Glycerolipid signals alter mTOR complex 2 (mTORC2) to diminish insulin signaling

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Increased flux through the glycerolipid synthesis pathway impairs the ability of insulin to inhibit hepatic gluconeogenesis, but the exact mechanism remains unknown. To determine the mechanism by which glycerolipids impair insulin signaling, we overexpressed glycerol-3-phosphate acyltransferase-1 (GPAT1) in primary mouse hepatocytes. GPAT1 overexpression impaired insulin-stimulated phosphorylation of Akt-S473 and -T308, diminished insulin-suppression of glucose production, significantly inhibited mTOR complex 2 (mTORC2) activity and decreased the association of mTOR and rictor. Conversely, in hepatocytes from $Gpat1^{-/-}$ mice, mTOR-rictor association and mTORC2 activity were enhanced. However, this increase in mTORC2 activity in $Gpat1^{-/-}$ hepatocytes was ablated when rictor was knocked down. To determine which lipid intermediate was responsible for inactivating mTORC2, we overexpressed GPAT1, AGPAT, or lipin to increase the cellular content of lysophosphatidic acid (LPA), phosphatidic acid (PA), or diacylglycerol (DAG), respectively. The inhibition of mTOR/rictor binding and mTORC2 activity coincided with the levels of PA and DAG species that contained 16:0, the preferred substrate of GPAT1. Furthermore, di-16:0-PA strongly inhibited mTORC2 activity and disassociated mTOR/rictor in vitro. Taken together, these data reveal a signaling pathway by which phosphatidic acid synthesized via the glycerol-3-phosphate pathway inhibits mTORC2 activity by decreasing the association of rictor and mTOR, thereby down-regulating insulin action. These data demonstrate a critical link between nutrient excess, TAG synthesis, and hepatic insulin resistance.

triacylglycerol | palmitate | hepatic steatosis

Insulin resistance is frequently associated with triacylglycerol (TAG) accumulation in nonadipose tissues, suggesting that insulin signaling might be antagonized by a lipid metabolite (1, 2). Glycerol-3-phosphate acyltransferase (GPAT; EC2.3.1.15) catalyzes the first and committed step in TAG and glycerolipid synthesis by acylating glycerol-3-phosphate with a long-chain fatty acyl-CoA to form LPA (3). LPA is further acylated by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) to form PA, and then a PA phosphohydrolase (PAP/lipin) hydrolyzes PA to form DAG, which is esterified by diacylglycerol acyltransferase (DGAT) to produce TAG.

Each of the four isoforms of GPAT is encoded by a separate gene and distinguished by subcellular location, substrate preference, and sensitivity to N-ethylmaleimide (NEM) (3, 4). The mitochondrial isoform, GPAT1, is of special interest because its mRNA and activity are up-regulated by SREBP1c when hepatic lipogenesis and TAG synthesis are enhanced by insulin stimulation (3), and down-regulated by glucagon, fasting, and streptozotocin-induced diabetes (5). GPAT1 activity and mRNA are highest in tissues that have a high capacity for TAG synthesis, such as the liver, where GPAT1 contributes 30–50% of the total GPAT activity. Unlike GPAT-2, -3, and -4, GPAT1 prefers saturated acyl-CoA substrates, particularly palmitate (16:0) (3).

Altering the expression of GPAT1 exemplifies the link between elevated hepatic TAG content and insulin resistance. $Gpat1^{-/-}$ mice have markedly lower hepatic content of DAG and TAG and are protected from hepatic insulin resistance (6). Conversely, adenovirus-mediated overexpression of hepatic GPAT1 in rats increases the intracellular content of DAG and TAG and induces hepatic and peripheral insulin resistance (7). Although the mechanisms by which excess glycerolipids contribute to insulin resistance are unclear, each of the three lipid intermediates produced in the TAG synthetic pathway, LPA, PA, and DAG, are known signaling molecules. Accordingly, increased DAG content corresponds with the activation of PKCε, an inhibitor of insulin signaling (2). Additionally, PA released from membrane phospholipids by phospholipase D (PLD) may play a role in regulating another modulator of insulin signaling, the mammalian target of rapamycin (mTOR) (8).

