Biochimica et Biophysica Acta 1862 (2017) 324–336

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Deficiency of glycerol-3-phosphate acyltransferase 1 decreases triacylglycerol storage and induces fatty acid oxidation in insect fat body

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ARTICLE INFO

Article history:
Received 21 July 2016
Received in revised form 6 December 2016
Accepted 8 December 2016
Available online 09 December 2016

Keywords:
Beta-oxidation
Fatty acid metabolism
Lipids
Triacylglycerol synthesis
Fatty acid oxidation
Insect physiology

ABSTRACT

Glycerol-3-phosphate acyltransferases (GPAT) catalyze the initial and rate-limiting step for the *de novo* synthesis of triacylglycerol (TAG). Four mammalian GPAT isoforms have been identified: the mitochondria-associated GPAT1 and 2, and the endoplasmic reticulum (ER)-associated GPAT3 and 4. In the insect *Rhodnius prolixus*, a vector of Chagas' disease, we previously predicted a mitochondrial-like isoform (RhoprGPAT1) from genomic data. In the current study, we clone the RhoprGPAT1 coding sequence and identify an ER-associated GPAT (RhoprGPAT4) as the second isoform in the insect. RhoprGPAT1 contributes 15% of the total GPAT activity in anterior midgut, 50% in posterior midgut and fat body, and 70% in the ovary. The *RhoprGpat1* gene is the predominant transcript in the midgut and fat body. To evaluate the physiological relevance of RhoprGPAT1, we generate RhoprGPAT1-deficient insects. The knockdown of *RhoprGpat1* results in 50% and 65% decrease in TAG content in the posterior midgut and fat body, respectively. *RhoprGpat1*-deficient insects also exhibits impaired lipid droplet expansion and a 2-fold increase in fatty acid β-oxidation rates in the fat body. We propose that the RhoprGPAT1 mitochondrial-like isoform is required to channel fatty acyl chains towards TAG synthesis and away from β-oxidation. Such a process is crucial for the insect lipid homeostasis.

1. Introduction

The synthesis of triacylglycerol (TAG) is initiated by the acylation of glycerol-3-phosphate (G3P) with a long-chain acyl-CoA in a rate-limiting step catalyzed by glycerol-3-phosphate acyltransferase (GPAT). Four GPAT isoforms have been identified in mammals: GPAT1 and 2 are mitochondria-associated proteins, whereas GPAT3 and 4 are located on the endoplasmic reticulum (ER). The contribution of each isoform to lipid metabolism varies in a tissue- and nutritionally-dependent manner. GPAT1 activity constitutes about 10% of total GPAT activity in most of tissues except for liver, where it corresponds to 30–50% of the total activity [1]. *Gpat1*$^{-/-}$ mice have reduced body weight, lower hepatic TAG content and increased hepatic β-oxidation [2]. When fed a high-fat diet, *Gpat1*$^{-/-}$ mice resist hepatic steatosis and maintain enhanced insulin sensitivity [3]. The abundance of *Gpat2* mRNA is 50-fold greater in testis than in lipogenic tissues [4]; in testis GPAT2 acts to incorporate arachidonoyl-CoA (20:4) into TAG, thereby regulating the availability of polyunsaturated acyl-chains within spermatogenic cells [5]. GPAT3 contributes nearly 80% of total GPAT activity in murine white adipose tissue [6]. GPAT3-deficient animals have reduced fat mass and increased hepatic TAG and cholesteryl ester content [7]. GPAT4 contributes 40–50% of total hepatic GPAT activity and is required for the incorporation of exogenous fatty acids into TAG [2]. GPAT4 is also the major GPAT isoform in brown adipose tissue (BAT), where it is important in the partitioning of exogenous acyl-CoAs into lipid droplet TAG and away from mitochondrial β-oxidation [8].

