of the supernatant were counted to measure radiolabeled acid-soluble metabolites (ASM), a measure of incomplete fatty acid oxidation. Cells were washed with 1% BSA and then with phosphate-buffered saline before lipids were extracted (16). Lipid extracts and standards were separated by thin layer chromatography on Partisil LK5D silica gel plates in a two-phase system: chloroform:methanol:ammonium hydroxide (65:25:4, v/v/v) run to 50% of the plate and then dried and followed by heptane:isopropyl ether:acetic acid (60:40:4, v/v/v) run to the top of the plate. Radiolabeled lipids were quantified with a Bioscan AR-2000 imaging scanner. Lipid standards were visualized by iodine staining.

**Blood Chemistries and Lipids**—Before blood collection, mice were either fasted for 24 h or fasted 24 h and then fed a high sucrose diet for 48 h. Plasma TAG (Stanbio, Boerne, TX), free fatty acid (Wako Diagnostics, Richmond, VA), glucose (glucose CII reagent, Wako Diagnostics), and β-hydroxybutyrate (Wako

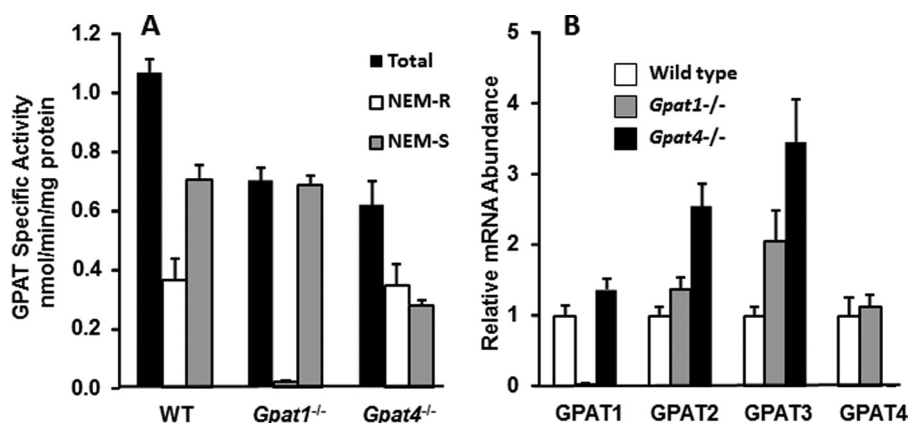


FIGURE 1. GPAT specific activity and mRNA expression in primary hepatocytes from wild type, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> mice. A, GPAT specific activity was measured in total particulate preparations of primary hepatocytes cultured for 16 h; results show triplicates from two independent experiments. Total, N-ethylmaleimide-resistant (NEM-R), and N-ethylmaleimide-sensitive (NEM-S) GPAT specific activities are shown. B, mRNA expression of each GPAT isoform was measured by RT-PCR. Total RNA was obtained from primary hepatocytes in triplicate from two experiments. Data are presented as means  $\pm$  S.E.

Diagnostics) were determined by enzymatic colorimetric methods.

**Lipid Extraction and TAG Measurement in Liver**—Liver lipids were extracted (12), dried under N<sub>2</sub> gas, resuspended in 200  $\mu$ l of *tert*-butanol:methanol:Triton X-100 (3:1:1, v/v), and analyzed colorimetrically as described above.

**Liver Acylcarnitine Measurement**—Livers were snap-frozen in liquid nitrogen, pulverized, and weighed. The pulverized liver (50–100 mg) was homogenized in acetonitrile:water:formic acid (50:49.7:0.3; v/v) at a final concentration of 50 mg/ml. Acylcarnitines were analyzed by flow injection tandem mass spectrometry (17). Samples were stored at  $-80^{\circ}\text{C}$  before analysis.

**Statistics**—Data represent means  $\pm$  S.E. of at least three independent experiments performed in triplicate unless indicated otherwise. *In vitro* models were analyzed by one-way analysis of variance comparing each genotype versus controls and post hoc comparisons of diet conditions within each genotype. Data were considered significant at  $p < 0.05$ .

