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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

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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.

1. 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 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

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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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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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.

2.5. Blood chemistries and lipids

Mice were fasted 4 h, anesthetized with Avertin, and bled retro-orbitally. 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/dl) per gram of liver.

2.6. 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).

2.7. [^{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.

2.8. 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].

2.9. Statistics

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

3. 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