Regulation of Mitochondrial sn-Glycerol-3-phosphate Acyltransferase Activity: Response to Feeding Status Is Unique in Various Rat Tissues and Is Discordant with Protein Expression

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Triacylglycerol plays a critical role in an organism’s ability to withstand fuel deprivation, and dysregulation of triacylglycerol synthesis is important in the development of diseases such as obesity and diabetes. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the initial and committed step of glycerolipid synthesis and is therefore a potential site for regulation of triacylglycerol synthesis. Because several studies suggest that triacylglycerol synthesis is linked to the mitochondrial isoform, we studied mitochondrial GPAT expression and the effect of feeding status on the regulation of mitochondrial GPAT in various rat tissues. Liver, adipose, and soleus muscle have high levels of GPAT mRNA, but low protein expression, whereas heart and adrenal, tissues with low GPAT mRNA abundance, have the highest GPAT protein expression. In addition, heart, which has the highest expression of mitochondrial GPAT protein, has low mitochondrial GPAT specific activity (0.02 nmol/min/mg). Liver and adipose have the highest mitochondrial GPAT specific activity (0.17 nmol/min/mg), but very low protein expression. Discrepancies between GPAT protein expression and activity suggest that mitochondrial GPAT may be regulated acutely. In response to a 48-h fast, liver and adipose mitochondrial GPAT protein expression and activity decrease 30–50%. After 24-h refeeding of either chow or high-sucrose diet, mitochondrial GPAT protein expression and activity overshoot normal levels 30–60%. In kidney, mitochondrial GPAT protein and activity increase 65 and 30%, respectively, with refeeding, whereas in the heart, mitochondrial GPAT activity increases 2.3-fold after a fast, with no change in protein expression. We also found that hepatic mitochondrial GPAT activity in the neonatal rat constitutes a lower percentage of the total GPAT activity than in the adult. We postulate that GPAT expression is modulated uniquely in each tissue according to specific needs for triacylglycerol storage.

Key Words: acyltransferase; triacylglycerol; nutritional regulation.

Triacylglycerol (TAG) is an important molecule for energy storage in mammals. TAG accumulates during times of excess energy, such as overfeeding, refeeding high carbohydrate, and preparing for hibernation. Stored TAG is hydrolyzed to FA during nutrient lack (fasting/starvation, hibernation) and energy expenditure (exercise, stress). Studies of the regulation of TAG synthesis have focused on the control of gene transcription by sterol regulatory element binding protein-1 (SREBP) and peroxisome proliferator-activated receptor γ (PPAR) (1, 2), but several studies suggest that glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15) may be regulated acutely (3–6).

GPAT catalyzes the acylation of sn-glycerol-3-phosphate at the sn-1 position to form lysophosphatidic acid (LPA) as the initial and committed step in the de novo synthesis of glycerolipids. The study of GPAT activity in mammalian cells is complicated by the fact that two isoenzymes exist, one in the endoplasmic reticulum

Abbreviations used: AICAR, 5-aminimidazole-4-carboxamide-1-beta-D-ribofuranoside; AMPK, AMP-activated kinase; CHO, Chinese hamster ovary; CPT-1, carnitine palmitoyltransferase-1; EDL, extensor digitorum longus; ER, endoplasmic reticulum; FA, fatty acid; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; NEM, N-ethylmaleimide; NIDDM, non-insulin-dependent diabetes mellitus; PVDF, polyvinylidene fluoride; SREBP, sterol regulatory element binding protein; TAG, triacylglycerol.

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(ER) and the other in the outer mitochondrial membrane (7). Activity in the ER is sensitive to sulphydryl reagents, such as N-ethylmaleimide (NEM), whereas mitochondrial activity is unaffected (3). Several studies have shown that microsomal and mitochondrial GPAT activities have different sensitivities to various reagents and have different fatty acid specificities, suggesting that GPAT activity in the ER and mitochondria arises from two distinct enzymes (3). In addition, antibodies raised against mitochondrial GPAT do not recognize anything in highly purified ER fractions (8, 9), indicating that mitochondrial and microsomal GPAT are distinct proteins. Early studies of GPAT activity used the sensitivity to NEM to determine that in rat tissues other than liver, microsomal GPAT specific activity is approximately 10 times higher than the mitochondrial (NEM-resistant) GPAT specific activity (3, 7). In rat liver, uniquely, mitochondrial activity is 30–50% of the total GPAT activity measured (7).