Information about GPAT enzymes is scarce among non-mammalian animals, such as arthropods. GPAT activity was detected in the Mediterranean fruit fly *Ceratitis capitata* [9] and inferred in the tobacco hornworm *Manduca sexta* based on lipid synthesis measurements [10]. In the silkworm *Bombyx mori*, the knockdown of a putative GPAT protein results in decreased TAG content in pheromone glands [11]. A mitochondrial GPAT activity was characterized in the shrimp *Macrobrachium borelii* [12], but the presence of multiple isoforms remains unknown.

Abbreviations: ACBP1, acyl-CoA-binding protein 1; ACSL1, acyl-CoA synthetase 1; BAT, brown adipose tissue; CPT1, carnitine palmitoyltransferase 1; DAG, diacylglycerol; ER, endoplasmic reticulum; G3P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LD, lipid droplet; LPA, lysophosphatidic acid; NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PL, phospholipid; TAG, triacylglycerol.

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http://dx.doi.org/10.1016/j.bbalip.2016.12.004
1388-1981/

The overexpression of *Drosophila melanogaster* mitochondrial-like GPAT increases lipid accumulation in larvae salivary glands [13]. Additionally, two microsomal-like isoforms (dmGPAT3 and 4) were identified in the fruit fly [14], and the migration of dmGPAT4 from ER to lipid droplets (LDs) is required for LD expansion [15].

In *Rhodnius prolixus*, a hematophagous insect and vector of Chagas' disease in Central and South America, lipids are required not only for basic cellular functions, but also for survival during prolonged starvation and for reproduction [16,17].

A mitochondrial-like GPAT (*RhoprGpat1*) gene, predicted from genomic data, was suggested to initiate the glycerolipid synthesis in the gut and in the fat body – an organ functionally analogous to both mammalian liver and adipose tissue – after a blood meal [18]. Here we show that an additional microsomal-like GPAT (RhoprGPAT4) is also present in this insect. Because RhoprGPAT1 comprised about 50% of total GPAT activity in the posterior midgut and fat body, was the predominant transcript in both organs, and was up-regulated after feeding, we hypothesized that RhoprGPAT1 must play a critical role in TAG synthesis in fed animals. To test this hypothesis, we evaluated lipid metabolism in insects after knocking down RhoprGPAT1. Our studies show that the lack of RhoprGPAT1 prevented fatty acid incorporation into TAG and increased fatty acid β-oxidation.

2. Material and methods

2.1. Insects

Insects were taken from a colony of *Rhodnius prolixus* maintained at 28 °C and 70–75% relative humidity, and were fed with live-rabbit blood at 3-week intervals [17]. All animal care and experimental protocols were conducted following the guidelines of the Committee for Evaluation of Animal Use for Research from the Federal University of Rio de Janeiro (CAUAP-UFRJ) and the NIH Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3). The protocols were approved by CAUAP-UFRJ. Technicians dedicated to the animal facility at the Institute of Medical Biochemistry (UFRJ) conducted all aspects related to rabbit husbandry under strict guidelines to ensure careful and consistent animal handling.

2.2. Gene identification

Genes were searched in the *R. prolixus* genome assembly (VectorBase, http://www.vectorbase.org, *R. prolixus* CDC annotation, RproC1) by similarity to the acyltransferase family consensus sequence (Pfam number PF01553) using FAT software [19]. Identified supercontigs were compared by tBlastn to GPAT protein sequences from different organisms available in GenBank. Coding region prediction, protein primary sequence and phylogenetic analyses were performed as described [17]. Subcellular localization was predicted by PSORT II Server [20].

2.3. Rapid amplification of cDNA 5′-end (5′-RACE)

The 5′ end of the *RhoprGpat1* was amplified using GeneRacer kit for full-length, RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (RLM-RACE, Invitrogen). Amplification of the full-length cDNA was performed using primers designed for specific amplification of target genes based on *in silico* predictions and the information obtained using RACE (Supplementary Table 1). PCR reactions were carried out as described [21]. The full length CDS sequences obtained for *RhoprGpat1* or *RhoprGpat4* were registered in the NCBI GenBank database under accession numbers KT328599 and KT328600, respectively.