mTOR is a Ser/Thr protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. mTOR is conserved throughout evolution and functions as a sensor that regulates cell growth and metabolism in response to nutrient availability and energy levels (9–11). mTOR exists in two distinct multiprotein complexes defined by their primary accessory proteins: mTOR complex 1 (mTORC1), which contains raptor (regulatory associated protein of Tor), and mTOR complex 2 (mTORC2), which contains rictor (rapamycin insensitive companion of T or) (12). mTORC1 activity is regulated by growth factors, nutrients, and energy levels (13). mTORC1 substrates S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) regulate cell growth, but activated S6K1 also provides feedback inhibition of insulin receptor substrate-1 (IRS1), thereby inhibiting insulin signaling (14). Although the regulation and substrates of mTORC2 are only now beginning to be deciphered, mTORC2 phosphorylates and activates Akt at Ser473, enhancing insulin signaling (15).

Because evidence suggests that exogenous PA and membrane-derived PA regulate mTORC1 and mTORC2 (15–17), we hypothesized that PA generated by de novo glycerolipid synthesis may also regulate mTOR signaling. In fact, overexpressing a microsomal GPAT isoform (GPAT3; LPAAT-theta) in HEK293T cells increases the phosphorylation of S6K1 (18). We postulate that lipid intermediates generated by the glycerolipid synthetic pathway allow mTOR to detect nutrient availability, not only from amino acids and glucose, but also from lipids. Nutrient-derived lipid activation of mTOR and subsequent inhibition of insulin signaling would provide a critical link between nutrient excess, TAG synthesis, and insulin resistance. Here, we present evidence that GPAT1-catalyzed glycerolipid synthesis produces lipid intermediate species that inhibit mTORC2 activity by decreasing the association between mTOR and rictor, thereby impairing insulin signaling.

MEDICAL SCIENCES

Results

Overexpressing GPAT1 in Hepatocytes Impairs Insulin Signaling. Ablation of *Gpat1* in mice results in diminished hepatic TAG content and protection against diet-induced hepatic insulin resistance (6), whereas overexpressing *Gpat1* increases hepatic TAG content and induces hepatic insulin resistance (7). To investigate the mechanisms by which increased glycerolipid synthesis affects insulin signaling, we overexpressed GPAT1 in primary mouse hepatocytes. Overexpression was confirmed by Western blotting (Fig. 1*A*) and by fourfold increases in total and NEM-resistant GPAT activity (Fig. 1*B*). Compared with EGFP-infected hepatocytes, GPAT1 overexpression impaired insulin-stimulated phosphorylation of Akt at both Thr308 and Ser473. Overexpressing GPAT1 also decreased the phosphorylation of targets directly downstream of Akt, Ser256-Foxo1 and Ser21-GSK3α, key enzymes involved in glucose metabolism (Fig. 1 *C* and *D*). Insulin suppresses hepatic gluconeogenesis through Foxo1 transcriptional regulation of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (19). In hepatocytes overexpressing GPAT1, insulin failed to suppress glucose output (Fig. 1*E*). In confirmation, GPAT1 overexpression also impaired insulin-stimulated phosphorylation of Akt and its downstream targets in 3T3-L1 and HEK 293T cells (Fig. S1). These data demonstrate that GPAT1 overexpression impairs insulin signaling.

GPAT1-Impaired Insulin Signaling Is Due to Decreased mTORC2 Activity. The effects of GPAT1 on hepatic insulin signaling are mediated, in part, by DAG activation of PKCε (20), but PA, which is derived from glycerolipid synthesis, may also regulate insulin signaling through mTOR. mTORC1 impairs insulin signaling via pS6K1-T389 phosphorylation and subsequent inactivation of IRS1. The impaired IRS1 signaling results in decreased insulin signaling through pAkt-T308. Although GPAT1 overexpression decreased pAkt-T308, mTORC1 signaling, characterized by phosphorylation of S6K1-T389 and IRS-S636/639, was not affected (Fig. S2 *A* and *B*). GPAT1 overexpression slightly reduced the amount of S6K1 protein ($P = 0.046$), but its