## RESULTS

**Isolated and Cultured Primary Hepatocytes Retained GPAT Specific Activity**—To compare the contributions of GPAT1 and GPAT4 to the total amount of GPAT activity, we isolated and cultured primary hepatocytes from control, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> mice and measured GPAT specific activity in the presence and absence of NEM, which inhibits GPAT2, 3, and 4 (Fig. 1A). When compared with wild type hepatocytes, total GPAT specific activity in *Gpat1*<sup>-/-</sup> hepatocytes was 35% lower and minimal residual NEM-resistant GPAT activity remained, whereas NEM-sensitive GPAT activity was unchanged. Total GPAT specific activity in *Gpat4*<sup>-/-</sup> hepatocytes was 42% lower than in control hepatocytes; NEM-resistant GPAT specific activity was unchanged, but NEM-sensitive GPAT activity was 60% lower. The residual NEM-sensitive GPAT activity is likely due to GPAT2 and -3. These results show that isolated and cultured primary hepatocytes retained GPAT activity and that the specific activity was similar in *Gpat1*<sup>-/-</sup> and *Gpat4*<sup>-/-</sup> hepatocytes.

To determine whether other GPAT isoforms were affected by deletion of either GPAT1 or GPAT4, we measured mRNA

expression of each GPAT isoform (Fig. 1B). In *Gpat1*<sup>-/-</sup> hepatocytes, *Gpat3* mRNA abundance was 2-fold higher than in wild type hepatocytes, but NEM-sensitive GPAT activity did not change, indicating a lack of compensation. In the absence of GPAT4, the mRNA expression of *Gpat2* and -3 increased 2.5- and 3.5-fold, respectively. GPAT2 activity is extremely low in liver (5), and even when the amount of *Gpat2* mRNA doubles, it probably does not contribute substantially to total NEM-sensitive GPAT activity. However, because we cannot differentiate between GPAT3 and GPAT4 protein or activity, the 42% lower NEM-sensitive GPAT activity (Fig. 1B) could reflect partial compensation by GPAT3.

**The Incorporation of *de Novo* Synthesized Fatty Acid into TAG Requires GPAT1**—Because GPAT1 is up-regulated by SREBP1c, we hypothesized that it might be functionally and specifically linked to *de novo* lipogenesis, such that newly synthesized fatty acids would be incorporated into TAG. Using [1-<sup>14</sup>C]acetate, we measured the ability of control, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> hepatocytes to incorporate *de novo* synthesized fatty acid into TAG and PL. The incorporation of acetate into TAG and PL was equal in hepatocytes from wild type and *Gpat4*<sup>-/-</sup> mice; however, in *Gpat1*<sup>-/-</sup> hepatocytes, the incorporation of [1-<sup>14</sup>C]acetate into PL was 78% lower than in controls, and the incorporation into TAG was virtually absent (Fig. 2, A and B). When wild type and *Gpat4*<sup>-/-</sup> hepatocytes were cultured in the presence of the fatty acid synthase inhibitor C75, [1-<sup>14</sup>C]acetate incorporation into TAG was inhibited 80–85% (Fig. 2A). However, in *Gpat1*<sup>-/-</sup> hepatocytes, C75 had no additional effect, indicating that the absence of GPAT1 in hepatocytes had abolished the incorporation of the *de novo* synthesized fatty acid. In wild type and *Gpat4*<sup>-/-</sup> hepatocytes, C75 decreased acetate incorporation into PL by 60%, but only by 27% in *Gpat1*<sup>-/-</sup> hepatocytes (Fig. 2B). In each group of hepatocytes, the addition of C75 decreased the incorporation of acetate into free fatty acids by more than 72% (data not shown). These results show that GPAT1, but not GPAT4, is required to incorporate *de novo* synthesized fatty acid into complex lipids.

**Both GPAT1 and GPAT4 Can Incorporate Exogenous Fatty Acids into TAG and Phospholipid**—In addition to *de novo* synthesis, liver fatty acids are also derived from exogenous sources.