2.4. Quantitative PCR (qPCR)

Organs were obtained as described [18], and total RNA was isolated from samples (pools from 3 to 5 organs), treated with RNase-free DNase I and used to synthesize cDNA. qPCR was performed using specific primers for the target genes (Supplementary Table 1) [21]. *Rhopr18S* gene amplification was used for normalization [22]. Primer efficiencies and qPCR inhibition were determined [23]. Amplification specificity analysis and qPCR controls to detect contaminations were conducted following the MIQE guidelines [24].

2.5. GPAT assay

Organs (obtained from 16 to 25 insects) were homogenized in a Potter-Elvehjem tube (30 strokes) in cold Medium I buffer (250 mM sucrose, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol). Samples were centrifuged at 3000 *g* for 15 min at 4 °C, and total membranes were isolated from the supernatant by centrifuging at 150,000 *g* for 1 h at 4 °C. *sn*-[2-^{3}H]glycerol 3-phosphate was synthesized enzymatically [25]. Efficiency of synthesis reaction was checked by paper chromatography, and obtained [3H]glycerol 3-phosphate was purified by anion exchange chromatography, as described [25]. GPAT activity was assayed for 10 min at room temperature in a 200 μL mixture containing 3 μCi [3H]glycerol 3-phosphate (specific activity 19 mCi/mmol), supplemented with 800 μM glycerol 3-phosphate, 80 μM palmitoyl-CoA, 75 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 1 mg/mL fatty acid-free albumin, 1 mM DTT, and 8 mM NaF [26]. After incubating samples on ice for 15 min in the presence or absence of 2 mM *N*-ethylmaleimide (NEM), the reaction was initiated by adding 4–8 μg of membrane protein to the assay mixture. The reaction products were extracted into $CHCl_3$ and 1% perchloric acid as described [27], and the radioactivity present in the organic phase, was determined by scintillation counting. >98% of the products extracted in this way were lysophosphatidic acid and also phosphatidic acid, rapidly synthesized from the former. NEM-resistant activity (RhoprGPAT1) was calculated by subtracting NEM-sensitive activity (RhoprGPAT4) from total activity.

2.6. Gene knockdown and insect treatment

dsRNA was synthesized by MEGAScript RNAi Kit (Ambion Inc., Austin, USA) using primers for *RhoprGpat1* specific gene amplification (Supplementary Table 1) [17]. RNAi specificity was confirmed by off-target search using dsCheck software [28]. Unfed adult females were injected with 0.5 μg dsRNA [17] and fed 3 days later. Knockdown efficiency was confirmed in each experiment by qPCR, and knockdown persistence was also determined (Suppl. Fig. 1). The bacterial *MalE* gene was used as a control dsRNA [29].

2.7. Phenotypic analyses

Adult females injected with dsRNA were fed with live-rabbit blood and immediately transferred to individual vials. The mortality rates and the number of laid eggs were recorded daily. Protein digestion analysis, enzymatic TAG determination, *de novo* lipid synthesis assay, and fatty acid oxidation assay were performed as described [21]. Briefly, for fatty acid oxidation assay, ten days after blood meal the insects were dissected and five fat bodies were obtained from each experimental group. Organs were washed in 0.15 M NaCl and homogenized in a Potter-Elvehjem tube (15 strokes) in 200 μL of cold buffer H (10 mM Hepes-KOH, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT) containing 0.002% v/v protease inhibitor cocktail (Sigma-Aldrich Co., St. Louis, USA). The homogenate (150 μg protein) was incubated with 8 μCi ^{3}H-palmitate (0.1 μCi/μL; Perkin-Elmer) in 75 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 2 mg/mL fatty acid free albumin, 5 mM ATP, 5 mM DTT, 0.2 mM CoASH, 10 mM L-carnitine, 20 mM palmitate (200 μL final volume) at 28 °C for 30 min. Reactions were stopped with 200 μL cold