effect on total IRS1 protein was not statistically significant ($P = 0.054$) (Fig. S2 *A* and *B*). Because insulin action is depressed by enhanced mTORC1/S6K1 signaling (14), the reduced total S6K1 protein is not likely to account for the effect of GPAT1 overexpression. To understand whether the IRS1/PI3K node mediates the impairment in insulin signaling caused by GPAT1 overexpression, we examined IRS1-associated p85 and IRS1 tyrosine phosphorylation. GPAT1 overexpression did not alter either IRS1-associated p85 protein amount or tyrosine phosphorylation of IRS1 (Fig. S2 *C* and *D*). However, GPAT1 overexpression significantly suppressed mTORC2 kinase activity, marked by diminished phosphorylation of Akt-S473 (Fig. 2 *A* and *B*), and the reduced mTORC2 activity appeared to be due to decreased binding of mTOR and rictor (Fig. 2 *A* and *B*). GPAT1 overexpression reduced mLST8-rictor binding in a manner similar to that of mTOR-rictor, but did not affect Sin1-rictor binding (Fig. 2 *A* and *B*), although Sin1 is thought to play an important role in maintaining mTORC2 assembly (21). These effects were consistent in 3T3-L1 and HEK 293T cells (Fig. S3 *A* and *B*). Total protein levels of mTOR, rictor, Sin1 and mLST8 were not altered (Fig. 2 *A* and *B*), indicating that GPAT1 diminished only the association of mTOR-rictor and/or mLST8-rictor. To further confirm that mTORC2 mediated the inhibitory effect of GPAT1 overexpression on insulin signaling, we examined phosphor-NDRG1 (*N*-myc downstream-regulated gene 1), which occurs downstream of the mTORC2 target SGK1 and has been used as a specific readout of mTORC2 signaling (22). GPAT1 overexpression significantly blocked insulin stimulation of NDRG1 phosphorylation (Fig. 2 *C* and *D*), supporting the purported role of mTORC2.

When purified Flag-GPAT1 protein was added to the in vitro mTORC2 kinase activity assay, neither Akt phosphorylation nor mTOR-rictor binding was affected (Fig. S4 *A* and *B*), indicating that the critical feature of GPAT1 is its enzymatic activity. Taken together, these results suggest that one or more intermediates in the pathway of glycerolipid synthesis decreases mTOR-rictor assembly, thereby blocking mTORC2 activity and its downstream effects on insulin signaling.

***Gpat1*^{−/−} Hepatocytes Showed Increased mTORC2/Akt Signaling and Enhanced Association of Rictor and mTOR.** In contrast to GPAT1 overexpression, the absence of GPAT1 protects *Gpat1*^{−/−} mice against high-fat diet-induced hepatic insulin resistance (6). Compared with wild-type mice, primary hepatocytes cultured from high-fat fed *Gpat1*^{−/−} mice had enhanced insulin-stimulated phosphorylation of Akt-S473, coupled with increased association of mTOR and rictor and enhanced mTORC2 activity (Fig. 3 *A* and *B*). Knocking down rictor, however, decreased mTOR-rictor binding and nullified the *Gpat1*^{−/−}-enhanced insulin signaling (Fig. 3 *C* and *D*). These data again demonstrate that glycerolipids generated in the GPAT1-initiated pathway interfere with the association of mTOR and rictor and that the effects of GPAT1 on insulin signaling are facilitated through functional mTORC2 activity.