## Unique Functions of GPAT1 versus GPAT4 Isoforms

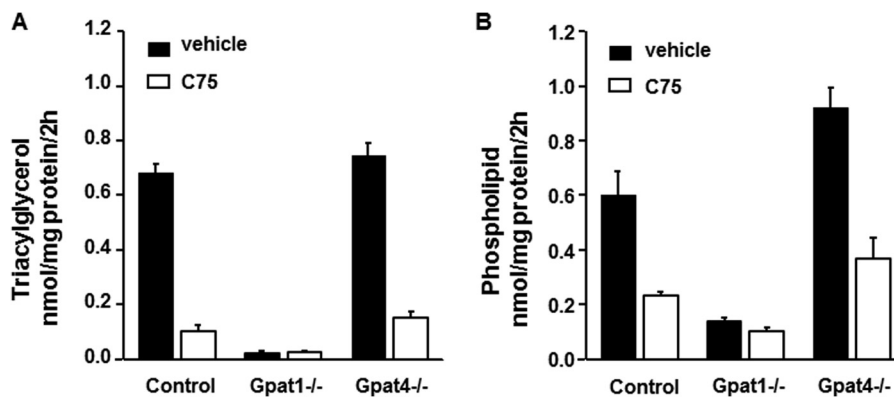


FIGURE 2. *Gpat1*<sup>-/-</sup> hepatocytes incorporated less [<sup>14</sup>C]acetate into glycerolipids. Hepatocytes were labeled with 500  $\mu$ M [<sup>14</sup>C]acetate in the presence or absence of 20  $\mu$ g/ml C75 for 2 h. A and B, [<sup>14</sup>C]acetate incorporation into triacylglycerol (A) or phospholipids (B) in control, *Gpat1*<sup>-/-</sup>, or *Gpat4*<sup>-/-</sup> hepatocytes. Data show means  $\pm$  S.E. of two experiments performed in triplicate.

To distinguish between the functions of GPAT1 and GPAT4 in the use of exogenous fatty acids, we measured the incorporation of [<sup>14</sup>C]palmitate into glycerolipids in primary hepatocytes. In contrast to fatty acids derived from *de novo* synthesis, the incorporation of [<sup>14</sup>C]palmitate into TAG and PL was not altered by the absence of either GPAT1 or GPAT4 (Fig. 3, A and B), indicating that neither isoform was essential for the use of exogenous fatty acid. Thus, in both *Gpat4*<sup>-/-</sup> and *Gpat1*<sup>-/-</sup> hepatocytes, the remaining GPAT isoforms can fully incorporate exogenous fatty acid into glycerolipids.

*In Hepatocytes, the Absence of GPAT1, but Not GPAT4, Increased Fatty Acid Oxidation*—Previous studies have strongly suggested that GPAT1 competes with CPT1 for acyl-CoAs at the outer mitochondrial membrane (12). To confirm this competition for acyl-CoAs and to determine whether GPAT4 located on the endoplasmic reticulum competes similarly, we measured the incorporation of [<sup>14</sup>C]palmitate into ASM, a measure of incomplete fatty acid oxidation. *Gpat1*<sup>-/-</sup> hepatocytes oxidized [<sup>14</sup>C]palmitate at a rate 200% higher than control hepatocytes, but the incorporation of [<sup>14</sup>C]palmitate into ASM by *Gpat4*<sup>-/-</sup> hepatocytes was unchanged (Fig. 3C). Of the total ASM extracted from the media and cells, intracellular ASM comprises only 5% of the total ASM (Fig. 3C, inset); thus, media ASM reflect 95% of the total ASM produced. These data show that only GPAT1 competes with CPT1 for acyl-CoAs, thereby diminishing the flux of fatty acid into the mitochondria for oxidation.

*Hepatic GPAT1 Diminished Fatty Acid Transport into Mitochondria in Vivo*—To confirm the disparate effects of each GPAT isoform on the  $\beta$ -oxidation of exogenous versus *de novo* synthesized fatty acid *in vivo*, we measured hepatic acylcarnitine content in wild type, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> mice after food was removed for 24 h. Because fasting increases the flux of adipose-derived fatty acids into the liver, more fatty acids are transported to the mitochondria via CPT1, thereby elevating the content of hepatic long-chain acylcarnitines. These metabolites are subsequently reconverted to acyl-CoAs in the mitochondrial matrix, where they enter the  $\beta$ -oxidation pathway. In fasting mice of each genotype, the content of free carnitine (C0), acetylcarnitine (C2), and short-chain (C3–C6) and medium-chain (C8–C12) acylcarnitine species was similar (Fig. 4, A and C). Consistent with the hypothesized ability of GPAT1 to

compete with CPT1 for acyl-CoAs, total long-chain (C14–C22) acylcarnitine content in fasted *Gpat1*<sup>-/-</sup> liver was 3-fold higher than in control liver (Fig. 4C). The most abundant long-chain acylcarnitine species, 14:0, 16:1, 16:0, 18:2, 18:1, and 18:0, were 2–6-fold higher than in control liver (Fig. 4E). In contrast, and consistent with the *in vitro* data, the acylcarnitine content in livers of fasted *Gpat4*<sup>-/-</sup> mice was similar to that of control mice.

