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Long-chain acyl-CoA synthetase 2 knockdown leads to decreased fatty acid oxidation in fat body and reduced reproductive capacity in the insect *Rhodnius prolixus*

Michele Alves-Bezerra [a,b], Eric L. Klett [b,c], Iron F. De Paula [a], Isabela B. Ramos [a], Rosalind A. Coleman [b], Katia C. Gondim [a,*]

[a] Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil
[b] Department of Nutrition, University of North Carolina, Chapel Hill, NC 27599, USA
[c] Department of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA

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ABSTRACT

Long-chain acyl-CoA esters are important intermediates in lipid metabolism and are synthesized from fatty acids by long-chain acyl-CoA synthetases (ACSL). The hematophagous insect *Rhodnius prolixus*, a vector of Chagas' disease, produces glycerolipids in the midgut after a blood meal, which are stored as triacylglycerol in the fat body and eggs. We identified twenty acyl-CoA synthetase genes in *R. prolixus*, two encoding ACSL isoforms (*RhoprAcsl1* and *RhoprAcsl2*). *RhoprAcsl1* transcripts increased in posterior midgut on the second day after feeding, and *RhoprAcsl2* was highly transcribed on the tenth day. Both enzymes were expressed in *Escherichia coli*. Recombinant RhoprACSL1 and RhoprACSL2 had broad pH optima (7.5–9.5 and 6.5–9.5, respectively), were inhibited by triacsin C, and were rosiglitazone-insensitive. Both showed similar apparent K_m for palmitic and oleic acid (2–6 μM), but different K_m for arachidonic acid (0.5 and 6 μM for RhoprACSL1-Flag and RhoprACSL2-Flag, respectively). The knockdown of *RhoprAcsl1* did not result in noticeable phenotypes. However, RhoprACSL2 deficient insects exhibited a 2.5-fold increase in triacylglycerol content in the fat body, and 90% decrease in fatty acid β-oxidation. *RhoprAcsl2* knockdown also resulted in 20% increase in lifespan, delayed digestion, 30% reduced oviposition, and 50% reduction in egg hatching. Laid eggs and hatched nymphs showed remarkable alterations in morphology. In summary, *R. prolixus* ACSL isoforms have distinct roles on lipid metabolism. Although RhoprACSL1 functions remain unclear, we propose that RhoprACSL2 is the main contributor for the formation of the intracellular acyl-CoA pool channeled for β-oxidation in the fat body, and is also required for normal reproduction.

1. Introduction

Fatty acids are required for the synthesis of a variety of complex lipids, including stored triacylglycerol (TAG), membrane phospholipids and bioactive lipids. Alternatively, fatty acids can be oxidized in the mitochondria for energy production. The use of a fatty acid for metabolism requires its activation to an acyl-CoA intermediate by a thioesterification reaction catalyzed by one of the acyl-CoA synthetase family members (ACS; EC 6.2.1.*x*). Long-chain acyl-CoA synthetases (ACSL) activate long-chain fatty acids (12–20 carbons) to acyl-CoA, allowing acyl chains to enter metabolic pathways. The five mammalian isoforms have specific tissue distributions, subcellular locations and substrate preferences [1]. Additionally, the ACSL isoforms appear to partition their acyl-CoA products into different metabolic pathways [1,2].

The blood-feeding insect *Rhodnius prolixus* is a vector of Chagas' disease. This disease is caused by the protozoan *Trypanosoma cruzi* and currently affects about 7–8 million people in Central and South America [3]. Apart from its medical relevance, *R. prolixus* has also been used as a model to study lipid metabolism in arthropods. Mechanisms involved in lipid synthesis [4–7], transport [8–11], mobilization [12,13] and the endocrine regulation of lipolysis [14] have been described.

In *R. prolixus*, dietary TAG is hydrolyzed in the midgut lumen by TAG lipase activity. The midgut epithelium absorbs the released fatty acids, which are used via the glycerol-3-phosphate pathway for the synthesis of glycerolipids, such as phospholipids (PL), diacylglycerol (DAG) and TAG [5,7]. These newly synthesized lipids are distributed to other tissues by the shuttle system mediated by lipophorin, a major circulating lipoprotein present in the hemolymph [5,10,15]. Lipids carried by lipophorin are stored mainly as TAG in the fat body [12], an organ functionally analogous to both mammalian liver and adipose tissue [16]. During prolonged starvation, which may be as long as five months for

Abbreviations: ACS, acyl-CoA synthetase; ACSS, short-chain ACS; ACSM, medium-chain ACS; ACSBG, *bubblegum* ACS; ACSL, long-chain ACS; ACSVL, very long-chain ACS; DAG, diacylglycerol; FATP, fatty acid transport protein; PL, phospholipid; TAG, triacylglycerol.

* Corresponding author at: Av. Carlos Chagas Filho, 373, Ilha do Fundão, Rio de Janeiro, RJ, 21941-902, Brazil.
E-mail address: katia@bioqmed.ufrj.br (K.C. Gondim).

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R. prolixus [17], TAG is mobilized to sustain the energy needs of the insect [12,17], probably by the oxidation of released fatty acids. TAG, DAG and PL are also synthesized by the ovaries during oogenesis [5,8,13]. In this organ, TAG accumulation is required for egg growth and nymph development [13]. In addition, arachidonic acid may potentially be used for the synthesis of eicosanoids, which act as mediators for the nutrient endocytosis in growing eggs [18]. Because ACSL activity could control the formation of acyl-CoA pools used in most of these pathways, we asked whether different ACSL isoforms are required for specific physiological purposes.

