Glycerol-3-phosphate acyltransferase-4-deficient mice are protected from diet-induced insulin resistance by the enhanced association of mTOR and rictor

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Zhang C, Cooper DE, Grevengoed TJ, Li LO, Klett EL, Eaton JM, Harris TE, Coleman RA. Glycerol-3-phosphate acyltransferase-4-deficient mice are protected from diet-induced insulin resistance by the enhanced association of mTOR and rictor. Am J Physiol Endocrinol Metab 307: E305–E315, 2014. First published June 17, 2014; doi:10.1152/ajpendo.00034.2014.—Glycerol-3-phosphate acyltransferase (GPAT) activity is highly induced in obese individuals with insulin resistance, suggesting a correlation between GPAT function, triacylglycerol accumulation, and insulin resistance. We asked whether microsomal GPAT4, an isoform regulated by insulin, might contribute to the development of hepatic insulin resistance. Compared with control mice fed a high-fat diet, Gpat4−/− mice were more glucose tolerant and were protected from insulin resistance. Overexpression of GPAT4 in mouse hepatocytes impaired insulin-suppressed gluconeogenesis and insulin-stimulated glycogen synthesis. Impaired glucose homeostasis was coupled to paired insulin-suppressed gluconeogenesis and insulin-stimulated resistance. Overexpression of GPAT4 in mouse hepatocytes inhibited rictor’s association with the mammalian target of rapamycin (mTOR), and mTOR complex 2 (mTORC2) activity. Compared with overexpressed GPAT3 in mouse hepatocytes, GPAT4 overexpression increased phosphatidic acid (PA), especially di16:0-PA. Conversely, in Gpat4−/− hepatocytes, both mTOR/rictor association and mTORC2 activity increased, and the content of PA in Gpat4−/− hepatocytes was lower than in controls, with the greatest decrease in 16:0-PA species. Compared with controls, liver and skeletal muscle from Gpat4−/−-deficient mice fed a high-fat diet were more insulin sensitive and had a lower hepatic content of di16:0-PA. Taken together, these data demonstrate that a GPAT4-derived lipid signal, likely di16:0-PA, impairs insulin signaling in mouse liver and contributes to hepatic insulin resistance.

lipid metabolism; phosphatidic acid; glycerolipids; diabetes; insulin signaling; mammalian target of rapamycin complex 2

THE METABOLIC SYNDROME is a cluster of derangements that includes central obesity, glucose intolerance, dyslipidemia, hypertension, and insulin resistance (16). Insulin resistance is not only a feature of the metabolic syndrome but has also been proposed to be a unifying condition that underlies the pathophysiology of the other metabolic syndrome elements (25). Excessive triacylglycerol (TAG) accumulation in nonadipose tissues, particularly in liver (27), is strongly associated with insulin resistance, suggesting the presence of a mechanistic link between hepatic lipid synthesis and insulin signaling. We have shown that the glycerolipid synthetic pathway initiated by the glycerol-3-phosphate acyltransferase isoform 1 (GPAT1) produces lipid intermediates that inhibit insulin signaling and impair the ability of insulin to diminish hepatic gluconeogenesis (22, 39).

The pathway of de novo TAG biosynthesis produces three potential signaling lipid intermediates, lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG). Glycerol-3-phosphate is acylated by glycerol-3-phosphate acyltransferase (GPAT) to form LPA (8). LPA is esterified by acyl-GPAT to form PA, which is hydrolyzed by phosphatidic acid phosphohydrolase (LIPIN) to form DAG. The DAG is esterified by DAG acyltransferase to produce TAG. Reverse reactions in this pathway are catalyzed by adipose TAG lipase and DAG kinase to form DAG and PA, respectively (8).

Among the enzymes that participate in de novo glycerolipid synthesis, the four GPAT isoforms are particularly important because they catalyze the rate-limiting step (8). Gpat1 is a target of the transcription factors sterol regulatory element-binding protein-1 (SREBP1), which is regulated by insulin, and of carbohydrate-responsive element-binding protein (ChREBP), which is regulated by carbohydrate (8). The absence of Gpat1 in ob/ob mice markedly diminishes hepatic steatosis (38). Hepatic GPAT activities increase 55% in diet-induced obese mouse models, and GPAT1 activity is 2.2-fold higher in ob/ob mice than in lean controls (8, 38), suggesting an association between GPAT activity and obesity-related metabolic disorders. GPAT1 resides in the outer mitochondrial membrane and contributes 30–50% of total GPAT activity in liver (8). Compared with wild-type mice, Gpat1−/− liver contains less hepatic DAG and TAG and is protected from insulin resistance induced by a high-fat diet (24). Conversely, adenovirus-mediated hepatic overexpression of GPAT1 in rats increases the hepatic content of DAG and TAG and induces hepatic insulin resistance within 1 wk without weight change or a high-fat diet (22). A GPAT1-derived lipid signal, believed to be DAG, appeared to interrupt hepatic insulin signaling by activating PKCe.

