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Mice deficient in mitochondrial glycerol-3-phosphate acyltransferase-1 have diminished myocardial triacylglycerol accumulation during lipogenic diet and altered phospholipid fatty acid composition

Tal M. Lewin[1,3], **Hendrik de Jong**[1], **Nicole J. M. Schwerbrock**[1], **Linda E. Hammond**[1], **Steven M. Watkins**[2], **Terry P. Combs**[1], and **Rosalind A. Coleman**[1]

[1] Department of Nutrition, University of North Carolina, Chapel Hill, NC 27599

[2] Lipomics Technologies, Inc., 3410 Industrial Boulevard, Suite 103, West Sacramento, CA 95691

Abstract

Glycerol-3-phosphate acyltransferase-1 (GPAT1), which is located on the outer mitochondrial membrane comprises up to 30% of total GPAT activity in the heart. It is one of at least four mammalian GPAT isoforms known to catalyze the initial, committed, and rate limiting step of glycerolipid synthesis. Because excess triacylglycerol (TAG) accumulates in cardiomyocytes in obesity and type 2 diabetes, we determined whether lack of GPAT1 would alter the synthesis of heart TAG and phospholipids after a 2-week high sucrose diet or a 3-month high fat diet. Even in the absence of hypertriglyceridemia, TAG increased 2-fold with both diets in hearts from wildtype mice. In contrast, hearts from *Gpat1*$^{-/-}$ mice contained 20–80% less TAG than the wildtype controls. In addition, hearts from *Gpat1*$^{-/-}$ mice fed the high-sucrose diet incorporate 60% less [^{14}C]palmitate into heart TAG as compared to wildtype mice. Because GPAT1 prefers 16:0-CoA to other long chain acyl-CoA substrates, we determined the fatty acid composition of heart phospholipids. Compared to wildtype littermate controls, hearts from *Gpat1*$^{-/-}$ mice contained a lower amount of 16:0 in phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine/phosphatidylinositol and significantly more C20:4n6. Phosphatidylcholine and phosphatidylethanolamine from *Gpat1*$^{-/-}$ hearts also contained higher amounts of 18:0 and 18:1. Although at least three other GPAT isoforms are expressed in the heart, our data suggest that GPAT1 contributes significantly to cardiomyocyte TAG synthesis during lipogenic or high fat diets and influences the incorporation of 20:4n6 into heart phospholipids.

Keywords

obesity; type 2 diabetes; lipotoxicity; diabetic cardiomyopathy; arachidonic acid

Introduction

Regulation of triacylglycerol (TAG) and phospholipid synthesis plays a critical role in diseases such as obesity, type 2 diabetes, and atherosclerosis. In the heart, excess TAG

[3] Corresponding Author: Tal M. Lewin, CB# 7461, University of North Carolina, Chapel Hill, NC 27599, Telephone: 919-843-2719, FAX: 919-966-7216, Email: tal_lewin@unc.edu.

accumulation in cardiomyocytes is considered to be a marker for "lipotoxicity," a condition resulting in apoptosis of cardiomyocytes and ultimately, myocardial dysfunction [1–6]. Phospholipids, the major lipid component of cell membranes, not only serve a barrier function, but also provide a major reservoir of signaling molecules such as lysophosphatidic acid, phosphatidic acid, diacylglycerol and the arachidonate- and eicosapentanoate-derived eicosanoids. In the heart, a change in the polyunsaturated fatty acid composition of membrane phospholipids alters downstream signaling cascades [7,8], and mitochondrial function in aging hearts [9], and is correlated with membrane dysfunction and decreased contractility in rodent models of type 1 diabetes [10].

The initial and rate-limiting step in TAG and phospholipid synthesis is the GPAT catalyzed acylation of *sn*-glycerol-3-phosphate with long-chain acyl-CoA. Historically, heart GPAT activity was believed to result from the actions of two isoenzymes, one in the outer mitochondrial membrane (GPAT1) and the other in the endoplasmic reticulum (microsomal GPAT) [11]. GPAT1 activity is easily distinguished because, unlike microsomal GPAT, it is not inhibited by sulfhydryl reagents such as *N*-ethylmaleimide (NEM) [12]. We recently identified a second NEM-sensitive GPAT activity (GPAT2) in liver mitochondria from *Gpat1*$^{-/-}$ mice [13] and cloned it [14], but it has not been characterized in the heart. The NEM-sensitive endoplasmic reticulum isoforms, GPAT3 [15,16] and GPAT4 [17,18] are also expressed in heart, but their contribution to heart glycerolipid synthesis has not been studied. In heart total membrane preparations, NEM-sensitive activity comprises 70–90% of total GPAT activity measured [11,19]. Unlike GPAT2, 3, and 4 which are NEM-sensitive GPAT activities that do not exhibit chain length or saturation specificity for long chain acyl-CoAs, GPAT1 prefers 16:0 three- to ten-fold more than other long-chain saturated or unsaturated acyl-CoA substrates [20–22].

Although microsomal GPAT activity (GPAT 3 and 4) and the terminal enzymes for TAG synthesis are all present in the endoplasmic reticulum, several studies suggest that the critical isoform that regulates TAG synthesis is the mitochondrial GPAT1. In general, *Gpat1* mRNA, protein, and activity in liver and adipose tissue increase with carbohydrate feeding and with insulin stimulation via SREBP-1c, whereas microsomal activity does not change [19,23–26]. The role for GPAT1 in up-regulating TAG synthesis is also supported by studies of overexpressed rat GPAT1 in Chinese hamster ovary (CHO) cells and primary rat hepatocytes. In CHO cells, over-expression of GPAT1 results in a 3.8-fold increase in NEM-resistant GPAT activity, a 4-fold increase in [^{14}C]oleate incorporation into TAG, and a 30% decrease in [^{14}C]oleate incorporation into phospholipids [27]. Similar results are observed in primary rat hepatocytes, where a 13-fold increase in GPAT1 activity increases both the TAG mass and [^{14}C]oleate incorporation into TAG more than 2-fold [28]. *Gpat1* null mice have lower hepatic TAG content, lower plasma TAG and very low density lipoprotein TAG, and decreased secretion of TAG from liver [29]. Female *Gpat1*$^{-/-}$ mice weigh less than controls and have reduced gonadal fat pad weights. These data strongly suggest that GPAT1 is required for the normal synthesis of TAG in both fat cells and hepatocytes.

The role of GPAT1 in cardiomyocytes has not been established. Because the product of GPAT, lysophosphatidic acid, is a universal precursor for all glycerolipids, we hypothesized that diminished GPAT1 activity would alter the metabolism of myocardial TAG and phospholipids, which are critically required for energy production and the synthesis of myocardial membranes.