Because GPAT1 was essential for the incorporation of *de novo* synthesized fatty acids into complex lipids *in vitro*, we predicted that the absence of hepatic GPAT1 *in vivo* would also increase the transport of *de novo* synthesized fatty acids into mitochondria. To test this hypothesis, we fasted mice for 24 h and then refed them with a high sucrose diet for 48 h to stimulate *de novo* lipogenesis. The total long-chain (C14–C22) acylcarnitine content of *Gpat1*<sup>-/-</sup> liver remained 3.5-fold higher than that of controls, and the most abundant long-chain acylcarnitine species, 14:0, 16:1, 16:0, 18:2, 18:1 and 18:0, ranged from 2- to 5-fold higher (Fig. 4, D and F). In contrast, no differences were observed in the hepatic acylcarnitine content of *Gpat4*<sup>-/-</sup> mice that had been fasted and then refed (Fig. 4, B, D, and F). Thus, GPAT1, but not GPAT4, was required *in vivo* to diminish CPT1-mediated transport of both *de novo* synthesized and exogenous fatty acid.

*Hepatic GPAT1 Was Essential for the Incorporation of De Novo Synthesized Fatty Acid into TAG*—Because the absence of GPAT1 increased hepatic  $\beta$ -oxidation of *de novo* synthesized fatty acids *in vivo*, we predicted that the liver TAG content would be low. After a 24-h fast, hepatic TAG content in control, *Gpat4*<sup>-/-</sup>, and *Gpat1*<sup>-/-</sup> liver was similar. After refeeding a high sucrose diet to enhance *de novo* lipogenesis, the TAG content in control and *Gpat4*<sup>-/-</sup> liver increased 2–2.5-fold (Fig. 5A); however, the hepatic TAG content of *Gpat1*<sup>-/-</sup> liver did not increase. These data support the conclusion that GPAT1 is essential for the incorporation of *de novo* synthesized fatty acid into TAG and for diverting those fatty acids away from mitochondrial oxidation.

*The Absence of GPAT1, but Not GPAT4, Increased Ketogenesis during Fasting*—When the concentration of plasma glucose decreases during an extended fast, adipocyte-derived fatty acids flood into the liver, their oxidation rate increases, and ketone bodies are synthesized and secreted into the blood. The accu-

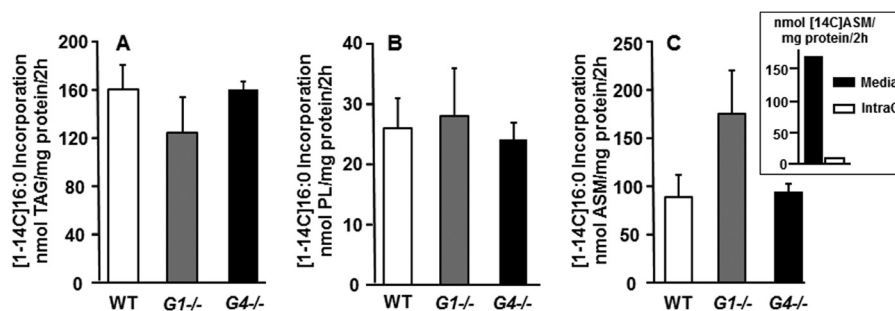


FIGURE 3. **GPAT1 was not required for the incorporation of exogenous fatty acid, and  $\beta$ -oxidation was higher in  $Gpat1^{-/-}$  hepatocytes.** Hepatocytes from wild type,  $Gpat1^{-/-}$  ( $G1^{-/-}$ ), or  $Gpat4^{-/-}$  ( $G4^{-/-}$ ) mice were labeled with 500  $\mu$ M [ $1-^{14}$ C]palmitic acid for 2 h. A–C, [ $1-^{14}$ C]palmitic acid incorporation into triacylglycerol (A), phospholipids (B), and ASM (C). Data show means  $\pm$  S.E. of two experiments performed in triplicate. *Inset*, comparison of cell media and intracellular (*IntraC*) ASM. Data show means of one experiment performed in quadruplicate.

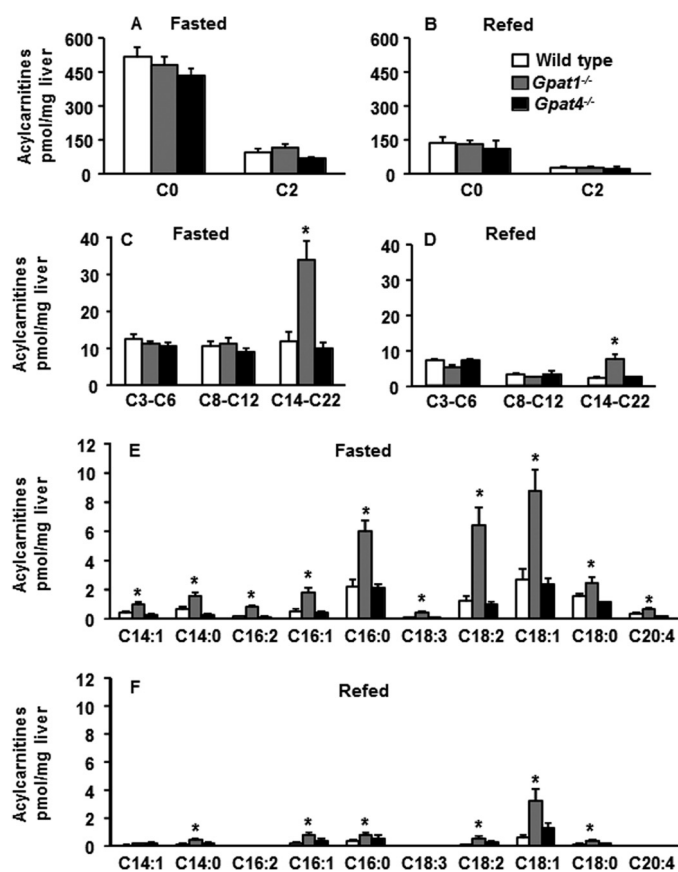


FIGURE 4. **Long-chain acylcarnitine content was higher in  $Gpat1^{-/-}$  liver than controls after a 24-h fast and after 48 h of refeeding.** Wild type,  $Gpat1^{-/-}$ , and  $Gpat4^{-/-}$  mice were fasted for 24 h or fasted for 24 h and then refed with a high sucrose diet for 48 h. A and B, hepatic content of free carnitine (C0) and acetylcarnitine (C2). C and D, short-, medium-, and long-chain acylcarnitine content of mouse liver. E and F, major hepatic long-chain acylcarnitine species from mice. Data are shown as means  $\pm$  S.E. Significant differences are indicated by \* for genotype. ( $p < 0.05$ , analysis of variance) ( $n = 6$  diet, genotype).

mulation of long-chain acylcarnitines in the liver together with an elevated plasma concentration of ketone bodies suggests an increased rate of hepatic fatty acid oxidation. Because GPAT1 diminishes the oxidation of exogenous fatty acid *in vitro* and the absence of GPAT1 in liver results in the accumulation acylcarnitines, we predicted that fasting would result in a higher rate of hepatic  $\beta$ -oxidation in  $Gpat1^{-/-}$  mice than in controls. To confirm this hypothesis, we measured plasma ketone bodies in control,  $Gpat1^{-/-}$ , and  $Gpat4^{-/-}$  mice. After a 24-h fast, the

concentrations of plasma glucose and TAG in control and  $Gpat1^{-/-}$  mice were similar (Fig. 5, B and C), but the plasma fatty acid concentration in  $Gpat1^{-/-}$  mice was 1.5-fold higher than controls (Fig. 5D). Consistent with elevated fatty acid oxidation in the  $Gpat1^{-/-}$  mice, the plasma concentration of  $\beta$ -hydroxybutyrate was 2-fold higher than controls (Fig. 5E).

**Ketogenesis Remained Elevated in  $Gpat1^{-/-}$  Mice during Refeeding**—After a 24-h fast, refeeding with high sucrose drives hepatic *de novo* lipogenesis, promotes TAG storage, and blocks fatty acid oxidation (18). Because our data suggested that GPAT1 esterifies *de novo* synthesized fatty acids, we asked whether the rate of hepatic fatty acid oxidation would remain elevated during short-term high sucrose refeeding. Although a high carbohydrate diet diminishes PPAR $\alpha$  gene targets related to  $\beta$ -oxidation and ketone body formation, in the absence of GPAT1, the plasma  $\beta$ -hydroxybutyrate concentration in  $Gpat1^{-/-}$  mice was 3-fold higher than in control or  $Gpat4^{-/-}$  mice (Fig. 5D), consistent with a continued higher rate of hepatic fatty acid oxidation. Consistent with diminished hepatic TAG synthesis in both  $Gpat1$  and  $Gpat4$  null mice, when compared with controls, the plasma TAG concentration was 48% lower in  $Gpat1^{-/-}$  mice and 25% lower in  $Gpat4^{-/-}$  mice (Fig. 5C). Plasma glucose and fatty acid concentrations in each genotype were similar (Fig. 5, B and E).

## DISCUSSION

With the discovery of four independent GPAT isoforms that differ in tissue distribution, subcellular location, acyl-CoA preference, and transcriptional regulation, questions have arisen as to the specific function of each isoform (1).  $Gpat2$  mRNA is most strongly expressed in testes and probably plays a minor role in liver, but the mRNA expression and activities in liver of the remaining GPAT isoforms are high (1, 5, 6, 19). Although studies suggest that GPAT3 is regulated by PPAR $\gamma$  and PPAR $\delta$  in keratinocytes (20) and by insulin-mediated phosphorylation in 3T3-L1 adipocytes (21), the regulation and specific contributions of hepatic GPAT3 to glycerolipid synthesis are unknown. The availability of  $Gpat1$  and  $Gpat4$  knock-out mice, however, has allowed us to directly compare them and to delineate the specific roles of each isoform.

The function of GPAT1 in the esterification of fatty acids has been best studied. GPAT1 esterification rates for 16:0-CoA are 3–10 times higher than those observed with 18:0-, 18:1-, 18:2n6-, and 18:3n3-CoAs (21–24). Examination of the glycer-

## Unique Functions of GPAT1 versus GPAT4 Isoforms

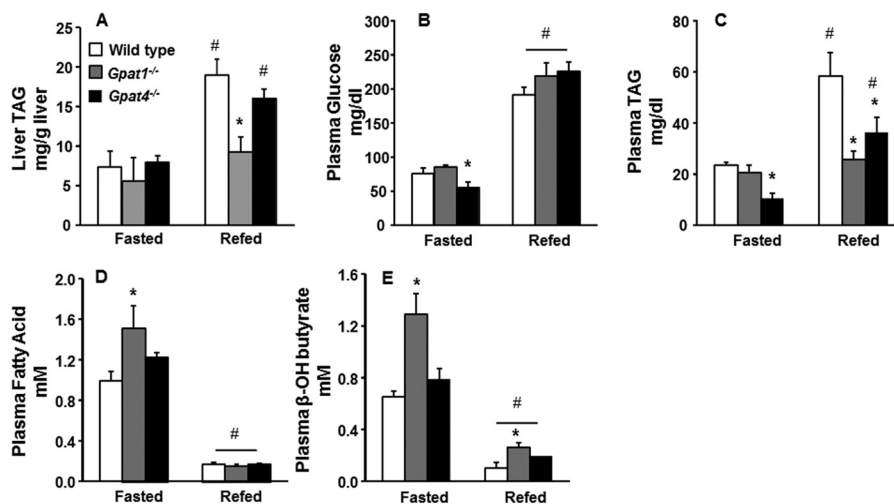


FIGURE 5. **Liver and plasma metabolites.** A, liver TAG content of wild type, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> mice fasted for 24 h or fasted for 24 h and then refed with a high sucrose diet for 48 h. B, plasma glucose; C, plasma TAG; D, plasma fatty acid; E, plasma β-hydroxybutyrate. Data are shown as means ± S.E. Significant differences are indicated by \* for genotype and by # for diet challenge. ( $p < 0.05$ , analysis of variance) ( $n = 6$  diet, genotype).

olipid species in *Gpat1*<sup>-/-</sup> liver have confirmed the preference of GPAT1 for 16:0-CoA because the absence of GPAT1 results in decreases in the amounts of 16:0 esterified to phosphatidylcholine and phosphatidylethanolamine in membranes from total liver (2) and from liver mitochondria (25). The 16:0 deficiencies at the *sn*-1 position of phosphatidylcholine and phosphatidylethanolamine are compensated by increases in the content of 18:1 at the *sn*-1 position and of 20:4ω6 at the *sn*-2 position.