The endoplasmic reticulum (ER) isoforms GPAT3 and GPAT4 contribute about 20 and 50%, respectively, of total GPAT activity in liver (23). The phenotype of GPAT3-deficient mice has not been reported, but Gpat4−/− mice have 45% less TAG in liver than controls and are protected from diet-
induced and genetic obesity (34). Insulin causes both GPAT3 and -4 to be phosphorylated and activated in a wortmannin-sensitive manner (29).

Because GPAT3 and -4 both reside in the ER, where the terminal enzymes of glycerolipid synthesis are located (3, 35), we hypothesized that, like GPAT1, they would also link hepatic lipid synthesis and accumulation with insulin resistance. Instead, our results demonstrate that GPAT4, but not GPAT3, produces a signal that inhibits mammalian target of rapamycin (mTOR) complex 2 (mTORC2) kinase activity and

**Fig. 1.** Glycerol-3-phosphate acyltransferase-4-deficient (Gpat4-/-) mice were protected from high-fat diet (HFD)-induced insulin resistance. Oral glucose tolerance (A and B) and i.p. insulin tolerance (C and D) tests were performed on male and female Gpat4-/- mice and littermate controls fed a high-fat safflower oil diet; n = 5–10 for each group of each sex. AUC, area under the curve; AAC, area above the curve. *P < 0.05 vs. wild type (WT).

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**Fig. 2.** Overexpressing GPAT4, but not GPAT3, in mouse primary hepatocytes impaired hepatic glucose production and glycogen synthesis. Mouse primary hepatocytes were infected for 24 h with EGFP, Flag-Gpat1, Flag-Gpat3, or Flag-Gpat4 adenoviruses. Cells were lysed and subjected to Western blotting with anti-Flag antibodies (A) or scraped from the dish, centrifuged to obtain total particulate preparations, and assayed for GPAT activity (B). C: representative images of cells with overexpressed EGFP, GPAT3, or GPAT4 infected with CellLight ER-RFP to stain endoplasmic reticulum (ER; red) or incubated with MitoTracker CMXRos (red) to stain mitochondria. GPAT3 and GPAT4 were identified with antibodies to the Flag epitope (green). Cells were treated with or without insulin (100 nM) for 10 min and then assayed for glucose secretion (D) or glucose incorporation into glycogen (E). Glycogen content in livers from male Gpat4-/- mice and littermate controls (n = 5; F). CD, control diet. *P < 0.05 vs. EGFP total (B), EGFP basal (D), or basal of the same group (E); #P < 0.05 vs. EGFP N-ethylmaleimide (NEM)-resistant (B) or EGFP stimulated with insulin (D and E); *P < 0.05 vs. EGFP basal (E).
insulin signaling, thereby contributing to the development of hepatic insulin resistance.

RESEARCH DESIGN AND METHODS

Animals and dietary treatment. Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Gpat1−/− mice were generated as previously described (14), and Gpat4−/− mice (originally designated AGPAT6−/−) were provided by Dr. Karen Reue (UCLA) (34). Gpat1−/− and Gpat4−/− male and female mice and their wild-type littermates (C57BL/6J background; back-crossed 8–10 times) were housed in an air-conditioned facility with access to food (Prolab SP76 Isopro 3000, 5.4% fat by weight) and water ad libitum with a 12:12-h light-dark schedule. For high-fat diet (HFD) experiments, 8- to 10-wk-old mice were fed a safflower oil diet (59% fat-derived calories, no sucrose, no. 112245; Dyets, Bethlehem, PA) or a matched control diet (10% fat-derived calories, no sucrose, no. 110700, Dyets) for 3–5 wk. Animals were housed in an air-conditioned facility with access to food (Prolab 5P76 Lysates were mixed 1:1 with 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.3% Triton X-100. Hepatocytes were harvested in 20 mM Tris·HCl, pH 7.5, 0.1 mM Na3VO4, 25 mM NaF, 25 mM glycerophosphate, 2 mM EGTA, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.3% Triton X-100. Lysates were mixed 1:1 with 2× Laemmli sample buffer, boiled, and loaded on SDS-PAGE. Horseradish peroxidase-conjugated secondary antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate and X-ray film exposure. The film was converted to digital images with an Epson scanner (Perfection 2400), images were cropped using Photoshop CS2, and band densities were determined with ImageJ software.