Materials and Methods

Animals

Animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Heterozygous (*Gpat1*$^{-/+}$) mice were bred, and wildtype and *Gpat1*$^{-/-}$ littermates were genotyped as described previously [29]. Mice were housed on a 12-h/12-h light/dark cycle with free access to water and defined isocaloric diets from Research Diets, Inc. The control diet (D12328) contained 16.4% protein, 12% maltodextrin, 60% corn starch, 4% soybean oil, and 6.5% hydrogenated coconut oil. The high sucrose diet (D12329) contained 16.4% protein, 12% maltodextrin, 60% sucrose, 4% soybean oil, and 6.5% hydrogenated coconut oil. The high fat diet (D12330) contained 16.4% protein, 12% maltodextrin, 12.5% corn starch, 4% soybean oil, and 54% hydrogenated coconut oil. At 2 months of age, wildtype and *Gpat1*$^{-/-}$ mice were placed in one of 3 feeding groups: control diet for 2 weeks, high sucrose diet for 2 weeks, or high fat diet for 3 months. F2 mice (50% C57BL/6, 50% SvEv129) were used for the high fat diet study and to examine cardiac lipid composition. Mice backcrossed 6 generations to the C57BL/6 background were used in the high sucrose feeding study.

RNA isolation and reverse transcriptase PCR

Hearts from two wildtype, two heterozygous and two *Gpat1* knockout mice were trimmed of fat and flash frozen in liquid nitrogen. RNA was isolated using the RNeasy mini kit for fibrous tissue (Qiagen) according to manufacturer's protocol. Briefly, frozen whole hearts (≈ 150 mg) were homogenized using a rotor stator homogenizer in 1.5 ml Buffer RLT + β-mercaptoethanol. Three hundred μl homogenate was used for RNA isolation. Heart RNA was subjected to reverse transcriptase PCR to determine the presence or absence of *Gpat1* and neomycin (*neo*) RNA transcripts. The cDNA was generated using the Superscript first-strand synthesis system for RT-PCR (Invitrogen), with oligo(dT)$_{12–18}$ primer according to manufacturer's protocol. PCR primers used were *Gpat1* forward (5′ ACA GTT GGC ACA ATA GAC GT), *Gpat1* reverse (5′ GAA GAT CTC CAG GAA CTG CT), and *neo* reverse (5′ TTA TGG CGC GCC ATC GAT CT). PCR products were analyzed on a 1.2 % agarose gel.

Isolation of heart total membrane and mitochondria

Hearts were trimmed of valves and external fat, minced, and homogenized with 10 up-and-down strokes in a Teflon-glass homogenizer in 10 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM DTT, and 1 mM EDTA. Large debris and nuclei were removed by centrifugation for 5 min at 600 × *g*. Total membranes were obtained by centrifuging the supernatant for 1 h at 100,000 × *g*. For mitochondrial isolation, hearts from 4 mice were trimmed and homogenized as described above. Mitochondria were obtained by centrifuging the post-nuclear supernatant at 10,300 × *g* for 10 min. The microsomal fraction was acquired by centrifuging the supernatant for 1 h at 100,000 × *g*. Protein concentrations were determined by the bicinchonic acid method (Pierce) using bovine serum albumin as the standard. Purity of the subcellular fractions was determined by measuring the activity of marker enzymes, NADH cytochrome *c* reductase [30] and cytochrome *c* oxidase (cytochrome *c* oxidase kit, Sigma) for endoplasmic reticulum and mitochondria, respectively.

Assay for glycerol-3-phosphate acyltransferase

sn-[2-^{3}H]glycerol-3-phosphate was synthesized enzymatically from [2-^{3}H]glycerol (1 mCi/ml) and purified as described previously [31]. GPAT activity was assayed at room temperature in a 200 μl mixture containing 75 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 1 mg/ml bovine serum albumin (essentially fatty acid-free), 1 mM DTT, 8 mM NaF, 800 μM [^{3}H]

glycerol-3-phosphate and 80 μM palmitoyl-CoA [32]. The reaction was initiated by adding 20–60 μg mitochondrial protein that had been incubated on ice for 15 min in the absence or presence of the indicated concentrations of NEM.

Blood chemistries and lipids

Mice were fasted 4 h, anesthetized with Avertin, and bled retroorbitally. Plasma TAG (TG, Stanbio Laboratory), total cholesterol (Cholesterol CII, Wako Chemical), free fatty acids (NEFA, Wako Chemical), and glucose (Glucose Trinder, Sigma) were determined by enzymatic colorimetric methods. Insulin was determined by radioimmunoassay (Linco). To determine VLDL secretion rates, mice were fasted for 4 h and then given 10% fructose to drink. Plasma was collected for TAG measurements at time zero and at 20, 40, 60, 80, and 120 min after I.V. injection of 20 mg Triton WR-1339 (Tyloxapol, Sigma) in a volume of 200 μl. At the final time point, mice were euthanized and livers were collected and weighed. The rate of VLDL secretion was expressed as the concentration of TAG secreted (mg per dl) per gram of liver.

Heart triacylglycerol

Lipids were extracted [33] from frozen hearts. One ml of lipid extract was dried in a SpeedVac concentrator and dissolved in 200 μl isopropyl alcohol containing 1% Triton X-100. TAG concentration was determined using an enzymatic colorimetric method (Stanbio Laboratory).

[^{14}C]palmitate incorporation into heart triacylglycerol

[^{14}C]palmitate incorporation into heart TAG was performed as described previously [34,35]. Mice were injected retro-orbitally with 1 μCi [^{14}C]palmitate complexed with 5% BSA. Blood was collected from the tail vein 5 and 30 min after injection of radiolabeled fatty acid. [^{14}C] in plasma was determined by scintillation counting. At 32 min post-injection, mice were euthanized. The heart was exposed, nicked near the aorta, perfused with 0.9% saline through the left ventricle, excised, rinsed in 0.9% saline to remove all blood, and then frozen in liquid N_2. Frozen tissue was pulverized into a fine powder and homogenized as described above. An aliquot of homogenized tissues was set aside for to determine protein concentration. Lipids were extracted from homogenized tissue as described above. Neutral lipids were separated by TLC with authentic standards for FA, DAG, and TAG (Avanti Polar Lipids) on LKD5 silica plates (Whatman) developed in hexane:ethyl ether: aceteic acid (80:20:2 by vol). [^{14}C] incorporated into TAG was quantified using Bioscan instrumentation and normalized to the protein concentration and dose of [^{14}C] counted in plasma.

Fatty acid analysis

Lipids from wildtype and *Gpat1*$^{-/-}$ (SvEv129 background) plasma and heart (100 mg) were extracted after a 4 h fast in the presence of authentic internal standards [36] using chloroform: methanol (2:1 v/v). Individual lipid classes were separated by preparative thin layer chromatography [37]. Isolated lipid classes were transesterified in 3 N methanolic HCl in a sealed vial under nitrogen atmosphere at 100 °C for 45 min. The resulting fatty acid methyl esters were extracted with hexane containing 0.05% butylated hydroxytoluene and then separated and quantified by capillary gas chromatography using a gas chromatograph (Hewlett Packard model 6890) equipped with a 30 m DB 225MS capillary column (J&W Scientific) and a flame ionization detector [37].

Statistics

Data are presented as means ± SD. Significant differences between groups were analyzed by two-tailed Student's *t*-test.

Results and Discussion

GPAT catalyzes the initial, committed, and rate limiting step in glycerolipid synthesis. Previous studies show that GPAT1 controls TAG synthesis in liver and adipose tissue in response to physiological signals [16,19,23–25], and that when GPAT1 is absent in liver, the liver phospholipid fatty acid composition is altered [29,38]. Therefore, we asked whether the relatively low GPAT1 activity in heart was functionally important for TAG accumulation and phospholipid fatty acid composition.