Impaired mTOR-Rictor Binding and mTORC2 Activity Is Associated with Increased PA and DAG Species That Contain 16:0. To determine which lipid intermediate impairs mTORC2 activity and subsequent insulin signaling, we overexpressed GPAT1, AGPAT2, or PAPase/lipin 2 to manipulate the cellular content of LPA, PA, and DAG, respectively. Overexpression of these enzymes was confirmed by Western blotting (Fig. S4*C*) and by an approximately sevenfold increase in PAPase activity (Fig. S4*D*). Although overexpressing lipin 2 had no effect on insulin signaling, overexpressing either GPAT1 or AGPAT2 suppressed insulin-stimulated phosphorylation of S473-Akt and diminished mTOR-rictor binding (Fig. 4 *A* and *B*).
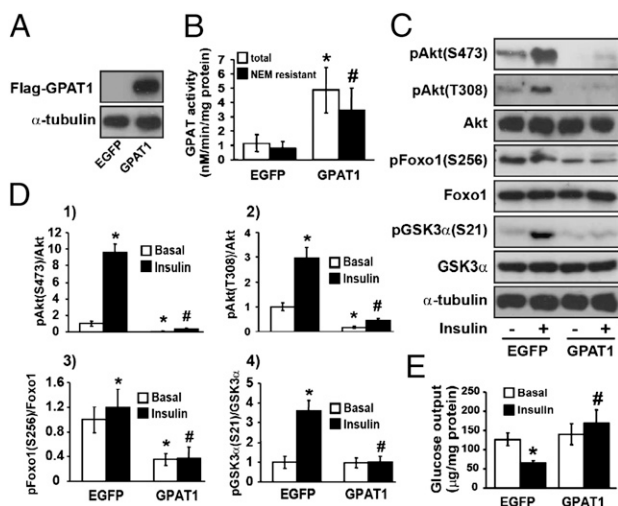
Fig. 1. Overexpressing GPAT1 in hepatocytes impairs insulin signaling. Primary mouse hepatocytes were infected for 24 h to overexpress either EGFP or Flag-GPAT1. (*A*) Representative Western blot image of Flag-GPAT1. (*B*) Total and NEM-resistant GPAT activity. (*C–E*) Mouse hepatocytes overexpressing EGFP or GPAT1 were treated with or without insulin (100 nM) for 10 min. (*C*) Representative Western blot. (*D*) Quantitative analysis of data from *C*. (*E*) Glucose output. Asterisk indicates significant differences ($P < 0.05$) from EGFP total (*B*) or EGFP basal (*D*). # indicates significance compared with EGFP NEM-resistant (*B*) or EGFP stimulated with insulin (*D*).

Fig. 2. GPAT1-impaired insulin signaling is due to decreased mTORC2 activity. Primary mouse hepatocytes infected to overexpress either EGFP or Flag-GPAT1 were treated with or without insulin (100 nM) for 10 min. (A) Representative images of Western blots of rictor-IP or cell lysates. (B) Quantitative analysis of data from A. (C) Representative images of Western blots of cell lysates. (D) Quantitative analysis of data f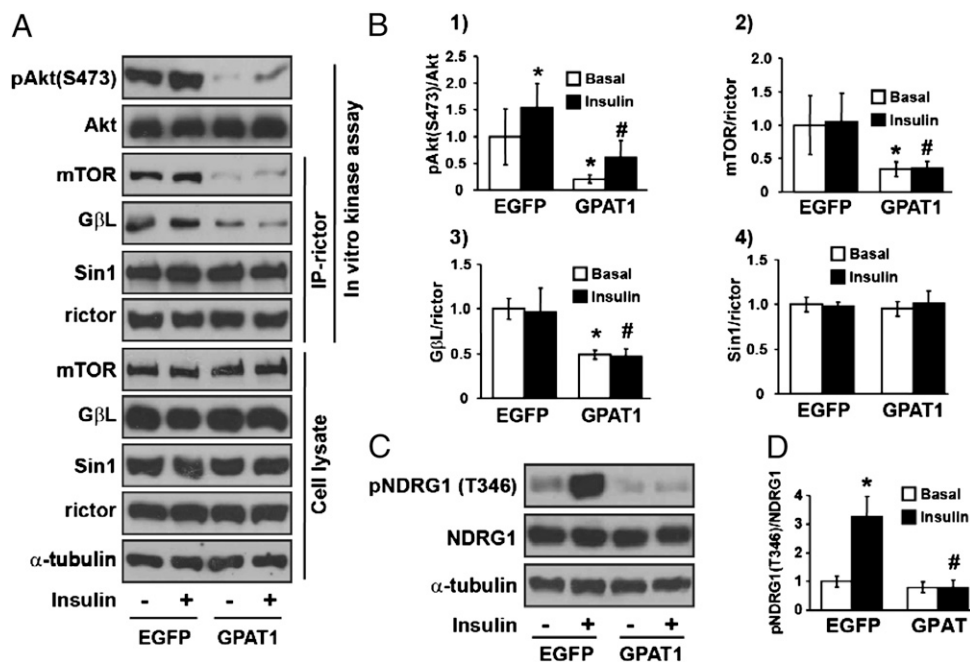rom C. Data in A and B represent results from five independent experiments performed in triplicate. Asterisk indicates significant differences (P < 0.05) from EGFP basal; #, significantly different from EGFP stimulated with insulin; GβL, mLST8.