Western blot analysis and reagents. Primary antibodies were from Cell Signaling Technology (Boston, MA) unless otherwise indicated. Anti-PI3K p85 antibody was from EMD Millipore. Secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate were from ThermoFisher Scientific (Pittsburgh, PA). Anti-β-actin, anti-tubulin antibodies, bovine serum albumin (BSA; fatty acid free), insulin (human recombinant), sodium D-lactate, phosphatase inhibitor cocktails 1 and 2, Percoll, ATP, and CHAPS were from Sigma. Type I collagenase was from Worthington Biochemical (Lake-wood, NJ). Protease inhibitor tablets were from Roche (Branford, CT). Inactive Akt1 was from SignalChem (British Columbia, Canada). Cell culture reagents were from Invitrogen (Carlsbad, CA). Hepatocytes were harvested in 20 mM Tris·HCl, pH 7.5, 0.1 mM Na3VO4, 25 mM NaF, 25 mM glycerophosphate, 2 mM EGTA, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.3% Triton X-100. Lysates were mixed 1:1 with 2× Laemmli sample buffer, boiled, and loaded on SDS-PAGE. Horseradish peroxidase-conjugated secondary antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate and X-ray film exposure. The film was converted to digital images with an Epson scanner (Perfection 2400), images were cropped using Photoshop CS2, and band densities were determined with ImageJ software.

Fig. 3. Overexpressed GPAT4, but not GPAT3, inhibited Akt phosphorylation and downstream signaling. Mouse primary hepatocytes overexpressing EGFP, Flag-GPAT4, or Flag-GPAT3 were treated with or without insulin (100 nM) for 10 min, followed by cell lystate preparation for Western blotting. A, C, E: representative Western blots from 3–4 independent experiments. B, D, F: quantitative analysis of data from A, C, and E. *P < 0.05 vs. basal within EGFP; #P < 0.05 vs. EGFP between the same group (basal or insulin treatment).
Hepatocyte infection. Recombinant Gpat1-FLAG adenovirus and Ad-EGFP were constructed and purified as described (18). Gpat3 cDNA was cloned from an adipocyte cDNA library, with a Flag-tag (Clontech, Mountain View, CA) at the carboxyl terminus. Gpat4 cDNA was cloned as described (23). Gpat3-Flag and Gpat4-Flag constructs were subcloned (AdEasy adenoviral vector system; Stratagene, La Jolla, CA). The UNC Gene Therapy Core Facility packaged and titered EGFP, Gpat1, Gpat3, and Gpat4 adenoviruses. MOIs (multiplicities of infection) of 20 were used for mouse hepatocyte infection, based on pilot experiments to obtain high infection efficiency without toxic effects. Hepatocytes were isolated from livers of 8- to 15-wk-old male mice (39) and cultured overnight in William’s E medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 2 mM glucose.

Confocal microscopy. Isolated hepatocytes grown on coverslips were infected with Ad-Gpat3 or Ad-Gpat4 for 24 h and with CellLight ER-RFP (Invitrogen) for 16 h. Live hepatocytes were incubated with 200 nM MitoTracker CMXRos (Invitrogen) for 30 min. Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked in 0.8% BSA, 1% gelatin for 30 min. Cells were incubated with mouse monoclonal anti-FLAG antibody (1:1,000) for 2 h at 37°C, washed, and incubated with secondary antibody (Alexa fluor 647 goat anti-mouse, Invitrogen) at 1:400 for 1 h at 21°C. Cells were mounted on glass slides using Prolong Gold (Invitrogen) and set overnight. Images were captured on a Zeiss 510 Meta Inverted Laser Scanning Confocal microscope and processed on Zeiss LSM Image Browser Software.

Glucose output and incorporation assays. Mouse primary hepatocytes overexpressing EGFP, Gpat1, Gpat3, or Gpat4 were cultured overnight in six-well plates in DMEM without FBS. For glucose output, the medium was replaced with 1 ml of glucose-free DMEM supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate, with or without insulin (100 nM) (39). After a 2-h incubation, medium was collected, and glucose concentration was measured colorimetrically. Glucose concentrations were normalized to total protein content in the cell lysates. For glucose incorporation, hepatocytes were incubated with 1.5 μCi of [U-14C]glucose (PerkinElmer, Santa Clara, CA) plus 100 nM insulin or vehicle for 2 h. Cells were washed three times with ice-cold PBS and scraped into 0.3 ml of 10 N KOH. After addition of carrier glycogen (4 mg), samples were boiled for 30 min, precipitated with two volumes of 95% ethanol overnight at 4°C, and then centrifuged at 10,000 g for 10 min. The pellets were washed once with 66% ethanol, resuspended in 0.5 ml of water, and subjected to scintillation counting (17).