Hearts from GPAT1$^{-/-}$ mice lack GPAT1 mRNA, but retain NEM-resistant GPAT activity in mitochondria

Mice lacking GPAT1 weigh less than controls, have reduced gonadal fat pad weights, and lower hepatic TAG, plasma TAG, and VLDL secretion [29]. These mice also have a higher rate of hepatic fatty acid oxidation [39], and remain insulin sensitive on a high fat diet [39]. In liver from knockout mice, *Gpat1* mRNA is undetectable and NEM-resistant GPAT activity is reduced >90% [29]. In *Gpat1*$^{-/-}$ mice, a novel NEM-sensitive mitochondrial GPAT activity (GPAT2) is detectable in liver mitochondria [13].

As expected, no *Gpat1* transcript was detected in hearts from the knockout animals (Fig. 1A). To confirm the absence of GPAT1 activity, we assayed heart total membrane fractions in the absence and presence of 1 mM NEM. In hearts from wildtype mice, the NEM-resistant GPAT activity was 0.13 nmol/min/mg, 27% of the total (0.47 nmol/min/mg) (Fig. 1B). In *Gpat1*$^{-/-}$hearts, total GPAT activity was diminished 40%, confirming the absence of GPAT1. However, 1 mM NEM failed to inhibit all the remaining GPAT activity, suggesting the presence of a novel NEM-resistant isoform.

To determine whether this new NEM-resistant activity was microsomal or mitochondrial, we assayed highly pure microsomal and mitochondrial fractions. GPAT activity from wildtype and *Gpat1*$^{-/-}$ mice was identical in heart microsomes (data not shown). In heart mitochondria, an NEM inhibition curve using 0 to 10 mM showed that maximal inhibition occurred with 1 mM NEM in both genotypes (Fig. 1D). In wildtype mitochondria, 43% of the GPAT activity was NEM-resistant (Fig. 1C). However, although the total mitochondrial activity in *Gpat1*$^{-/-}$ hearts was 57% less than the activity measured in mitochondria from wildtype mice, 1 mM NEM only decreased this activity 55% to 0.11 nmol/min/mg protein (Fig. 1C). These data are contrary to our findings in liver mitochondria, where we found that 1 mM NEM treatment of liver mitochondria from *Gpat1*$^{-/-}$ mice completely inhibited all GPAT activity indicating the absence of NEM-resistant GPAT activity [13]. Because the other cloned GPAT isoforms (2 – 4) are inhibited by NEM [14,15,18], our data suggest the presence of a novel non-GPAT1 NEM-resistant GPAT activity in heart mitochondria.

Absence of GPAT1 diminishes myocardial TAG accumulation in response to a high sucrose diet

Although our studies on heart TAG synthesis are complicated by the presence of multiple GPAT isoforms (GPAT1-4) [14,15,17,18,40], the *Gpat1*$^{-/-}$ mice still express significantly lower myocardial GPAT activity. Because GPAT1 has an established role in regulating TAG synthesis in liver, we asked whether GPAT1 might also regulate myocardial TAG accumulation under lipogenic conditions such as sucrose-induced TAG accumulation in the heart [41]. On the control diet, heart TAG content was similar in wildtype and *Gpat1* null

mice (Fig. 2A). Although 2 weeks of high sucrose feeding did not change GPAT1 mRNA expression or NEM-resistant activity in mitochondria (data not shown), myocardial TAG content increased 2-fold to 300 ng TAG/μg protein in male and female wildtype mice. In sucrose-fed male *Gpat1*$^{-/-}$ animals, heart TAG content did not increase as compared to mice fed the control diet (133 ng TAG/μg protein vs. 124 ng TAG/μg protein). In sucrose-fed female knockout mice, myocardial TAG only increased 45% to 180 ng TAG/μg protein. Myocardial TAG content in female *Gpat1*$^{-/-}$ may be greater than in male knockout littermates because the female mice appear to be developing insulin resistance, as indicated by significant increases in plasma insulin concentration (Table 1). The presence of diminished heart TAG content in sucrose-fed mice indicated that GPAT1 may be an important regulator of myocardial TAG synthesis.

Because *Gpat1*$^{-/-}$ mice have decreased VLDL secretion rates and lower plasma TAG than wildtype littermates [29], we postulated that diminished delivery of TAG to the heart might be responsible for the lower myocardial TAG content in heart from sucrose fed *Gpat1*$^{-/-}$ mice. In sucrose-fed male *Gpat1*$^{-/-}$ mice, plasma TAG and cholesterol were significantly lower than in wildtype mice (Fig. 3A, Table 1). However, in female *Gpat1*$^{-/-}$ mice, values for plasma free fatty acid (FFA), TAG, and cholesterol did not differ significantly from control or from treated wildtype mice (Fig. 3A, Table 1).

To determine whether the lower VLDL secretion rate reported in *Gpat1*$^{-/-}$ mice fed chow [29] remained low in mice fed a lipogenic diet, VLDL secretion was measured in mice fed a high sucrose diet for 2 weeks. The high sucrose diet increased liver TAG content 8-fold and, compared to our previous study [29], increased the rate of VLDL synthesis 64% in wildtype mice. Consistent with the diminished TAG synthesis observed in the absence of GPAT1, sucrose-fed female *Gpat1*$^{-/-}$ mice had 40% lower content of liver TAG (43.7±3.3 mg/g liver, n = 5) compared to wildtype controls (74.6±10.2 mg/g liver, n = 5). Although plasma TAG and cholesterol concentrations were lower in male *Gpat1*$^{-/-}$ mice fed the sucrose diet and unchanged in female mice (Table 1), VLDL secretion rates were 30% lower in both sexes (Fig. 4A, B).

Surprisingly, even in the absence of hypertriglyceridemia (Fig. 3A), cardiac myocytes from wildtype mice accumulated a significant amount of TAG (Fig. 2A). Although, myocardial TAG accumulation is typically observed in pathophysiological conditions such as obesity and diabetes [42–46], these data suggest that hypertriglyceridemia is not essential for heart TAG accumulation.

Taken together, our data suggest that the lower content of TAG in *Gpat1*$^{-/-}$ hearts (Fig. 2A) did not result from diminished entry of plasma fatty acid into the myocardial cells. Although male *Gpat1*$^{-/-}$ mice had 24% lower plasma TAG (Fig. 3A), the plasma TAG concentration in females was similar in knockout and wildtype animals, and VLDL secretion rates were 30% lower in both sexes. Thus, myocardial TAG content appeared to depend more on GPAT1-initiated synthesis than on fatty acid delivery to the myocardium.

To confirm that myocardial TAG synthesis is diminished in *Gpat1*$^{-/-}$ hearts, we monitored [^{14}C]palmitate incorporation into heart TAG in vivo. Because male *Gpat1*$^{-/-}$ mice failed to accumulate excess myocardial TAG with high-sucrose feeding (Fig. 2A), male wildtype and *Gpat1*$^{-/-}$ mice fed the high-sucrose diet for 2 wks were injected with [^{14}C]palmitate complexed to BSA. After 30 min, *Gpat1*$^{-/-}$ hearts incorporated 60% less [^{14}C] into TAG as compared to wildtype mice (Fig. 5). Both the availability of plasma fatty acids and the protein mediated uptake of fatty acids (ie via CD36, FABP) influence the rate of fatty acid entry into the cardiomyocyte [47]. We did not determine if absence of GPAT1 altered expression of proteins required for fatty acid entry, but plasma fatty acid concentrations

were similar between wildtype and *Gpat1*$^{-/-}$ mice fed the high-sucrose diet (Table 1), suggesting that the diminished rate of [^{14}C] incorporation into heart TAG results from the lack of GPAT1.