ACSL isoforms have been characterized in arthropods. A microsomal ACS activity for palmitate (16:0) activation was identified in the *Macrobrachium borelli* shrimp [19]. In *Drosophila melanogaster*, an ACSL isoform (dACSL) is required for normal embryonic segmentation and normal TAG accumulation in larvae [20,21]. However, the presence of additional isoforms and their specific physiological roles remain unclear. To our knowledge, none of the ACSL enzymes have been previously studied in vectors of human diseases. Here we show that *R. prolixus* genome encodes at least twenty acyl-CoA synthetases (ACS). Among them, two isoforms are ACSL and exhibit tissue-specific transcriptional regulation. To determine whether specific characteristics could result in differential roles for RhoprACSL1 and 2 isoforms, we investigated biochemical properties from recombinant enzymes and analyzed the phenotypes generated by dsRNA-mediated knockdown of each gene. We found that RhoprACSL2 is required for fatty acid β-oxidation in the fat body, and for oviposition and egg viability. These data provide compelling evidence for the central role of RhoprACSL2 in the insect energetic and reproductive physiology.

2. Material and methods

2.1. Insects and ethical statement

Insects were taken from a colony of *R. prolixus* maintained at 28 °C and 70–75% relative humidity. Adult insects were fed with live rabbit blood at 3-week intervals, beginning 15 days after the moult from 5th instar to adult. Adult females were studied after the second or third meal (fed condition), or three weeks after the first or second blood meal (unfed condition). 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 the 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) [22] by similarity to the AMP-binding family consensus sequence (Pfam number PF00501) using FAT software [23]. Retrieved sequences were manually analyzed to confirm the presence of additional ACS conserved motifs [24,25]. Identified supercontigs were compared to ACS protein sequences from different organisms available in GenBank by tBlastn [6]. Coding region prediction, protein primary sequence and phylogenetic analyses were performed as described [14].

2.3. RNA isolation and cDNA synthesis

For gene expression analysis in different organs, anterior and posterior midguts, abdominal fat bodies and ovaries were obtained from females on the fourth day after a blood meal. To analyze the time-dependent response, organs were dissected before feeding (day 0) and on different days after a blood meal. To analyze gene expression in follicles at different stages of growth, ovaries were dissected from females on the fourth day after a blood meal, and follicles were removed in 0.15 M NaCl under a stereomicroscope and grouped according to their length into four categories: 0.5, 1.0, 1.5 and 2.0 mm. Total RNA was isolated from samples (pools from 3 to 5 organs), treated with RNase-free DNase I and used to synthesize cDNA as described [14].

2.4. Rapid amplification of cDNA ends (RACE)

The 3′ end of the *RhoprAcsl1* cDNA and the 5′ end of the *RhoprAcsl1* and *RhoprAcsl2* cDNAs were amplified using 3′-RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, USA) and the GeneRacer kit for full-length, RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (RLM-RACE, Invitrogen), respectively, according to the manufacturers' recommendations. Amplification of the full-length cDNA was performed using primers designed for specific amplification of target genes based in in silico predictions and the information obtained using RACE (Supplementary Table 1). PCR reactions were carried out using iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, USA). PCR products were separated by electrophoresis in agarose gel and isolated with the QIAquick Gel Extraction Kit (Qiagen, Valencia, USA). The full length CDS sequences obtained for *RhoprAcsl1* or *RhoprAcsl2* were registered in the NCBI GenBank database under the accession numbers KY328601 and KT328602, respectively.

2.5. Quantitative PCR (qPCR)

qPCR was performed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) using SYBR Green PCR Master Mix (Applied Biosystems) under the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C. qPCR amplification was performed using specific primers for the target genes (Supplementary Table 1), and were designed using the Primer3 software [26]. *Rhopr18S* gene amplification was used for normalization [27]. Primer efficiencies and qPCR inhibition were determined as described [28]. Amplification specificity analysis and qPCR controls to detect contaminations were carried out following the MIQE guidelines [29].

2.6. Construction of recombinant plasmids

cDNA was synthesized from posterior midgut RNA and used as template to amplify the *RhoprAcsl1* and *RhoprAcsl2* open reading frames by PCR using the iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, USA). Primers were designed for *RhoprAcsl1* or *RhoprAcsl2* specific amplification with the addition of specific restriction sites (Supplementary Table 1). The amplified PCR product was digested with restriction enzymes (XhoI, EcoRI or HindIII; New England Biolabs, Ipswich, USA) and ligated into pFlag-CTS vector (Sigma-Aldrich Co., St. Louis, USA). The sequences of RhoprACSL-Flag fusion constructs were verified by the University of North Carolina Automated Sequencing Facility.

2.7. Expression of recombinant proteins in Escherichia coli

Recombinant RhoprACSL1-Flag and RhoprACSL2-Flag were expressed in *E. coli* JM109 strain after induction with 0.5 mM IPTG (Thermo Fisher Scientific, Waltham, USA) at an A600 of 0.5. Induced bacteria was grown in Terrific Broth (1.2% triptone, 2.4% yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, pH 7) supplemented with ampicillin (50 μg/mL) at 15 °C and shaken at 150 rpm. After a 6-h induction, cells were harvested by centrifuging at 3200 *g* for 15 min at 4 °C. The cell pellet was resuspended in 8.5 mL of lysis buffer (10 mM HEPES, pH 7.8, 1 mM ATP, 0.5 mM EDTA, 10 μM PMSF, 5% glycerol, 1% protease inhibitor cocktail (Sigma), 10 U/mL benzonase and 0.2 mg/mL lysozyme).