Glycogen content assay. Male Gpat4-deficient and littermate control mice fed a control diet or a HFD were fasted for 6 h and anesthetized. Livers were removed, frozen in liquid nitrogen, and powdered with a mortar and pestle. About 20 mg of powder from each liver was transferred to a 2-ml microcentrifuge tube and weighed. Then, 0.5 ml of 1 N HCl was added, and the mixture was homogenized with a Pro250 Scientific homogenizer at speed 5. For the blank, 100 μl of the homogenate was transferred to a new microcentrifuge tube, and 100 μl of 1 N NaOH was added. To hydrolyze glycogen, homogenate samples (100 μl) were transferred to microcentrifuge tubes and heated at 95°C for 90 min. The samples were cooled to room temperature and shaken on an orbital shaker at 120 rpm for 1 h. 1 N NaOH (100 μl) was added and vortexed, and samples and blanks were centrifuged at 13,000 rpm for 10 min. Glucose was measured in the supernatants (Autokit glucose assay kit; Wako, Richmond VA).

PI 3-kinase assay. Mouse hepatocytes were lysed on ice with cold lysis buffer (40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM Na3VO4, EDTA-free protease inhibitors, 1% phosphatase inhibitor mixture 2 and 3, 0.3% CHAPS). Cell lysates were centrifuged at 13,000 g for 10 min, and supernatants were transferred to new tubes. p85 Protein was immunoprecipitated and used for the PI 3-kinase assay by ELISA (Echelon:Pico, Echelon Biosciences, Salt Lake City, UT).

**Fig. 4.** Overexpressed GPAT4 did not alter mTOR complex 1 (mTORC1) or IRS-1/PI3K signaling. Mouse primary hepatocytes overexpressing EGFP or Flag-GPAT4 were treated with or without insulin (100 nM) for 10 min or with or without rapamycin (100 nM) for 45 min. Cells were lysed and subjected to Western blotting or immunoprecipitation (IP) followed by Western blotting or assay of PI 3-kinase. A and C: representative Western blots of lysates from 3–4 independent experiments. B and D: quantitative analysis of data from A and C. E: PI 3-kinase assay *P < 0.05 vs. basal of EGFP or GPAT4; #P < 0.05 vs. EGFP basal.
PA content. Total cell or tissue lipid was extracted (12), and PA content was analyzed by LC-MS (21). The amount of each PA species in the biological samples was calculated from the peak areas obtained, using software that controls the LC-MS system ( Analyst 1.5, Applied Biosystems). Raw peak areas were corrected for recovery and sample loading, and transformed into amounts of analyte, using standard curves made with commercially obtained glycerolipids. Glycerolipids were quantified with 0.1 nmol 17:0 LPA as an internal standard to correct for recovery. PA was quantified and normalized to protein concentrations of the cellular lysates.

**GPAT activity.** Samples were homogenized in 10 mM Tris, pH 7.4, 250 mM sucrose, 1 mM DTT, and 1 mM EDTA and centrifuged at 100,000 g for 1 h. The membrane pellet was rehomogenized in 10 mM Tris, pH 7.4, 250 mM sucrose, and 1.0 mM EDTA. Protein concentrations were determined by the bicinchoninic acid method with BSA as the standard. GPAT specific activity was assayed for 10 min at 25°C with 800 μM [3H]glycerol-3-phosphate and 82.5 μM palmitoyl-CoA (4). The reaction was initiated with 10–30 μg of total membrane protein after incubating the membrane protein on ice for 15 min in the presence or absence of 1 mM N-ethylmaleimide (NEM), which inhibits GPAT2, -3, and -4 but not GPAT1 (8). NEM-sensitive GPAT activity was calculated by subtracting the NEM-resistant GPAT1 activity from total GPAT activity.

**Immunoprecipitation and kinase activity.** Immunoprecipitation (IP) of rictor and the mTORC2 kinase assay were performed as described (28). Western blots were probed with phospho-Akt (Ser473) to indicate mTORC2 activity.

**Statistical analysis.** Values are expressed as means ± SE. Comparisons between groups (adenoviral-delivered gene overexpression of EGFP and GPAT1, -3, or -4, or different genotypes of wild-type, Gpat1<sup>−/−</sup>, or Gpat4<sup>−/−</sup>) of the same treatment (basal, insulin-stimulated, or NEM-resistant) or the same lipid species (LPA, PA, or DAG) were determined using Student’s two-tailed t-test (using EGFP-infected or wild-type cells or tissues as the control). Data represent at least three independent experiments. P ≤ 0.05 was considered significant.