Both the microsomal and mitochondrial GPAT isoforms have been linked to enhanced triacylglycerol synthesis (3). Only the mitochondrial GPAT isoform has been cloned (9, 10), and the microsomal isoform has not yet been identified. Therefore, changes in activity of the two isoforms can be compared, whereas changes in mRNA abundance or protein expression cannot. Although the lack of cloned microsomal GPAT limits comparisons, in general, mitochondrial GPAT appears to be more responsive to feeding status and insulin than the microsomal isoform (11–13), suggesting that mitochondrial GPAT may be linked to triacylglycerol synthesis. Our recent results further strengthen this hypothesis. Overexpression of rat mitochondrial GPAT in CHO cells resulted in a fourfold increase in mitochondrial GPAT activity and a fourfold increase in [14C]-oleate incorporation into triacylglycerol (14). We hypothesize that mitochondrial GPAT directs exogenous fatty acids toward TAG storage.

Because GPAT catalyzes the initial and committed step in glycerolipid, we chose to study the nutritional regulation of mitochondrial GPAT in various rat tissues. We found that mitochondrial GPAT protein expression and activity do not correlate in various rat tissues, suggesting that GPAT may be acutely regulated. We also found that mitochondrial GPAT protein expression and activity are regulated differently in various rat tissues in response to fasting and refeeding. We postulate that GPAT expression is modulated uniquely in each tissue according to specific acute needs for triacylglycerol storage.

EXPERIMENTAL PROCEDURES

Animals. Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Female (150 g) Sprague-Dawley rats were housed on a 12-h/12-h light/dark cycle with free access to water. Control animals had free access to Purina rat chow. Fasted animals were sacrificed after being without food for 48 h. Refed rats were sacrificed after being fed Purina rat chow or a high-sucrose diet (69.5% sucrose, Dyets, Inc.) for 24 h after a 48-h fast. All animals were sacrificed by decapitation after CO2 anesthesia.

Northern analyses. RNA was isolated using guanidine thiocyanate and ultracentrifugation (15–17). Total RNA (25 μg) was denatured in formamide, run out on a 1.2% agarose-formaldehyde gel, and transferred to a nylon membrane. The probe for GPAT encompasses 700 nucleotides within the coding sequence, which is unique for the mitochondrial GPAT enzyme (8, 9). Probes against GPAT and β-actin were labeled with [32P]CTP by primed random priming (Boehringer-Mannheim). The signal was quantified by densitometry or phosphor imaging.

Subcellular fractionation. Liver total membrane fraction, microsomes, and mitochondria were isolated from female rats by differential centrifugation (18) in the presence of protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Fractions were stored in aliquots at –80°C. Protein concentrations were determined by the BCA method (Pierce) using BSA as the standard.

Immunoblotting. Proteins were separated on an 8% polyacrylamide gel containing 1% SDS, transferred to a PVDF membrane (Bio-Rad), and incubated with antibody against rat mitochondrial GPAT (8). For chemiluminescent detection, the immunoreactive bands were visualized by incubating the membrane with HRP-conjugated goat anti-rabbit IgG and Picowest reagents (Pierce). For quantitative Western blot analyses, the PVDF membrane was incubated with 0.5 μCi [32P]Protein A (ICN), exposed to a phosphor screen, and quantified with the Molecular Dynamics Storm 840 and ImageQuant software.

Enzyme assays. GPAT was assayed at 23°C with 300 μM [14C]glycerol-3-P and 112.5 μM palmitoyl-CoA in the presence or absence of 2 mM N-ethylmaleimide to inhibit the microsomal isoform (19). Microsomal GPAT was estimated by subtracting the N-ethylmaleimide-resistant activity (mitochondrial GPAT) from the total. All assays measured initial rates. [3H]Glycerol-3-phosphate was synthesized enzymatically (20).