Absence of GPAT1 diminishes myocardial TAG accumulation in response to a high fat diet

High fat feeding, an established method for inducing obesity in rodents [48], is a second method of increasing myocardial TAG content [49]. In an initial characterization of the *Gpat1*$^{-/-}$phenotype, F2 mice (50% C57BL/6J, 50% 129SvEv) were fed a high fat diet (58% of kcal) for 3 months. The wildtype mice did not become obese or hypertriglyceridemic and body weight of wildtype and *Gpat1*$^{-/-}$ mice increased only moderately (6–10 g), probably because the study was performed on F2 mice which had a mixed background4 of 50% C57BL/6J (obesity-prone [48]) and 50% 129SvEv (obesity-resistant; personal communication Shelia Collins, Duke University). Despite the lack of obesity, the high fat diet increased plasma TAG 30–70% (Fig. 3B) and plasma cholesterol 70–80% (Table 2) in both wildtype and *Gpat1*$^{-/-}$ mice.

In male and female wildtype mice, the high fat diet increased myocardial TAG content 1.5- to 2.2-fold above baseline to 213 and 323 ng TAG/μg protein, respectively (Fig. 2B). In *Gpat1*$^{-/-}$mice, however, heart TAG increased only 35% to 168 ng TAG/μg protein in males and 80% to 223 ng TAG/μg protein in females (Fig. 2B). Greater myocardial TAG content in female *Gpat1*$^{-/-}$ mice as compared to male knockout littermates may be due to the significant rise in plasma insulin after 3 months of high-fat feeding (Table 2). Because the increase in diet-induced plasma TAG concentration was the same in wildtype and knockout mice, the lower cardiomyocyte TAG content in *Gpat1* null mice suggests that GPAT1, rather than an increase in fatty acid delivery, is required for the full increase in heart TAG accumulation in response to a high fat diet.

Absence of GPAT1 alters heart phospholipid fatty acid composition

Myocardial phospholipid composition is altered in rats with heart dysfunction due to type 1 diabetes [10] and in aging rodents [9]. In aging rats, cardiolipin (CL) content is diminished, resulting in impaired mitochondrial function, similar to that reported in liver from *Gpat1*$^{-/-}$ mice [38]. Because the fatty acid (FA) composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is altered in liver from *Gpat1*$^{-/-}$ mice [29], we measured the phospholipid FA composition in *Gpat1*$^{-/-}$ hearts. Compared to wildtype mice, the amount of 16:0 in GPAT1$^{-/-}$ heart PC, PE, and PS/PI was 25%, 40%, and 45% lower, respectively (Fig. 6 A–C), consistent with the preference of GPAT1 for 16:0-CoA [20–22]. PC from *Gpat1*$^{-/-}$ hearts also contained more 18:0, 18:1n9, 20:4n6, and 22:0 (increases of 20%, 47%, 20%, and 28%, respectively) than wildtype controls (Fig. 6A). In PE from *Gpat1*$^{-/-}$ hearts, 18:0, 18:1n9, and 20:4n6 were only 10–18% higher than controls (Fig. 6B). Different FA were altered in PI/PS, with a dramatic 3.5-fold increase in 20:4n6 in *Gpat1*$^{-/-}$ hearts compared to wildtype controls (Fig. 6C). PI/PS also contained 32% less 22:6n3. The fatty acid composition of CL, a lipid critical for mitochondrial function, remained virtually unchanged in *Gpat1*$^{-/-}$ hearts, but the minor species 22:0 was 85% higher than in wildtype hearts (Fig. 6D). Because the heart derives fatty acid substrates from plasma lipoproteins, and plasma TAG from *Gpat1*$^{-/-}$ mice shows similar changes in phospholipid FA composition, with 40% less C16:0 and 35, 21, and 6% more 18:0, 18:1n9, and 18:2n6, respectively, than wildtype controls (Fig. 6E), we cannot with certainty attribute these changes to the absence of heart GPAT1. However, compared to wildtype mice heart PC, PE, and PI/PS from *Gpat1*$^{-/-}$ mice contain 28%, 20%, and 350% more 20:4n6, respectively. Because these increases were not observed in *Gpat1*$^{-/-}$ plasma TAG, it appears that heart

[4] *Gpat1*$^{-/-}$ mice backcrossed onto the C57BL/6J background become obese when fed a high fat, high sucrose diet for 4 months [55].

GPAT1 plays an important role in establishing the fatty acid composition of its membrane phospholipids. Higher 20:4n6 content was previously reported in both total liver phospholipids [29] and liver mitochondrial phospholipids [38] from *Gpat1*$^{-/-}$ mice. In liver, because the elevated 20:4n6 was primarily found at the *sn*-2 position, it was hypothesized that the replacement of 16:0 with 18:0 and 18:1 at the *sn*-1 position might have altered lysophospholipid remodeling at the *sn*-2 position [29]. Analyses of phospholipids typically show 18:0 and 20:4 in the *sn*-1 and *sn*-2 positions, respectively, of the same phospholipid molecule [50].

In *Gpat1*$^{-/-}$ hearts, the increased incorporation of 20:4n6 into phospholipids, particularly PI and PS, could alter cardiac function. Arachidonic acid content in heart is important for normal cardiac function especially in the sarcolemmal membrane in which 20:4 is normally present on the cytoplasmic leaflet in PE, PS, and PI, and contributes to proper Ca^{2+} handling and contractile function [10]. In streptozotocin-diabetic rats with diabetic cardiomyopathy, a diminished 20:4 content is found in sarcolemmal PC, PE, and PS [10]. In addition, arachidonate-derived eicosanoids have been linked to the development of cardiac fibrosis [51]. The changes observed in myocardial PC, PE, and PI/PS provide strong evidence that GPAT1 influences the final fatty acid composition of membrane phospholipids.

Absence of GPAT1 may result only in a mild myocardial phenotype because several GPAT isoforms are present in the heart. Seventy percent of total heart GPAT activity is NEM-sensitive and located in the endoplasmic reticulum [11,19]. This microsomal GPAT activity, in part comprised of GPAT3 and 4 {Cao, 2006 #1199; Nagle, 2008 #1241}, is not regulated concomitantly with increased heart TAG synthesis [19]. In heart mitochondria from *Gpat1*$^{-/-}$mice, the residual NEM-sensitive GPAT activity is probably GPAT2 [13,40], whose contribution to TAG synthesis is unknown (Fig. 1C). In addition, the persistence of NEM-resistant GPAT activity in *Gpat1*$^{-/-}$ heart mitochondria (Fig. 1C, D) suggests that a third mitochondrial GPAT isoform exists. Although, additional GPAT isoenzymes in the heart could have partially substituted for the function of GPAT1, the changes in TAG content and phospholipid composition in hearts from both high-sucrose and high-fat fed mice strongly suggest that cardiomyocyte GPAT1 contributes significantly to the synthesis of TAG and phospholipids and to the storage of TAG.

Our data are consistent with the hypothesis that each GPAT isoenzyme has a distinct function within the cell. Several other functionally distinct isoforms have been described in glycerolipid synthesis, including 1-acyl-glycerol-3-phosphate acyltransferase-2 (AGPAT2) [52], and diacylglycerol acyltransferases-1 and -2 [53,54]. The phenotype of mice or humans deficient in each of these activities shows that the presence of catalytically similar isoforms does not functionally replace the missing enzyme and suggests that each isoform plays a specific and unique role in lipid metabolism.

Abbreviations

AGPAT	1-acyl-glycerol-3-phosphate acyltransferase
CL	cardiolipin
DGAT	diacylglycerol acyltransferase
FA	fatty acid
GPAT	glycerol-3-phosphate acyltransferase
LPL	lipoprotein lipase
NEM	*N*-ethylmaleimide

neo	neomycin
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
TAG	triacylglycerol

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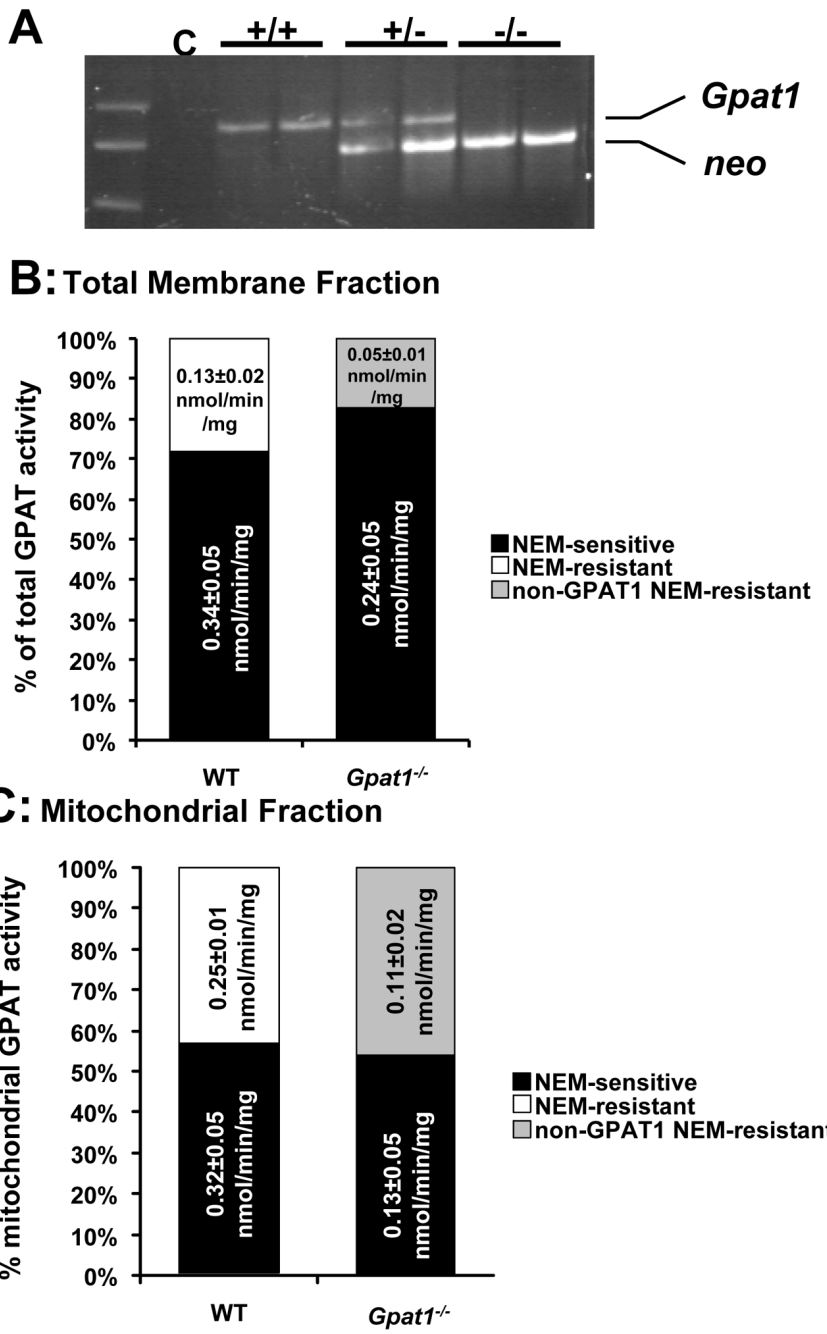

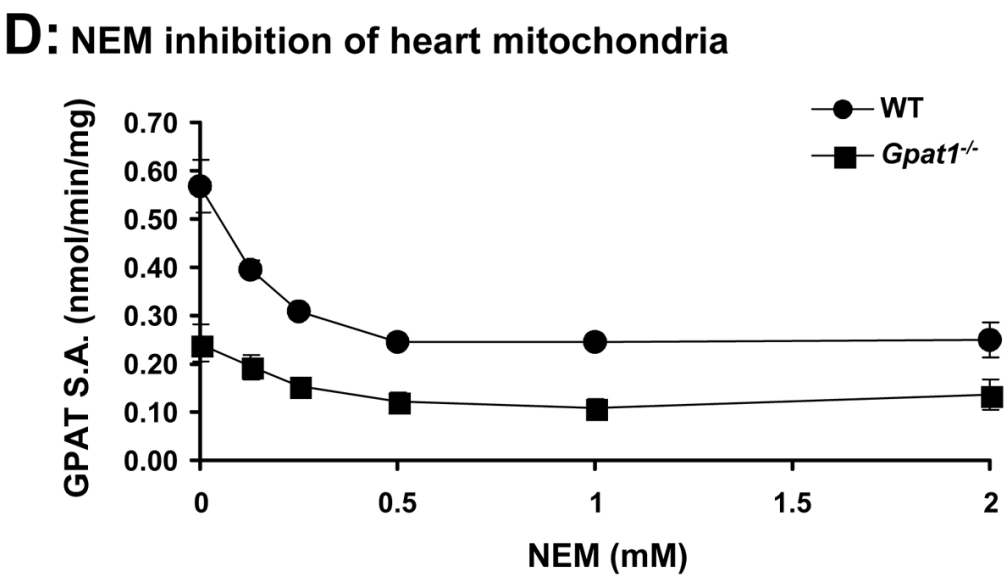

Figure 1. Hearts from *Gpat1*$^{-/-}$ mice lack *Gpat1* mRNA, but retain an NEM-resistant GPAT activity

A: RNA was isolated from backcrossed Bl/6 wildtype (+/+), heterozygous (+/−), and knockout (−/−) *Gpat1* mouse hearts (n = 2) and analyzed for the presence or absence of *neo* and *Gpat1* transcripts by reverse transcriptase PCR as described in Materials and Methods. The first lane contains DNA size markers (from top: 1000, 850, and 650 bp). The second lane (C) is a no template control reaction. Heart total membranes (*B)* and mitochondria (*C, D*) from wildtype and *Gpat1*$^{-/-}$ mice were isolated as described and assayed for GPAT activity in the absence or presence of *(B, C)* 1 mM NEM or *(D)* 0–10 mM NEM. Data not shown for 5 and 10 mM NEM, as further inhibition was not observed. Data are presented as the mean ± SD (n = 3 – 4).

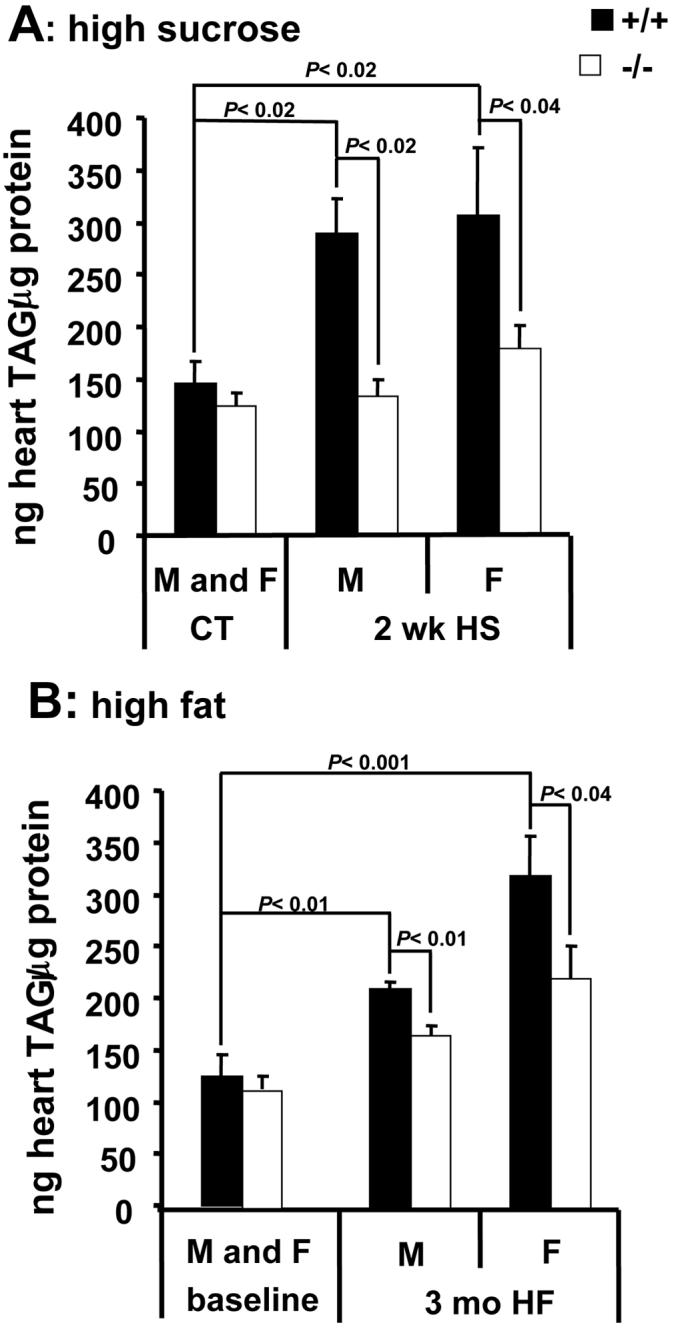

Figure 2. Absence of GPAT1 diminishes myocardial triacylglycerol accumulation

Lipids were extracted from hearts of male and female Bl/6 (*A*, high sucrose diet) or F2 (*B*, high fat diet) mice (n ≥ 6 for each group) and TAG content was determined as described in Materials and Methods. *A:* High sucrose (HS) vs control (CT) diet. *B:* High fat (HF) diet vs baseline. For hearts from mice at baseline or on the control diet, heart TAG did not differ between male and female mice. Data are presented as mean ± SD.

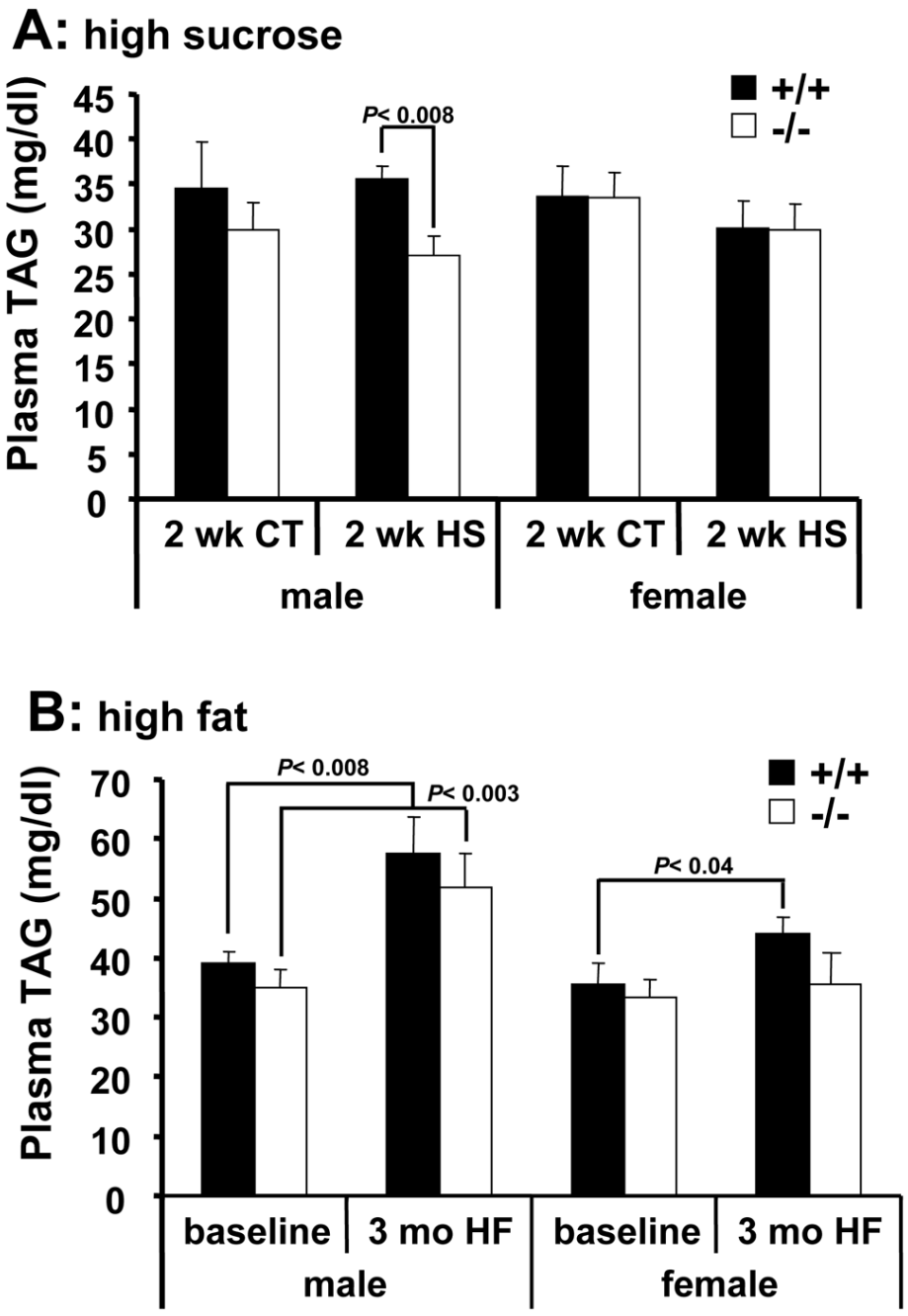

Figure 3. *Gpat1*$^{-/-}$ mice have lower plasma triacylglycerol than wildtype mice after high sucrose- or high fat-diet

Plasma lipids were extracted from male and female Bl/6 (*A*, high sucrose diet) or F2 (*B*, high fat diet) mouse hearts and TAG concentration was determined as described in Materials and Methods. *A:* High sucrose (HS) vs control (CT) diet. *B:* High fat (HF) diet vs baseline. Data are presented as the mean ± SD; n = 6 for each group.