**RESULTS**

Gpat4<sup>−/−</sup> mice fed HFD were protected from diet-induced glucose intolerance and insulin resistance. Because Gpat1<sup>−/−</sup> mice are protected from HFD-induced insulin resistance (24) and hepatocytes from Gpat1<sup>−/−</sup> mice show increased insulin signaling (39), we examined Gpat4<sup>−/−</sup> mice to determine whether a deficiency in the major ER GPAT isoform similarly improved glucose and insulin homeostasis. Livers from GPAT4-deficient mice do not show any compensatory increase in Gpat1 mRNA or in NEM-resistant GPAT activity (37). To induce insulin resistance, mice were fed a diet containing 59% kcal from fat (HFD), primarily safflower oil, or a matched control diet (24). Both genotypes fed the control diet had similar fasting glucose concentrations (males: control 115 ± 16, Gpat4<sup>−/−</sup> 123 ± 16 mg/dl; females: control 109 ± 19, Gpat4<sup>−/−</sup> 100 ± 16 mg/dl) and similar oral glucose tolerance curves (data not shown). When fed the HFD, Gpat4<sup>−/−</sup> mice were more glucose tolerant and insulin sensitive than controls (Fig. 1, A–D). These results show that GPAT4 deficiency protected mice from HFD-induced insulin resistance and suggested a common mechanism whereby hepatic GPAT4, like GPAT1 (39), modulates insulin sensitivity.

**Overexpression of GPAT4, but not GPAT3, impaired hepatic glucose production and glycogen synthesis.** We reported that overexpressing GPAT1, the mitochondrial isoform, in rat liver increases hepatic TAG and induces hepatic insulin resistance (22) and that GPAT1 overexpression in mouse hepatocytes diminishes insulin-mediated suppression of glucose secretion by blocking the formation of the mTORC2 complex (39). To determine whether the microsomal GPAT isoforms might also regulate hepatic insulin signaling and to confirm our observations in the Gpat4<sup>−/−</sup> mice, we overexpressed GPAT3 or GPAT4 in mouse hepatocytes. On the basis of our previous studies, we used the overexpression of GPAT1 as a positive control for impaired insulin signaling (39). Overexpression was confirmed by Western blotting (Fig. 2A) and by 5-fold (GPAT1), 3.5-fold (GPAT3), and 4.5-fold (GPAT4) higher total GPAT activity than in control cells (Fig. 2B). Overexpressing GPAT1, which is resistant to NEM inactivation, increased NEM-resistant GPAT activity; in contrast, overexpressing GPAT3 or GPAT4, which are inactivated by NEM, increased NEM-sensitive GPAT activity (Fig. 2B). Overexpressed GPAT3 and GPAT4 were both located on the ER (Fig. 2C). In control cells, insulin suppressed glucose output and stimulated glycogen synthesis nearly 50% and 65%, respectively (Fig. 2, D and E). Consistent with impaired insulin function, in hepatocytes that overexpressed GPAT1 or GPAT4, insulin failed to suppress glucose secretion or to stimulate glycogen synthesis. In contrast to GPAT4, GPAT3 overexpression did not diminish the effects of insulin (Fig. 2, D and E), and liver glycogen content was higher in Gpat4<sup>−/−</sup> mice than in controls fed the same low-fat diet (Fig. 2F), suggesting that the two microsomal GPAT isoforms perform distinct functions despite being located on the same subcellular organelle. Taken to-
together, these findings suggest that GPAT4-derived signals, but not those from GPAT3, modulate insulin signaling.

Overexpression of GPAT4, but not GPAT3, inhibited Akt-mediated signaling. Insulin suppresses hepatic gluconeogenesis by inhibiting forkhead box 01 (Foxo1)-mediated transcriptional inhibition of phosphoenolpyruvate carbox kinase and glucose-6-phosphatase (7), and it promotes glycerogen synthesis by activating glycogen synthase kinase-3 (GSK3) (36). To investigate the mechanism by which GPAT4 impaired the ability of insulin to suppress hepatic glucose production and insulin-stimulated glycerogen synthesis, we isolated primary hepatocytes from wild-type mice, overexpressed GPAT4 in these cells, and examined the effect of insulin on Foxo1 phosphorylation at Ser256 and GSK3α phosphorylation at Ser34, sites that are crucial for transcriptional activity of Foxo1 and enzymatic activity of GSK3α. Compared with EGFP overexpression, in hepatocytes that overexpressed GPAT4, phosphorylation at these sites was significantly inhibited (Fig. 3, A and B). Both Foxo1 and GSK3α respond to insulin action through Akt (2, 9), and GPAT4 overexpression blocked insulin-stimulated phosphorylation of Akt at Ser473 and Thr308, the two sites required for full activation (1, 28) (Fig. 3, C and D). In contrast, GPAT3 overexpression did not alter insulin-stimulated phosphorylation of Akt at Ser473 or Thr308 (Fig. 3F). Thus, overexpression of GPAT4 impaired hepatic glucose metabolism by interrupting insulin-stimulated Akt signaling, but overexpression of GPAT3 did not.