Because both GPAT1 and AGPAT2 inhibited mTORC2, but only GPAT1 overexpression increased LPA content (Fig. 5A and Table S1), mTORC2 signaling was unlikely to have been disrupted by LPA. However, the impaired mTORC2 and insulin signaling by both GPAT1 and AGPAT2 overexpression was accompanied by greater than twofold increases in total cell palmitate (16:0)-containing PA species (Fig. 5B). The increases in total 16:0-containing PA resulted from individual PA species increased by GPAT1 or AGPAT2: 16:0–16:0 PA (14.1- and 7.2-fold, respectively), 16:0–18:0 PA (4.6- and 2.3-fold), 16:0–18:1 PA (1.2- and 2.0-fold), and 16:0–18:2 PA (1.7- and 1.4-fold) (Table S1). Other PA species did not exhibit this trend (Fig. 5B and Table S1).

In a similar association, both GPAT1 and AGPAT 2 overexpression increased the cell content of total and individual DAG species that contained 16:0, but not other species. GPAT1 and AGPAT2 increased total 16:0-containing DAG by 3.4- and 1.7-fold, respectively, as well as individual species: 16:0–16:0 DAG (17- and 3.1-fold), 16:0–18:1 DAG (2.3- and 1.6-fold), and 16:0–18:2 DAG (7.2- and twofold) (Fig. 5C and Table S1). Collectively, these results indicate that the synthesis of 16:0-containing PA and DAG is associated with GPAT1- and AGPAT2-induced inhibition of rictor-mTOR assembly and mTORC2 activity. Because, unlike other GPAT isoforms, GPAT1 has a strong substrate preference for 16:0-CoA, these PA and DAG species are most likely to be the molecules that inhibit mTORC2 signaling in this system.

Di-16:0-PA Inhibited Phosphorylation of Akt-S473 and Promoted mTOR/Rictor Disassembly in Vitro. To provide direct evidence that lipid species derived from the GPAT pathway interfere with mTOR/rictor assembly and inhibit mTORC2 activity, we performed an in vitro mTORC2 kinase assay after adding specific lipids. The two LPA species and the four DAG species tested showed no effect on mTOR/rictor assembly or on mTORC2 activity (Fig. 6A). In contrast, 16:0–16:0-PA strongly inhibited

both mTOR/rictor assembly and mTORC2 activity in a dose-dependent manner (Fig. 6 A and B), whereas three other PA species had no effect (Fig. 6A). Of the LPA, PA, and DAG species tested, only di-16:0-PA disrupted the association between rictor and mLST8. These results show that certain PA species can disassociate a previously formed mTORC2 complex.

PLD1-Derived PA Influences Akt Phosphorylation Through a Mechanism Other than mTOR/Rictor Assembly. PA hydrolyzed from membrane phospholipids by PLD exhibits diverse effects on mTOR signaling. Thus, we determined whether PLD1-derived PA inhibited mTORC2 and insulin signaling. In hepatocytes, serum activation of PLD-mediated phosphorylation of Akt-S473 was similar to insulin (Fig. 7). However, inhibiting PLD with 1-butanol abolished the insulin-stimulating effect of serum. Although PLD1-derived PA affects Akt signaling, neither activating nor inhibiting PLD1 altered mTOR-rictor assembly. This result suggests that, unlike GPAT1- and AGPAT2-derived PA, PA derived from PLD1 influences Akt phosphorylation through a mechanism other than mTOR/rictor assembly.

Discussion

The major finding of this study is that overexpressing GPAT1 generates lipid intermediates that decrease mTORC2 activity and impair insulin signaling (Fig. S5). Our previous data demonstrated that overexpressing GPAT1 in rats induces insulin resistance (7), and conversely, that the absence of Gpat1 protects mice from high-fat-diet-induced hepatic insulin resistance (6). In both studies, the changes in insulin sensitivity probably occurred because of the altered flux through the pathway of glycerolipid synthesis initiated by GPAT. Although hepatic TAG content is strongly associated with insulin resistance, TAG is a neutral lipid sequestered in lipid droplets and is unlikely to inhibit insulin action. On the other hand, synthesis of the lipid intermediates LPA, PA, and DAG is tightly regulated and maintained at low levels because these molecules are critical intermediates for de
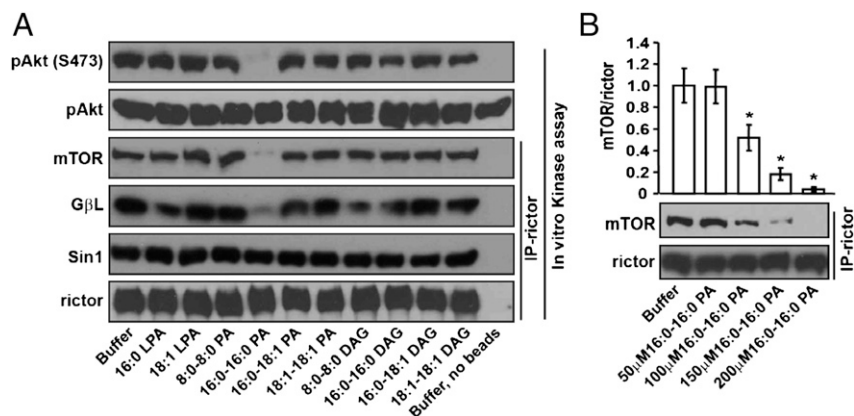
Fig. 3. $Gpat1^{-/-}$ hepatocytes have increased mTORC2/Akt signaling and enhanced association of rictor and mTOR. Hepatocytes were isolated from WT or $Gpat1^{-/-}$ mice fed a high-fat diet for three weeks. Isolated hepatocytes were treated with or without insulin (100 nM) for 10 min. (A) Representative images of Western blots from cell lysates and rictor-IP product. (B) Quantitative analysis of data from A. Asterisk indicates significant difference compared with EGFP basal; #, significant difference compared with EGFP stimulated with insulin. Mouse primary hepatocytes of WT or $Gpat1^{-/-}$ mice fed a high-fat diet were infected with LacZ-shRNA or rictor-shRNA adenovirus for 72 h and treated with or without insulin (100 nM) for 10 min. (C) Representative images of Western blots from cell lysates. (D) Quantitative analysis of data from C. Asterisk indicates significant differences ($P < 0.05$) from EGFP basal; #, significantly different from EGFP stimulated with insulin. Different letters represent significant differences within basal or insulin treatment.

novo synthesized glycerolipids as well as bioactive signaling molecules. Although the levels of lipid intermediates change transiently during normal physiological responses, increases in their steady-state content are likely to result in metabolic dysfunction.

GPAT1-mediated changes in insulin signaling have been attributed to DAG activation of novel isoforms of PKC (2) which phosphorylate serine residues of IRS1 and impair insulin signaling. In muscle and liver, fatty acid-induced insulin resistance is partially mediated through DAG activation of PKCθ (23) and PKCε (24), and decreasing the hepatic content of DAG reduces PKCε activation and protects rats against dietary fat-induced insulin resistance (25). Because in GPAT1-overexpressing rats, an increase in hepatic DAG corresponded with increased activation of PKCε, whereas in $Gpat1^{-/-}$ mice both hepatic DAG and activated PKCε were low (6, 7), we concluded that DAG activation of PKC mediates insulin resistance. In the current

Fig. 4. Overexpressing GPAT1 or AGPAT2, but not Lipin2, in mouse hepatocytes impairs mTOR/rictor association and insulin signaling. Primary hepatocytes overexpressing GPAT1, AGPAT2, or Lipin2, were treated with or without insulin (100 nM) for 10 min. (A) Representative images of Western blots from cell lysates and rictor-IP product. (B) Quantitative analysis of data from A. Asterisk indicates significant differences ($P < 0.05$) from EGFP basal; #, significantly different from EGFP stimulated with insulin.

Fig. 5. Overexpressing GPAT1 and AGPAT2 in mouse hepatocytes increased the levels of PA and DAG species that contained 16:0. Lipids were quantified from primary hepatocytes overexpressing GPAT1, AGPAT2, or Lipin2. (A) LPA. (B) PA. (C) DAG content. Asterisk indicates significant differences ($P < 0.05$) from EGFP.

Fig. 6. Di-16:0-PA inhibits the phosphorylation of Akt at S473 and promotes mTOR/rictor disassembly in vitro. Lipid vesicles were added to the rictor-IP beads and then mTORC2 kinase was assayed. (*A*) Representative images of Western blots from rictor-IP and kinase assay product. (*B*) Representative images of Western blots from rictor-IP product and quantitative analysis. Lipid vesicles of each species were added at 150 uM. Asterisk indicates *P* < 0.05 compared with the control incubated with buffer alone; GβL, mLST8.

study, elevated DAG induced by overexpressing GPAT1 or AGPAT2 paralleled the inhibition of mTORC2 activity, thus supporting a possible second role for DAG-mediated insulin resistance via inactivation of mTORC2 signaling. Despite these strong associations, much of the DAG produced via de novo glycerolipid synthesis, as opposed to membrane hydrolysis, is likely to be sequestered in lipid droplets (26), in theory, rendering the DAG less available for signaling functions; PKCε activation would require DAG to escape these lipid droplets.

Unlike DAG, water-soluble LPA and PA, which are generated by de novo glycerolipid synthesis, are not confined to lipid droplets. LPA interferes with insulin signaling through PKC-dependent phosphorylation and inactivation of GSK3 (27). In our model, however, LPA is an improbable signal for glycerolipid-mTORC2 signaling because of the disconnect between LPA content and impaired mTORC2 activity. However, PA content was strongly associated with the dissociation of mTOR and rictor and the decrease in mTORC2 activity. Both exogenous PA and the PA derived from PLD membrane hydrolysis activate mTORC1 and mTORC2, each of which has distinct effects on insulin signaling. mTORC1 provides feedback inhibition of insulin signaling through p70S6K phosphorylation and inactivation of IRS1 (14), whereas mTORC2 phosphorylates S473-Akt and augments insulin signaling (15). A critical role for mTORC2 in insulin signaling is evident in both muscle-specific (MRic^{−/−}) and adipose-specific (FRic^{−/−}) rictor knockout mice. Defective glucose transport and insulin signaling in muscle from *MRic^{−/−}* mice cause mild glucose intolerance (28). Likewise, *FRic^{−/−}* mice have impaired glucose transport in adipocytes, resulting in hepatic and whole-body insulin resistance (29).

PA derived from PLD-mediated hydrolysis of membrane phospholipids directly activates mTORC1 by interacting with the FKBP12-rapamycin-binding (FRB) domain of mTOR and stabilizing mTORC1 (8, 17, 30, 31). In fact, PA is critical for mTORC1 activation by nutrients like glucose and amino acids (32). Similarly, suppressing PLD-derived PA inhibits the association of mTOR and rictor and leads to diminished mTORC2 activity (15, 33). Our data suggesting that PA produced via the glycerolipid synthesis pathway decreases mTOR/rictor association and mTORC2 activity creates an interesting paradox that could be resolved if PA derived from glycerolipid synthesis were functionally different from PA derived from membrane hydrolysis. How can the same molecule, albeit derived from different sources, engender opposite effects on mTORC2 signaling? Presumably, PA from both pools would interact with the same FRB domain of mTOR. To mediate opposing effects on function, one

might invoke differences in PA structure. In support of this hypothesis, our data show that only PA species that contained 16:0 were associated with the inhibition of mTORC2 activity and only 16:0–16:0-PA directly caused dissociation of the mTOR/rictor complex. The PA species predominant in PLD-derived PA activation of mTORC1 and -2 are not known. However, the molecular species of PA released by PLD vary, probably depending on the agent used to stimulate PLD and the cell type used (34–38). In no case, however, did species containing 16:0 predominate. Characterizing the nature of the hepatic pools of PA derived from PLD and from de novo synthesis will be needed to understand normal and dysfunctional nutrient lipid signaling, particularly as it relates to nutrient oversupply and insulin resistance.