A second function of GPAT1 is related to *de novo* lipogenesis. This role is implied by the fact that the specific activity of GPAT1 is low in liver during fasting, in part because of inhibition by AMP-activated kinase (26), and is up-regulated transcriptionally when lipogenesis from carbohydrate precursors is enhanced. Similar to acyl-CoA carboxylase and fatty acid synthase, *Gpat1* mRNA, protein, and specific activity increase in response to SREBP1c up-regulation, which, in turn, is stimulated by insulin and depressed via liver X receptor by polyunsaturated fatty acids (9). Thus, GPAT1 is markedly elevated in *ob/ob* mice, and a deficiency in *ob/ob* mice of either *Gpat1* itself (10) or of SREBP1c (14) protects against the development of a fatty liver. In addition to promoting the synthesis of TAG, indirect evidence suggests that GPAT1 diverts acyl-CoAs away from CPT1 and β-oxidation because long-chain acylcarnitines and β-hydroxybutyrate increase in *Gpat1*<sup>-/-</sup> mice fed an obesogenic diet that is high in sucrose and palm oil (12). Thus, under conditions when the synthesis of fatty acid is elevated, the presence of GPAT1 would ensure that the resulting fatty acids are esterified rather than oxidized. Our current data directly support this idea.

In isolated hepatocytes, incubation with [<sup>14</sup>C]acetate showed that only the deficiency of GPAT1 blocked the incorporation of *de novo* synthesized fatty acids into glycerolipids. The incorporation was equivalent in control and *Gpat4*<sup>-/-</sup> hepatocytes and was diminished equally by the fatty acid synthase inhibitor C75. These data suggest that fatty acids synthesized *de novo* from glucose require GPAT1 to be incorporated into TAG. Perhaps because of the preference of GPAT1 for 16:0-CoA, the predom-

inant species synthesized *de novo* by fatty acid synthase, GPAT1 was also responsible for most of the acetate-derived fatty acid that was incorporated into PL. Inhibition of fatty acid synthesis by C75 had a weaker effect on PL synthesis than it did on TAG synthesis, suggesting that *de novo* synthesized fatty acids may be preferentially directed to TAG storage, consistent with the fact that lipogenesis is up-regulated when high carbohydrate intake and insulin concentrations activate SREBP1c. The final step in the synthesis of TAG is catalyzed by DGAT1 and DGAT2. Stable isotope studies show that only DGAT2 incorporates fatty acids synthesized *de novo* (27).

Because *Gpat1*<sup>-/-</sup> hepatocytes failed to incorporate label from [<sup>14</sup>C]acetate into TAG, we concluded that GPAT1 is required for the synthesis of TAG from *de novo* lipogenesis. Confirming this interpretation, after the fasting-refeeding protocol to promote hepatic *de novo* lipogenesis, *Gpat1*<sup>-/-</sup> liver contained 53% less TAG than did controls. When compared with fasted mice, the hepatic TAG content in both refed control and refed *Gpat4*<sup>-/-</sup> mice increased 2- and 3-fold, respectively, but liver TAG content in refed *Gpat1*<sup>-/-</sup> mice did not change, indicating that *Gpat1*<sup>-/-</sup> failed to incorporate *de novo*-derived fatty acid into hepatic TAG. A direct comparison of hepatic β-oxidation revealed that when compared with controls, long-chain acylcarnitines and β-hydroxybutyrate in *Gpat1*<sup>-/-</sup> mice were 3.5- and 2-fold higher, respectively. Thus, when hepatic GPAT1 was absent, the *de novo* synthesized fatty acids were oxidized rather than incorporated into TAG. These data are consistent with our *in vitro* findings and confirm that GPAT1 is required to incorporate *de novo* synthesized fatty acids into TAG and diminish fatty acid oxidation.

In humans, VLDL secretion is stimulated by dietary sugars, and after a high carbohydrate load, *de novo* synthesized fatty acids comprise roughly 37% of plasma VLDL-TAG (28, 29). Consistent with a role for GPAT1 in incorporating *de novo* synthesized fatty acids into VLDL, *Gpat1*<sup>-/-</sup> mice fed a diet containing 60% of calories from sucrose were protected from hypertriglyceridemia and showed diminished VLDL secretion rates (30). After the fasting-refeeding protocol, plasma TAG



concentrations in *Gpat1*<sup>-/-</sup> mice did not change, suggesting that GPAT1 is essential for the postprandial lipemia that is stimulated by carbohydrate feeding. In contrast, plasma TAG in re-fed control and *Gpat4*<sup>-/-</sup> mice increased 2- and 3.5-fold, respectively, consistent with the continued presence and function of GPAT1 in using *de novo* synthesized TAG in VLDL.

The absence of either GPAT1 or GPAT4 did not block the incorporation of exogenous fatty acid into TAG or PL. Normal incorporation seems surprising because TAG content in both *Gpat1*<sup>-/-</sup> and *Gpat4*<sup>-/-</sup> liver is 40–50% lower than controls (2, 31). However, our hepatocyte studies measured TAG synthesis from [<sup>14</sup>C]palmitate in the absence of insulin. Normally, after a 24-h fast, when insulin levels are low, fatty acids released from adipocytes constitute the majority of the fatty acid entering the liver to be stored or incorporated into VLDL-TAG, a process that is not sensitive to plasma insulin levels (28). Under these conditions, fatty acids entering the liver might be preferentially oxidized for energy, and the remaining low GPAT activity would suffice for a minimal demand for TAG synthesis for storage or for VLDL secretion. Consistent with this idea and with our *in vitro* results, liver TAG content was similar in fasting control, *Gpat1*<sup>-/-</sup>, and *Gpat4*<sup>-/-</sup> mice. After feeding in humans, concomitant with a rise in plasma insulin, both dietary fatty acids and those derived from *de novo* synthesis are incorporated into TAG (28). Because the TAG content in *Gpat1*<sup>-/-</sup> liver was lower than in controls after high sucrose refeeding, it is likely that insulin-induced SREBP1c activity drove fatty acid flux toward TAG storage; however, in the absence of GPAT1, the remaining GPAT activity was unable to meet the demand for higher TAG synthesis.

Because *Gpat1*<sup>-/-</sup> mice fed low or high fat diets for 4 months had a higher content of plasma and liver acylcarnitines and higher concentrations of plasma  $\beta$ -hydroxybutyrate, we had speculated that GPAT1 might normally divert fatty acids away from CPT1 and  $\beta$ -oxidation (12). The present results directly support this hypothesis by showing that when compared with controls, *Gpat1*<sup>-/-</sup> hepatocytes produced twice as much ASM from [<sup>14</sup>C]palmitate and that plasma  $\beta$ -hydroxybutyrate and hepatic acylcarnitines were markedly elevated in *Gpat1*<sup>-/-</sup> mice under both fasted and re-fed conditions. In contrast, ASM production was similar in *Gpat4*<sup>-/-</sup> and control hepatocytes under both conditions, indicating that lack of GPAT4 did not increase the use of excess acyl-CoAs for  $\beta$ -oxidation.

To directly compare  $\beta$ -oxidation metabolites from the two *Gpat* null genotypes, we investigated acylcarnitines. After a 24-h fast, the hepatic content of long-chain acylcarnitines was equivalent in control and *Gpat4*<sup>-/-</sup> mice. Confirming the hypothesis that GPAT1 competes with CPT1 for acyl-CoAs and diverts them away from mitochondria, the content of long-chain acylcarnitines in *Gpat1*<sup>-/-</sup> livers was 3-fold higher than controls. Thus, despite the observation that exogenous fatty acid incorporation into TAG was unaffected by a deficiency of either GPAT1 or GPAT4, these results further support the unique function of GPAT1 in diverting fatty acids away from  $\beta$ -oxidation.

These studies clearly differentiate the roles of the major hepatic isoforms, GPAT1 and GPAT4. GPAT1, but not GPAT4, reciprocally regulates hepatic *de novo* glycerolipid syn-

thesis and fatty acid oxidation. In this role, the absence of GPAT1 protects mice from high fat diet- and genetic-induced hepatic steatosis and hepatic insulin resistance (10). Conversely, the increased expression of GPAT1 may contribute to insulin resistance, both by diverting fatty acids away from oxidation and toward glycerolipid synthesis and by increasing lipid intermediates such as diacylglycerol and phosphatidic acid, which impair hepatic insulin signaling (11, 32).

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