GPAT4 overexpression altered IRS-1/Pi3K signaling. Signaling to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1 activation to Akt includes positive inputs from mTORC2 and PI3K and negative inputs via mTORC1

Fig. 6. Gpat4−/− hepatocytes showed increased mTORC2/Akt signaling and enhanced rictor-mTOR association. Primary hepatocytes from CD-fed Gpat4−/− mice or littermate controls were treated with or without insulin (100 nM) for 10 min. Cells were lysed and subjected to Western blotting or IP. IP lysates were assayed for mTORC2 kinase activity (phosphorylation of exogenously added Akt) followed by Western blotting. A and C: representative Western blots from 3–4 independent experiments. B and D: quantitative analysis of data from A and C. *P < 0.05 vs. basal within EGFP; #P < 0.05 vs. EGFP between the same group (basal or insulin treatment).

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E310 GPAT4- DERIVED LIPOIDS CONTRIBUTE TO INSULIN RESISTANCE

Fig. 7. Gpat4−/− hepatocytes showed decreased pFoxo1 (S256) and pGSK3α (S21) and increased pAkt (S473). Hepatocytes from wild-type mice, overexpressed GPAT4, and littermate controls were treated with or without insulin (100 nM) for 10 min. Cells were lysed and subjected to Western blotting. A: representative Western blots from 3–4 independent experiments. B: quantitative analysis of data from A. *P < 0.05 vs. basal within EGFP; #P < 0.05 vs. EGFP between the same group (basal or insulin treatment).
cause Akt phosphorylation at Thr\textsuperscript{308} is dependent on prior phosphorylation at Ser\textsuperscript{473} (26).

**GPAT4 overexpression inhibited mTORC2 activity.** To investigate the third major positive input to Akt signaling, we tested the mTORC2 pathway. GPAT4 overexpression markedly reduced rictor-associated mTOR protein and significantly inhibited mTORC2 activity, as represented by reduced Akt phosphorylation at Thr\textsuperscript{308} (Fig. 5, A and B). To further confirm that mTORC2 mediated the inhibition by GPAT4 of insulin signaling, we examined phospho-NDRG1 (N-myc downstream-regulated gene 1), which lies downstream of the mTORC2 target SGK1 and is a specific readout of mTORC2 activity (13). GPAT4 overexpression significantly blocked insulin-stimulated NDRG1 phosphorylation (Fig. 5, C and D), consistent with disrupted signaling by mTORC2. These results indicate that GPAT4 overexpression inhibited insulin signaling by disrupting the mTOR/rictor association, thereby inhibiting mTORC2 activity.

**mTORC2/Akt signaling and rictor/mTOR association were higher in Gpat4\textsuperscript{+/−} hepatocytes.** To confirm the physiological role of GPAT4 in response to insulin, we tested hepatocytes from Gpat4\textsuperscript{+/−} mice and littermate controls. Compared with controls, hepatocytes lacking GPAT4 showed enhanced insulin-stimulated phosphorylation of Akt(Ser\textsuperscript{473}), of GSK3α (Ser\textsuperscript{21}) of Foxo1 (Ser\textsuperscript{256}), and of NDRG1 (Thr\textsuperscript{346}) (Fig. 6, A and B). These changes were accompanied by enhanced association of mTOR and rictor and higher mTORC2 activity (Fig. 6, C and D). The results are consistent with a model whereby GPAT4-derived lipid signals interfere with the association of mTOR and rictor and alter downstream insulin signaling.

**Insulin sensitivity was greater in Gpat4\textsuperscript{+/−} liver and skeletal muscle.** To confirm that the liver is involved in the GPAT4 deficiency-initiated improvement in glucose tolerance, we examined insulin signaling in liver and gastrocnemius muscle from Gpat4\textsuperscript{+/−} mice and littermate controls fed a HFD. In Gpat4\textsuperscript{+/−} liver, basal and insulin-stimulated phosphorylation of Akt at Ser\textsuperscript{473} increased (Fig. 7, A and B), indicating that liver is indeed involved in GPAT4-related regulation of insulin signaling and glucose homeostasis. Plasma insulin 15 min after oral glucose was similar for both genotypes (Fig. 7C), showing that the improved glucose tolerance in Gpat4\textsuperscript{+/−} mice was not due to an increase in insulin secretion. Interestingly, Gpat4\textsuperscript{+/−} mice also had increased insulin-stimulated phosphorylation of Akt at Ser\textsuperscript{473} in the gastrocnemius muscle (Fig. 7, A and B), suggesting that the global GPAT4 knockout enhanced insulin signaling in multiple tissues, although we cannot exclude the possibility that the enhanced insulin sensitivity in the muscle was a consequence of the increased insulin signaling in liver.