Although we found that only 16:0–16:0-PA inhibited mTORC2 activity in vitro, it is possible that other PA and DAG species may be able to inhibit mTORC2 activity in living cells. Our experiment required disassociation of the mTORC2 complex after it had already formed; it may be easier for an inhibitory lipid to prevent the initial association than to disrupt an existing complex. The relatively high concentration of 16:0–16:0-PA that was required for inhibition may also reflect this difficulty in promoting
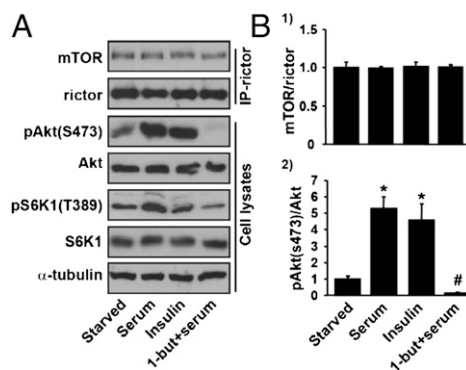

Fig. 7. PLD1-derived PA influences Akt phosphorylation through a mechanism other than mTOR/rictor assembly. Primary mouse hepatocytes were serum-starved for 24 h and then stimulated with serum (30%) or insulin (100 nM) and with or without 1-butanol (0.5%) for 30 min. (*A*) Representative images of Western blots from cell lysate and rictor-IP products. (*B*) Quantitative analysis of data from *A*. Asterisk indicates significant differences (*P* < 0.05) compared with serum starvation (starved); #, significantly different compared with serum stimulation.

complex dissociation. However, even in vivo, the concentration of 16:0–16:0-PA is unlikely to be uniform within the cell, and because we added PA in vesicles, we cannot know its concentration at the surface of the mTORC2 complex. Similarly, because of the difficulty in solubilizing long-chain DAGs and presenting them effectively to a protein complex, we cannot rule out the possibility that, in vivo, DAG might also exert an effect on mTORC2.

Our findings demonstrate that mTOR is a molecular sensor for multiple types of nutrients, including glucose, amino acids, and now lipids. The data reveal a signaling pathway in which lipid intermediates synthesized via the glycerolipid synthetic pathway inhibit mTORC2 activity by dissociating rictor and mTOR, thus impairing insulin action. Our data also suggest that the PA molecular species derived from PLD hydrolysis and de novo glycerolipid synthesis comprise functionally distinct lipid pools that have opposing effects on mTOR signaling. Taken together, this study demonstrates that 16:0-containing PA is strongly associated with the inhibition of mTORC2 activity and downstream insulin

signaling and that PA provides a critical link between excess nutrient intake, hepatic TAG synthesis, and insulin resistance.

Materials and Methods

GPAT Activity. Hepatocytes were homogenized in 10 mM Tris (pH 7.4), 250 mM sucrose, 1 mM DTT, 1.0 mM EDTA. Membranes were isolated by centrifuging at $100,000 \times g$ for 1 h. Protein was measured by the bicinchoninic acid method with BSA as the standard. GPAT specific activity was assayed for 10 min at 25 °C with 800 μM [^{3}H]glycerol-3-phosphate and 100 μM palmitoyl-CoA in the presence or absence of 2 mM *N*-ethylmaleimide (NEM), which inhibits the isoforms GPAT-2, 3 and 4.

Antibody reagents animal care, cell culture methods, enzyme activity and glucose output assays, recombinant adenoviruses, cell lipid measurements and analyses, immunoprecipitations and kinase activity assays, and statistical analyses are detailed in *SI Materials and Methods*.

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