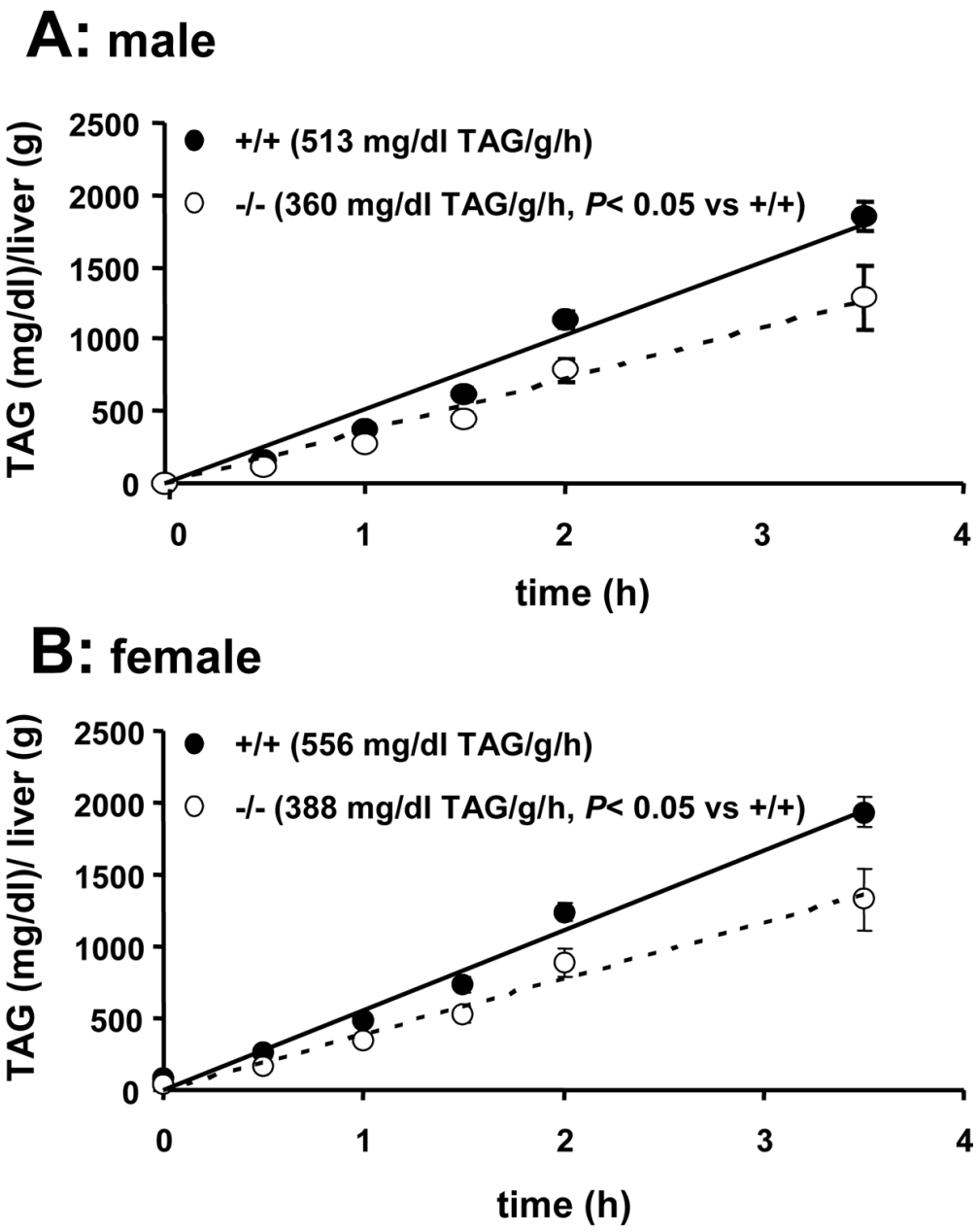

Figure 4. Male and female *Gpat1*$^{-/-}$ mice have lower VLDL secretion rates than wildtype mice after 2 week high sucrose diet

After a 4 h fast, male (A) and female (B) wildtype and *Gpat1* null Bl/6 mice were injected with Tyloxapol as described in Materials and Methods and plasma was collected at the times shown. Rates of VLDL secretion were calculated as the concentration of TAG (mg/dl) per unit of liver weight (g) per hour. Data are presented as the mean ± SD; n ≥ 5 for each group.

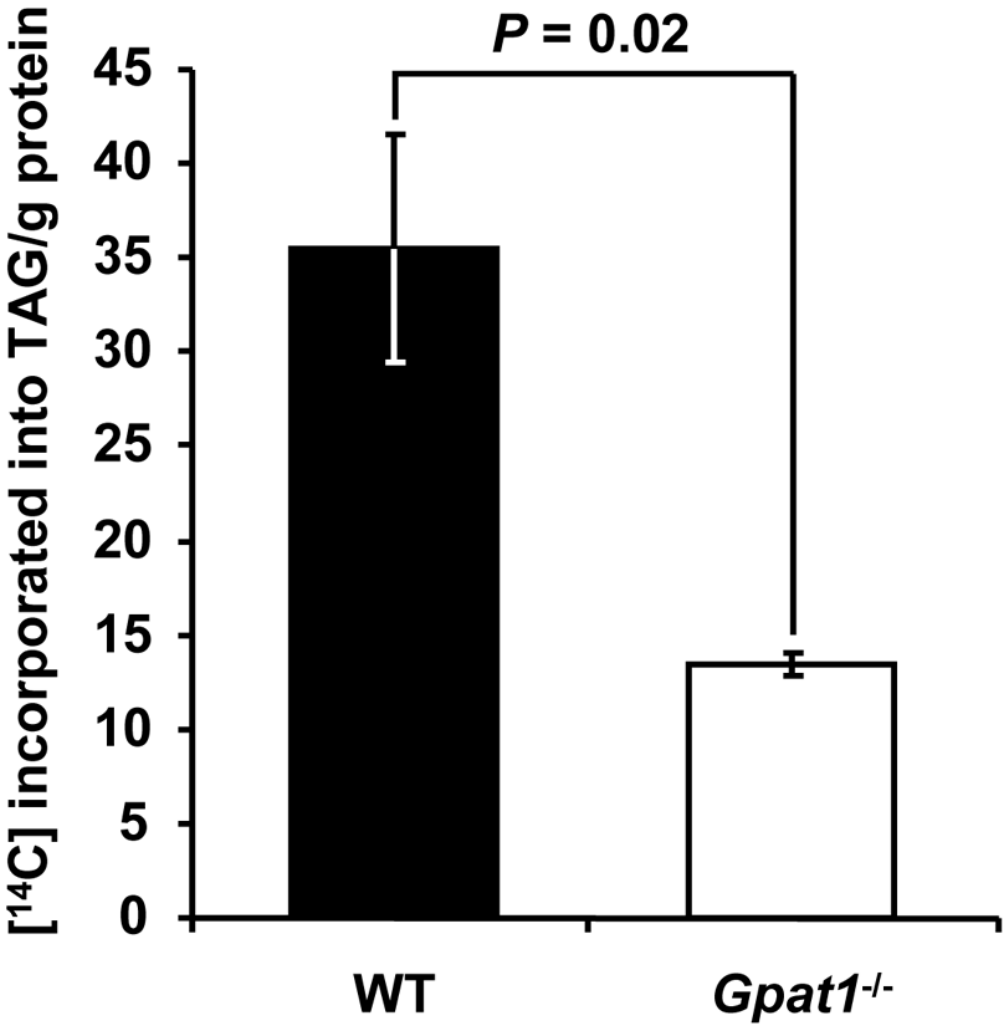

Figure 5. Absence of GPAT1 decreases [^{14}C]palmitate incorporation into heart triacylglycerol in vivo

Wildtype and *Gpat1*$^{-/-}$ mice were fed a high-sucrose diet for 2 wks and injected with [^{14}C]palmitate complexed to BSA as described in Materials and Methods. Heart lipids were extracted and separated by TLC and the amount of [^{14}C] incorporated into TAG was quantified by Bioscan analysis. Data are presented as cpm [^{14}C] incorporated into TAG/g protein/cpm [^{14}C] in plasma; n = 3.

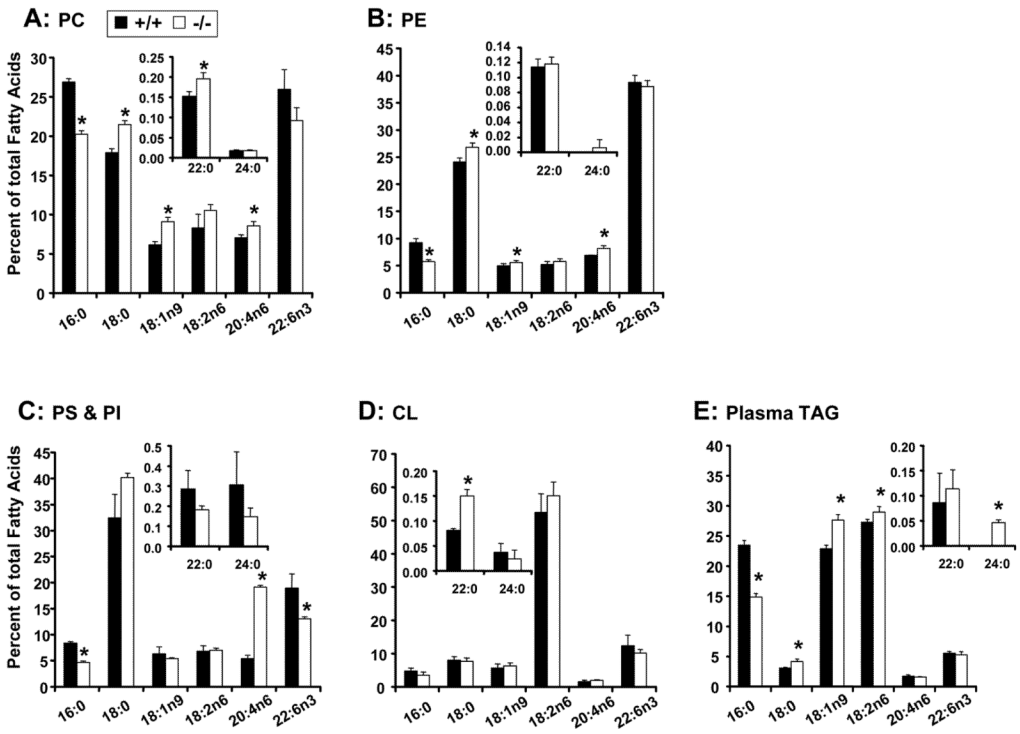

Figure 6. Absence of GPAT1 alters heart phospholipid fatty acid composition

Lipids were extracted from hearts and plasma of F2 female wildtype (+/+) and *Gpat1*$^{-/-}$ (−/−) mice (n = 4/genotype) and fatty acid composition was determined as described in Materials and Methods. *A*, phosphatidylcholine (PC); *B*, phosphatidylethanolamine (PE); phosphatidylserine (PS) and phosphatidylinositol (PI); *D*, cardiolipin (CL) *E*, plasma TAG. Data are presented as mean ± SD. **P* ≤ 0.05 relative to wildtype control.

Table 1

Weight and plasma values for Bl/6 mice fed a high sucrose diet for 2 weeks

			Weight (g)	FA (mM)	Cholesterol (mg/dl)	Glucose (mg/dl)	Insulin (ng/ml)
Male	+/+	**2 wk control**	24.8±0.8	0.56±0.1	83.6±6.9	190±11	0.86±0.1
		2 wk sucrose	23.1±0.9	0.82±0.1	96.1±10.1	172±18	0.82±0.3
	−/−	**2 wk control**	24.0±0.6	0.69±0.1	74.3±6.3	194±7	0.66±0.2
		2 wk sucrose	23.5±1.6	0.72±0.2	74.1±10.4[a]	202±16	0.91±0.4
Female	+/+	**2 wk control**	19.4±0.7	0.67±0.1	70.3±3.7	182±7	0.70±0.1
		2 wk sucrose	19.9±0.8	0.69±0.1	73±6.9	187±18	0.57±0.3
	−/−	**2 wk control**	18±0.71	0.82±0.1	62.6±4.5	180±12	0.44±0.1[a]
		2 wk sucrose	19.3±1.0	0.76±0.1	66.8±6.7	215±18	0.81±0.2 [b]

Data are presented as the mean ± SD; n = 5–8 for each group.

[a] −/− vs +/+ $P \leq 0.05$;

[b] diet vs control $P \leq 0.05$

Table 2

Weight and plasma values for f2 mice fed a high fat diet for 3 months

			Weight gain (g)	FA (mM)	Cholesterol (mg/dl)	Glucose (mg/dl)	Insulin (ng/ml)
Male	+/+	**baseline**	NA	0.57±0.06	74.9±4.9	189±10	ND
		3 mo HF	8.0±1.5	0.69±0.1	137.2±9.2 [a]	167±14	ND
	−/−	**baseline**	NA	0.67±0.1	65.4±5.2	205±6	ND
		3 mo HF	9.6±1.1	0.74±0.1	117.5±9.1 [a]	195±14	ND
Female	+/+	**baseline**	NA	0.69±0.7	65.2±2.8	185±1	0.70±0.1
		3 mo HF	5.7±1.3	0.66±0.1	110±6.5 [a]	167±16	1.09±0.2 [a]
	−/−	**baseline**	NA	0.74±0.1	54.2±5.0	167±18	0.54±0.1
		3 mo HF	6.5±1.4	0.59±0.1	94.9±6.3 [a]	167±9	0.9±0.1 [a]

Data are presented as mean ± SD; n ≥ 5 for each group.

[a] diet vs baseline $P \leq 0.05$; NA= not applicable ND = not determined.

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