**di16:0-PA increased in hepatocytes overexpressing GPAT4 and was low in Gpat4\textsuperscript{+/−} hepatocytes.** PA is the product of phospholipase D-mediated hydrolysis of membrane phospholipids, and it has been suggested that 1) this PA is essential for activation of mTORC1 signaling (10, 11), and 2) that insulin activation of the mTORC1/S6K1 pathway causes serine phosphorylation of IRS-1, thereby inhibiting insulin-mediated activation of PI 3-kinase and Akt (20, 32). PA is also synthesized by the GPAT-initiated pathway of TAG synthesis (8). GPAT1 has a preference for 16:0-CoA, and the inhibitory effect of GPAT1 on mTOR correlates closely with the formation of PA species that contain 16:0, particularly di16:0-PA (39). GPAT4...
also has a mild preference for 16:0-CoA compared with 18:0-CoA, whereas GPAT3 exhibits no acyl-CoA specificity (6, 39). To determine whether the inhibition of insulin signaling mediated by GPAT4 also correlates with PA content, we analyzed mouse hepatocytes after overexpressing EGFP, GPAT3, or GPAT4. GPAT4 overexpression increased total PA content about twofold, which was totally attributable to an increase in PA species that contained 16:0 (2.6-fold increase); the content of other PA species did not change (Fig. 8, A and B). Among the 16:0-containing PA species, the largest increase was of di16:0-PA (7.7-fold) (Fig. 8B and Table 1), suggesting an association between impaired hepatic insulin signaling and increased 16:0-PA. In contrast, GPAT3 overexpression increased the total content of 16:0-containing PA only 20%, and very little di16:0-PA was present (Fig. 8, A and B, and Table 1). In vivo measurements in GPAT-deficient mice were consistent with these observations (Fig. 8, C and D, and Table 1). Compared with wild-type hepatocytes from control diet-fed mice, Gpat1−/− and Gpat4−/− hepatocytes contained 45% and 38% less 16:0-containing PA, respectively, and 64% and 59% less di16:0-PA, respectively, consistent with the association of diminished di16:0-PA and improved hepatic insulin signaling. These results suggest that 16:0-PA species, especially di16:0-PA, mediate the inhibition of insulin signaling and contribute to hepatic insulin resistance.

**Improved insulin sensitivity and protection from HFD-induced insulin resistance in Gpat4−/− mice is associated with decreased hepatic di16:0-PA content.** Because these studies showed a correlation between impaired insulin signaling and increased PA content, we examined PA in liver from HFD-fed mice. Although the difference in total PA content in Gpat4−/− mice compared with controls was not statistically significant (Fig. 9A), Gpat4−/− male and female liver contained 47% less di16:0-PA (Fig. 9B), and the content of PA species that contained at least one 16:0 was 15 and 21% lower for females and males, respectively (Fig. 9C). These data are consistent with the cell culture data showing that Gpat1−/− and Gpat4−/− hepatocytes contained less 16:0 and di16:0-PA (Fig. 8, C and D) and provide additional evidence that cellular 16:0-PA, especially di16:0-PA, is associated with impaired hepatic insulin signaling and the dissociation of rictor and mTOR (39).

**DISCUSSION**

TAG biosynthesis is critical for energy homeostasis, absorption of dietary lipids and fat-soluble vitamins, and

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**Table 1. Content of PA species in mouse primary hepatocytes overexpressing EGFP, GPAT3, or GPAT4 or from WT or GPAT1 and GPAT4 knockout mice (pmol/mg protein)**