Statistics. Data are presented as means ± SD. Differences between control and fasted or refed groups were analyzed by paired Student’s t test.

RESULTS

Mitochondrial GPAT mRNA levels, protein expression, and activity are discordant. To determine mitochondrial GPAT message levels, we isolated RNA from several rat tissues and performed Northern blot analyses. As expected, expression of mitochondrial GPAT mRNA was highest in liver and adipose (Fig. 1A), the two tissues with the highest mitochondrial GPAT activity (Fig. 1C). Mitochondrial GPAT mRNA expression was next highest in brain and was very low in spleen, kidney, extensor digitorum longus (EDL), heart, and adrenal (Fig. 1A).

To compare mitochondrial GPAT mRNA levels and protein expression, we isolated the total membrane fraction from the same tissues and performed Western blot analyses. Surprisingly, we found very little correlation between mitochondrial GPAT mRNA abundance and protein expression. Mitochondrial GPAT protein levels were highest in the heart and adrenal (Fig. 1B), tissues with low GPAT mRNA expression (Fig. 1A). Liver, adipose, soleus, and brain, which had the highest mitochondrial GPAT mRNA levels, showed low mi-
tochondrial GPAT protein expression relative to the heart and adrenal. However, other tissues with low mitochondrial GPAT mRNA abundance (spleen, kidney, EDL) also had low protein expression.

To determine whether the high amount of mitochondrial GPAT protein in the heart might be due to a greater abundance of mitochondria, we isolated mitochondria from liver, heart, and kidney and measured mitochondrial GPAT protein expression by quantitative Western blot. We found the same relative amounts of GPAT protein expression as seen in the total membrane fraction (Fig. 2). This indicates that the large amount of mitochondrial GPAT protein detected in the heart compared to that in the liver is not due to a greater abundance of mitochondria.

We also assayed GPAT activity in total membrane fractions (Fig. 1C). As previously reported (3), mitochondrial GPAT specific activity was highest in liver and adipose (0.17 nmol/min/mg). Adrenal had the next highest mitochondrial GPAT specific activity (0.08 nmol/min/mg). All other tissues assayed (brain, spleen, kidney, soleus, EDL, heart) had very low GPAT activities (0.02 to 0.04 nmol/min/mg). These data show a striking discordance between mitochondrial GPAT protein expression and activity. For example, heart, which had the highest amount of GPAT protein (100 units), had low mitochondrial GPAT specific activity (0.02 nmol/min/mg), and adipose, which had low protein expression (4.2 units), had the highest specific activity (0.17 nmol/min/mg). The discordance between GPAT

FIG. 1. Mitochondrial GPAT mRNA levels, protein expression, and activity do not correlate. (A) Total RNA (25 µg) isolated from various rat tissues was analyzed by Northern blot with probes against mitochondrial GPAT and β-actin. Signal was quantified by densitometry. The signal from the 6.8-kb GPAT band was normalized against β-actin. The bar graph represents relative mitochondrial GPAT mRNA expression. Values are representative of three independent experiments. (B) Total membrane fraction (200 µg protein) isolated from various rat tissues was analyzed by quantitative Western blot with mitochondrial GPAT antiserum and 125I-Protein A. Blots were exposed to a phosphor screen and the signal was quantified with a Molecular Dynamics Storm 840 system. The bar graph represents relative mitochondrial GPAT protein expression. Values shown are representative of four independent experiments. (C) Total membrane fraction isolated from various rat tissues was assayed for GPAT activity in the presence of 2 mM NEM. Values are means of four independent experiments. SD was less than 20% in each tissue.

FIG. 2. GPAT protein expression and activity are discordant in mitochondria isolated from liver, heart, and kidney. Mitochondria were isolated from rat liver, heart, and kidney. Protein (100 µg) was analyzed by quantitative Western blot with mitochondrial GPAT antiserum and 125I-Protein A. Blots were exposed to a phosphor screen and the signal was quantified with a Molecular Dynamics Storm 840 system. Values in the bar graph are averages of two independent mitochondrial isolations from each tissue and represent relative mitochondrial GPAT protein expression.
protein expression and activity suggests that post-translational modification might activate or inactivate GPAT protein in different tissues.

GPAT protein expression and activity are regulated differently by fasting and refeeding in various rat tissues. GPAT is transcriptionally regulated by fasting and high-carbohydrate refeeding, but discrepancies between mRNA abundance and activity have been previously noted. For example, 16 h after mice were refed a high-carbohydrate, fat-free diet, liver mitochondrial GPAT mRNA levels increased 30-fold over fasting levels (12), but refeeding increased GPAT specific activity only 6-fold over fasting levels (9). Since we also found that GPAT mRNA, protein expression, and activity are discordant, we measured GPAT protein expression and activity in liver, adipose, heart, and kidney from rats fed normally, fasted for 48 h, or fasted for 48 h and then refed either Purina rat chow or a high-sucrose (69%) diet for 24 h.

The most striking discordance between mitochondrial GPAT activity and protein expression was observed in the heart. After a 48-h fast, heart mitochondrial GPAT activity increased 2.3-fold despite only a modest 35% increase in protein expression (Fig. 3). Thus, heart mitochondrial GPAT appears to be acutely responsive to increased FA present during a fast. Mitochondrial GPAT protein levels and specific activity remained virtually unchanged after refeeding either normal chow or high-sucrose diet compared to normally fed animals. Heart microsomal GPAT activity was unaffected by changes in feeding status.

Hepatic and heart mitochondrial GPAT responded differently to fasting and refeeding. In liver, mitochondrial GPAT protein expression and activity decreased approximately 30% compared to control animals after a 48-h fast (Fig. 4), consistent with the 30% decrease observed in GPAT mRNA abundance (data not shown). With chow refeeding, mitochondrial GPAT mRNA abundance (data not shown) and activity increased 60%, whereas protein levels only increased 30%. Sucrose refeeding resulted in a 3-fold increase in mitochondrial GPAT activity and a 2.4-fold increase in protein expression. In general, the increases in mitochondrial GPAT activity resulting from refeeding were slightly greater than the increases observed in GPAT protein expression. Feeding status did not induce changes in the activity of the microsomal GPAT isoform.

In adipose tissue we observed a pattern of GPAT regulation similar to that in liver. Following a 48-h fast, mitochondrial GPAT protein expression and activity decreased 50 and 30%, respectively (Fig. 5). Refeeding normal rat chow increased protein expression 58%, and mitochondrial GPAT activity increased a similar amount (52%). In contrast to these similar changes, sucrose refeeding contributed to a 3.5-fold increase in protein expression of mitochondrial GPAT whereas activity only increased 60% compared to normally fed animals. Thus, in adipose tissue, the changes in mitochondrial GPAT protein expression and activity in response to feeding status were the same, except following sucrose refeeding when the small increase in mitochondrial GPAT activity and the large increase observed in protein expression were discordant. Although it appears that microsomal GPAT activity increased following both refeeding regimens, the changes were not statistically significant.

In the kidney, mitochondrial GPAT is only upregulated following refeeding. Mitochondrial GPAT activity and protein expression increased 30 and 65%, respec-
tively, with 24 h refeeding after a 48-h fast (Fig. 6). A 48-h fast had no effect on mitochondrial GPAT in the kidney. Microsomal GPAT activity was unaffected by feeding status.

Developmental regulation of liver mitochondrial GPAT. Although the microsomal GPAT specific activity has been reported to increase 74-fold in 5-day-old rats compared to fetuses 2–3 days before birth, the mitochondrial GPAT activity changes minimally (19). Cloning of the mitochondrial GPAT enabled us to determine the corresponding changes in mitochondrial GPAT mRNA levels. Mitochondrial GPAT mRNA abundance in rats at prenatal day −3 was only 30% of that found in adults (Fig. 7A). At postnatal day 3, mitochondrial GPAT expression had fallen to 9% of that measured in adult rats. GPAT expression remained at this low level until day 28, when GPAT mRNA levels were similar to those in adults.

As observed in liver from adult rats, we found that mitochondrial GPAT mRNA abundance did not correlate with mitochondrial GPAT activity. Although mitochondrial GPAT mRNA levels decreased at postnatal day 3 compared to prenatal day -3, both mitochondrial and microsomal activities increased twofold by postna-
tal day 3 compared to prenatal day 3 (Fig. 7B). On day 28, both mitochondrial GPAT mRNA abundance and activity (0.17 nmol/min/mg) were similar to those measured in the adult. Before day 28, mitochondrial GPAT constituted only 15% of total GPAT activity, the distribution normally found in nonhepatic tissues of adult animals (3, 7), but strikingly by day 28, hepatic mitochondrial activity constituted 50% of total GPAT activity, the percentage observed in adult rats.

**DISCUSSION**

Regulation of enzyme activity is a complex process that occurs at several molecular levels. For mitochondrial GPAT, regulation has been shown to occur at the level of transcription in response to changes in differentiation state, feeding status, the hormones insulin and glucagon, and the transcription factor SREBP-1 (11–13, 21–23). GPAT protein may also be modified by phosphorylation/dephosphorylation, resulting in decreased or increased acyltransferase activity (3–6, 24). Finally, like all enzymes, GPAT activity is subject to substrate availability.

GPAT activity is present in all mammalian tissues. In general, the microsomal (NEM-sensitive) specific activity in rat tissues is 10-fold greater than the mito-
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In addition to regulation at the level of transcription, translation, and protein degradation, the activity and function of proteins can be altered through posttranslational modifications such as phosphorylation, glycosylation, and proteolytic cleavage. Such posttranslational modifications may explain the differences observed between mitochondrial GPAT protein expression in liver and adipose. The specific activity of mitochondrial GPAT is identical in liver and adipose and mRNA levels are very similar (Fig. 1). However, the abundance of GPAT protein in the liver is four times higher than that detected in adipose tissue (Fig. 1B). In tissues with low GPAT activity relative to protein expression, GPAT might be modified in a manner that would activate or inactivate the enzyme. Consistent with this hypothesis, increases in hepatic GPAT activity resulting from refeeding were greater than the increases observed in GPAT protein expression (Fig. 3). It seems that in rat liver, GPAT protein may be acutely activated in response to refeeding, in addition to the already characterized transcriptional up-regulation in response to insulin (9, 12). In adipose tissue, such a mechanism does not seem to operate, since changes in both GPAT protein expression and activity were similar in response to feeding status (Fig. 4).

Acute activation of mitochondrial GPAT may also occur in the heart. After a 48-h fast, GPAT activity increased sevenfold more than did protein expression (Fig. 6). This marked discrepancy between an increase in GPAT activity and protein expression is consistent with the abundance of GPAT protein in the heart and the idea that response to sudden changes in energy demand or supply may require an acute mechanism. During a fast, the supply of fatty acids entering the heart increases (30). The resulting fatty acyl-CoAs may exceed the supply required for β-oxidation, and since fatty acyl-CoAs at high concentrations disrupt membranes, the acyl-CoAs may have to be esterified and stored safely as TAG. TAG can also serve as an internal store of fatty acids for energy via β-oxidation (31).

Acute regulation of GPAT activity may also occur by a phosphorylation/dephosphorylation mechanism. Several studies have shown alterations in GPAT activity following treatment with various kinases and phosphatases. Hepatic microsomal GPAT is inactivated by a cyclic-AMP-dependent mechanism (3), and adipose microsomal GPAT is inactivated by a partially purified tyrosine kinase (5). Liver mitochondrial GPAT activity decreases 50–60% when incubated with ATP, MgCl₂, and cyclic-AMP-dependent kinase (3). In the heart, total GPAT activity decreases 50% after adrenaline treatment or incubation of membrane fractions with cyclic-AMP-dependent kinase (4). Both insulin and orthovanadate stimulate the translocation of PKC-β and increase total GPAT activity in BC3H-1 myocytes (24). Finally, we have shown that recombinant AMP-activated kinases-1 and -2 (AMPK) inactivate liver mitochondrial GPAT in isolated mitochondria in a time- and ATP-dependent manner (6). Regulation of liver mitochondrial GPAT by phosphorylation in liver is consistent with the observation that incubating hepatocytes with 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an allosteric activator of AMPK, not only decreases mitochondrial GPAT activity, but also increases [14C]oleate oxidation 78% and decreases [14C]oleate incorporation in TAG 43% (6). Inactivation of GPAT by AMPK might also occur in the heart, which expresses high AMPK activity (32).

Although our data suggest that mitochondrial GPAT activity may be regulated acutely, mitochondrial GPAT...
has also clearly been shown to be transcriptionally regulated as well. Mitochondrial GPAT is regulated by SREBP-1, a key regulator of several lipogenic enzymes (2). Mitochondrial GPAT expression increases 6.7-fold following ectopic expression of SREBP-1c in 3T3-L1 adipocytes (23), and in liver, SREBP-1a overexpression increases mitochondrial GPAT mRNA levels 10-fold (22). The murine mitochondrial GPAT promoter sequence contains three SREBP-1 sites and one NF-Y site, which are responsible for SREBP-1- and NF-Y-mediated transactivation (23). In addition, refeeding fasted animals a high-carbohydrate diet increases hepatic mitochondrial GPAT mRNA levels approximately 20-fold over fasted levels as a result of an enhanced transcription rate (12). Administration of dibutyryl-cAMP at the time of refeeding abolishes the increase in mitochondrial GPAT transcription, suggesting that glucagon, which stimulates adenylate cyclase and increases cAMP levels, decreases mitochondrial GPAT transcription (12). In streptozotocin-diabetic mice, the increase in GPAT mRNA after refeeding is not observed unless insulin is administered (12).

Although the lack of cloned microsomal GPAT limits comparisons, in general mitochondrial GPAT appears to be more responsive than the microsomal isoform to feeding status and insulin. In epididymal fat pads, streptozotocin-induced diabetes decreases mitochondrial activity 62% and microsomal activity 32%; insulin administration restores these activities to normal levels (11). In perfused rat livers, insulin increases mitochondrial GPAT activity 34%, whereas the microsomal activity increases only 9% (33). In addition, when fasted rats are fed a high-carbohydrate low-fat diet, hepatic mitochondrial specific GPAT activity increases sixfold with little change in microsomal activity (9). These data are consistent with our results in liver and adipose (Figs. 3 and 4) and our study showing that overexpression of mitochondrial GPAT in CHO cells results in a fourfold increase in TAG mass (14). Taken together, these data strongly suggest that mitochondrial GPAT is linked to TAG synthesis.

TAG synthesis is also linked to increased microsomal GPAT activity. In differentiating 3T3-L1 cells, microsomal GPAT activity increases 70-fold whereas the mitochondrial GPAT mRNA and activity increase about 10-fold (34). Microsomal GPAT activity also increases 74-fold in 5-day-old neonatal liver, compared to day -3 (3). Compared to that in adult liver, microsomal GPAT specific activity was 2- to 3-fold higher postnatally (Fig. 7). Interestingly, however, the usual distribution of GPAT activity in adult liver (30–50% mitochondrial) was not observed until day 28. The adult distribution of activity was achieved by a 2-fold increase in the mitochondrial activity observed on days -3 to 15 and a corresponding decrease in the microsomal activity (Fig. 7). When the liver is rapidly expanding in the neonatal period, the requirement for microsomal GPAT appears to be greater than in the adult.

We have shown that mitochondrial GPAT activity and protein expression are discordant, particularly in liver and heart, suggesting a mechanism of acute regulation. One possible mechanism for such regulation is phosphorylation, particularly by AMPK; however, no studies have shown a direct modification of mitochondrial GPAT protein. Future studies elucidating the mechanism of acute regulation of mitochondrial GPAT will clarify the response of mitochondrial GPAT to sudden changes in substrate availability in different cell types. Changes in mitochondrial GPAT regulation may contribute to the etiology of diseases characterized by dysregulation of lipid metabolism, such as obesity and NIDDM. Several studies suggest that intracellular triacylglycerol accumulation might be linked to the pathogenesis of disordered energy homeostasis (35). Although the mechanism of tissue TAG accumulation and metabolic dysregulation has not been elucidated, an important step in regulating cellular TAG is likely to be mitochondrial GPAT.

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