<table>
<thead>
<tr>
<th>PA Species</th>
<th>EGFP</th>
<th>GPAT3</th>
<th>GPAT4</th>
<th>WT</th>
<th>Gpat1−/−</th>
<th>Gpat4−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0–16:0</td>
<td>0.62 ± 0.02</td>
<td>0.95 ± 0.10*</td>
<td>4.76 ± 0.31*</td>
<td>0.69 ± 0.11</td>
<td>0.25 ± 0.1*</td>
<td>0.28 ± 0.13*</td>
</tr>
<tr>
<td>16:0–18:0</td>
<td>0.70 ± 0.02</td>
<td>0.84 ± 0.01*</td>
<td>1.81 ± 0.07*</td>
<td>0.83 ± 0.05</td>
<td>0.54 ± 0.11*</td>
<td>0.47 ± 0.14*</td>
</tr>
<tr>
<td>16:0–18:1</td>
<td>8.66 ± 0.20</td>
<td>9.94 ± 0.19*</td>
<td>17.97 ± 0.67*</td>
<td>8.71 ± 0.94</td>
<td>5.05 ± 1.51*</td>
<td>6.37 ± 1.27*</td>
</tr>
<tr>
<td>16:0–18:2</td>
<td>1.62 ± 0.06</td>
<td>2.22 ± 0.08*</td>
<td>5.56 ± 0.31*</td>
<td>2.37 ± 0.30</td>
<td>0.65 ± 0.22*</td>
<td>0.66 ± 0.15*</td>
</tr>
<tr>
<td>18:0–18:0</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.35 ± 0.14*</td>
<td>0.17 ± 0.02</td>
<td>0.12 ± 0.05</td>
<td>0.09 ± 0.02*</td>
</tr>
<tr>
<td>18:0–18:1</td>
<td>1.07 ± 0.24</td>
<td>1.20 ± 0.06*</td>
<td>3.47 ± 0.25*</td>
<td>1.27 ± 0.21</td>
<td>1.54 ± 0.49</td>
<td>0.94 ± 0.21</td>
</tr>
<tr>
<td>18:1–18:1</td>
<td>4.76 ± 0.26</td>
<td>6.51 ± 0.27*</td>
<td>1.16 ± 0.00*</td>
<td>2.14 ± 0.22</td>
<td>1.94 ± 0.24*</td>
<td>2.34 ± 0.52</td>
</tr>
</tbody>
</table>

Values represent means ± SE of 3 independent experiments performed in triplicate. PA, phosphatidic acid; GPAT, glycerol-3-phosphate acyltransferase. *P < 0.05 vs. EGFP or wild-type (WT) control by Student’s t-test.
lipoprotein generation, but lipid accumulation in nonadipose tissues is highly associated with insulin resistance and type 2 diabetes (27). Several mechanisms for lipid-induced insulin resistance have been proposed, including interference with insulin signaling by excess acyl-CoA (19) or ceramide (5), or by one of the lipid intermediates produced in the pathway of TAG biosynthesis, LPA (30), PA (39), or DAG (24, 27). In the current study, we show that GPAT4, like GPAT1, produces di16:0-PA, which can dissociate the mTOR/rictor complex (39) and contribute to the development of hepatic insulin resistance. Because each GPAT isoform catalyzes the same enzymatic reaction, we asked whether increased expression of both microsomal isoforms, GPAT3 and -4, might cause hepatic insulin resistance. The current study shows that overexpressing GPAT4, but not GPAT3, inhibited insulin signaling in hepatocytes and that the Gpat4−/− mice were protected from HFD-induced insulin resistance.

To investigate the role of GPAT4 in hepatic insulin resistance, control and Gpat4−/− mice were fed a diet containing 59% kcal from fat (primarily safflower oil). Mice fed this diet for 3 wk do not become obese, but they develop impaired hepatic insulin sensitivity (24). Gpat4−/− mice fed the HFD remained more glucose tolerant and insulin sensitive than controls, suggesting a physiological role for GPAT4 in modulating the hepatic response to insulin.

To identify the mechanism by which the lack of GPAT4 protects mice from diet-induced hepatic insulin resistance, we investigated mTOR signaling. Compared with controls, hepatocytes lacking GPAT4 showed an increased association between rictor and mTOR, higher mTORC2 kinase activity, and greater basal and insulin-stimulated phosphorylation of Akt (Ser473), Akt (Thr308), Foxo1 (Ser256), and GSK3α (Ser21). In primary hepatocytes, GPAT1 overexpression disrupts the mTORC2 complex and impairs insulin-stimulated glycogen accumulation and insulin-suppressed glucose output (39). Overexpression of GPAT1 causes a 17-fold increase in PA, and a comparison of several species of LPA, PA, and DAG showed that only di16:0-PA was able to directly disrupt the mTORC2 complex. These results are not mutually incompatible; in each case, the different diets and incubation conditions are likely to have produced lipid intermediates with differing acyl-chain lengths or degrees of saturation that may determine the potency of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule.

When GPAT1 is overexpressed in rat liver, activated PKCe implicates DAG as a mediator of the lipid-induced insulin resistance (27). In isolated hepatocytes, overexpressed GPAT1 or GPAT4 increased the content of a PA that mediates lipid-induced insulin resistance via the dissociation of the mTORC2 complex. These results are not mutually incompatible; in each case, the different diets and incubation conditions are likely to have produced lipid intermediates with differing acyl-chain lengths or degrees of saturation that may determine the potency of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule. GPAT1 prefers acyl-CoA of a lipid intermediate as a signaling molecule.
GPAT4-DERIVED LIPIDS CONTRIBUTE TO INSULIN RESISTANCE
drate, whereas GPAT4 tends to use diet-derived fatty acids (37). GPAT1 and GPAT4 are likely to contribute to hepatic insulin responsiveness under different physiological conditions.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES