Comparative Biochemistry and Physiology, Part A 203 (2017) 109–114

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Molecular drivers of mitochondrial membrane proliferation in response to cold acclimation in threespine stickleback

Kelly Keenan, Megan Hoffman, Kristin Dullen, Kristin M. O'Brien *,[1]

University of Alaska Fairbanks, Institute of Arctic Biology, Fairbanks, AK 99775, United States

ARTICLE INFO

Article history:
Received 29 June 2016
Received in revised form 1 September 2016
Accepted 1 September 2016
Available online 06 September 2016

Keywords:
Mitochondrial biogenesis
Cold acclimation
Threespine stickleback
Membrane biosynthesis

ABSTRACT

Little is known about how the synthesis of mitochondrial phospholipids is integrated into mitochondrial biogenesis in fish or mammals. Glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15) catalyzes the addition of fatty acyl CoA to the *sn*-1 position of glycerol-3-phosphate, in what is considered the rate-limiting step in phospholipid biosynthesis. Previous studies have shown that mitochondrial volume density increases in oxidative skeletal muscle but not liver of *Gasterosteus aculeatus* (threespine stickleback) in response to cold acclimation. We hypothesized that maximal activity of GPAT would increase in oxidative skeletal muscle but not liver during cold acclimation, coinciding with mitochondrial biogenesis. GPAT activity was measured in liver and oxidative skeletal (pectoral adductor) muscle of threespine stickleback acclimated to 8 °C or 20 °C. In addition, mRNA levels of enzymes involved in phospholipid synthesis, including cytidine diphosphodiacylglycerol synthase-1 (CDS1), CDS2, GPAT1, GPAT2 and 1-acylglycerol 3-phosphate acyltransferase-2 (AGPAT2), were quantified in liver and pectoral muscle of stickleback harvested during cold acclimation. GPAT activity and transcript levels of AGPAT2 increased in response to cold acclimation in pectoral muscle but not liver. Transcript levels of GPAT1 increased in liver but not pectoral muscle. Overall our results suggest that the activity of GPAT, and possibly AGPAT as well, increase during cold acclimation and may contribute to mitochondrial phospholipid biosynthesis required for mitochondrial biogenesis.

1. Introduction

Mitochondrial density increases in response to cold acclimation in oxidative skeletal muscle of many cold-active teleosts, including goldfish, striped bass and stickleback (Egginton and Sidell, 1989; Johnston and Maitland, 1980; Orczewska et al., 2010; Tyler and Sidell, 1984). Mitochondria are chimeric organelles, possessing proteins encoded in both the mitochondrial and nuclear genomes. Thus, mitochondrial biogenesis requires coordination of the two genomes to synthesize mitochondrial proteins and lipids, and to replicate the mitochondrial genome. In mammals, the co-transcriptional activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is considered the master regulator of mitochondrial biogenesis. In addition to regulating the transcription of nuclear-encoded genes destined for mitochondria, it also transactivates the expression of mitochondrial transcription factor A (TFAM), which is then translated on cytosolic ribosomes and imported into the mitochondrion where it induces mitochondrial DNA (mtDNA) replication and transcription, leading to an increase in mtDNA and proteins (Scarpulla, 2008). Less is known about mitochondrial biogenesis in fishes but several studies suggest that nuclear respiratory factor-1 (NRF-1) and PGC-1β, rather than PGC-1α, orchestrate the process (Bremer et al., 2015; Bremer et al., 2012; Orczewska et al., 2010). While mammalian studies have clearly delineated how mtDNA is replicated and mitochondrial proteins are synthesized during mitochondrial biogenesis, the molecular drivers mediating the synthesis of mitochondrial phospholipids remain largely unknown (O'Brien and Mueller, 2010).

Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first, committed step in glycerolipid synthesis (Wendel et al., 2009). GPAT catalyzes the addition of fatty acyl coenzyme A (CoA) to the *sn*-1 position of glycerol-3-phosphate (G3P), producing lysophosphatidic acid (LPA). Downstream of GPAT, 1-acylglycerol-3-phosphate acyltransferase (AGPAT) [or lysophosphatidic acid acyltransferase (LPAAT)] catalyzes the addition of fatty acyl CoA to the *sn*-2 position of LPA, producing phosphatidic acid (PA). PA is then hydrolyzed to form diacylglycerol (DAG). PA is the precursor for synthesizing

Abbreviations: AGPAT, 1-acylglycerol 3-phosphate acyltransferase; CDP-DAG, cytidine diphosphodiacylglycerol; CDS, cytidine diphosphodiacylglycerol synthase; CL, cardiolipin; DAG, diacylglycerol; EF-1α, elongation factor-1 alpha; G3P, glycerol 3-phosphate; GPAT, glycerol 3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; mTOR, mechanistic target of rapamycin; mtDNA, mitochondrial DNA; NRF-1, nuclear respiratory factor 1; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TAG, triacylglycerol; TFAM, mitochondrial transcription factor A.

* Corresponding author at: University of Alaska Fairbanks, Institute of Arctic Biology, P.O. Box 757000, Fairbanks, AK 99775, United States.
E-mail address: kmobrien@alaska.edu (K.M. O'Brien).

[1] All authors contributed equally to this work.

http://dx.doi.org/10.1016/j.cbpa.2016.09.001
1095-6433/

phosphatidylinositol (PI), phosphatidylglycerol (PG) and cardiolipin (CL); DAG is the precursor for synthesizing the most abundant membrane phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Takeuchi and Reue, 2009). The synthesis of CL, an essential phospholipid associated with the complexes of the mitochondrial respiratory chain, is synthesized from PA by cytidine diphosphodiacylglycerol (CDP-DAG) synthase (CDS) (Horvath and Daum, 2013; Ren et al., 2014). There are two isoforms of CDS and both are localized to the endoplasmic reticulum (ER) (D'Souza et al., 2014). Recent studies suggest that Tam41, also a CDS but localized to the mitochondrion, may play a more prominent role in the synthesis of CL than CDS1 or CDS2 (Tamura et al., 2013). There are four isoforms of GPAT, localized to the outer mitochondrial membrane (GPAT 1 and 2) and the ER membrane (GPAT 3 and 4) (Bell and Coleman, 1980). AGPAT1 and AGPAT2 are localized to the ER and have been well characterized, but as many as 11 isoforms have been reported in mammals (Takeuchi and Reue, 2009). Recent studies have revealed the first linkages between the synthesis of mitochondrial proteins and phospholipids. Knocking out PGC-1α and PGC-1β in mice results in altered mitochondrial morphology and a decrease in mRNA levels of CDS1 and levels of CL, PC and PE in hearts (Lai et al., 2014). Overexpression of PGC-1α in extensor digitorum longus (EDL) muscle of mice alters phospholipid profiles, increasing content of 18:0/22:6 PC and 18:0/22:6 PE, similar to changes observed in response to exercise, and these changes are prevented by knocking out PGC-1α (Senoo et al., 2015). Transcript levels of GPAT1, GPAT3, and LPAAT2 (AGPAT2) also increased in EDL of PGC-1α null mice, suggesting that one more of these enzymes may drive phospholipid remodeling, although principle component analysis did not support this (Senoo et al., 2015).

To shed light on how mitochondrial membrane synthesis is stimulated during cold-induced mitochondrial biogenesis in fish, we measured the maximal activity of GPAT in liver and oxidative pectoral adductor muscle of threespine stickleback held at 20 °C and cold-acclimated to 8 °C. Previous studies in our lab have shown that mitochondrial density increases in pectoral adductor muscle but not liver in response to cold acclimation (Orczewska et al., 2010). We anticipated that if GPAT drives increases in mitochondrial membrane biosynthesis, GPAT activity would increase in oxidative muscle but not liver. Additionally, we measured transcript abundance of key genes of the phospholipid biosynthetic pathway: CDS1, CDS2, GPAT1, GPAT2 and AGPAT2.

2. Materials and methods

2.1. Animal care

Threespine stickleback, *Gasterosteus aculeatus* were collected in 2013 from Kashwitna Lake, AK (61°50′ N, 150°00′ W; 13.1 °C) using minnow traps. Fish were transported to the University of Alaska Fairbanks, housed in environmental chambers, and maintained on a 10:14 h light:dark cycle in aerated, filtered 114 L aquaria filled with 0.35% Instant Ocean in distilled water. Animals were held at 20 °C for 20 weeks then half of the animals (~60) were cold acclimated by decreasing the temperature in the environmental chamber to 15 °C on day 1, 10 °C on day 2, and 8 °C on day 3. Warm-acclimated fish were maintained at 20 °C for the duration of the experiment. Fish were held at 8 °C or 20 °C for an additional 11 weeks for measuring GPAT activity. For measuring transcript levels, fish from 8 °C and 20 °C were harvested 1, 4, and 9 weeks after the start of cold acclimation. Animals were harvested prior to feeding at the same time each morning. Fish were euthanized with an overdose of tricaine methanesulfonate (250 mg l^{-1}; pH 7) followed by cervical cord transection for assaying GPAT activity, and flash frozen in liquid nitrogen for measuring mRNA levels. All protocols were approved by the UAF Institutional Animal Care and Use Committee (436131-10).

2.2. Maximal activity of GPAT (EC 2.3.1.15)

Liver and oxidative pectoral adductor muscle were excised on an ice-cold stage. Tissues from two individuals were pooled and homogenized in 8 volumes of buffer (10 mM Tris–HCl, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT; pH 7.4 at 4 °C) using a Tenbroeck homogenizer. Protein concentrations were determined using a Bradford assay with bovine serum albumin (BSA) as a standard (Bradford, 1976). Initial assays were carried out in a 500 μl reaction mixture containing 40 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 2 mg ml^{-1} BSA (essentially fatty acid-free), 2 mM KCN, 100 μM palmitoyl-CoA or palmitoleoyl-CoA, 0.1–10 mM G3P and 0.1 μCi [^{14}C] glycerol-3-phosphate (Perkin Elmer, Waltham MA, USA) (Vancura and Haldar, 1994). Reactions were initiated by the addition of 150–400 μg of protein. Maximal GPAT activity was linear for 20 min and proportional to the amount of protein added. These assays indicated that 5 mM G3P was sufficient to saturate the enzyme and activities were equivalent using either palmitoyl-CoA or palmitoleoyl-CoA as a substrate (data not shown). GPAT activity in warm- and cold-acclimated stickleback was assayed at 14 °C in a 500 μl reaction mixture containing 40 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 2 mg ml^{-1} BSA (essentially fatty acid-free), 2 mM KCN, 100 μM palmitoleoyl-CoA, 5 mM G3P, 0.1 μCi [^{14}C] glycerol-3-phosphate, and 400 μg protein. Reactions were stopped after 20 min by the addition of 0.5 ml H_2O-saturated 1-butanol. Samples were centrifuged at 12,100 *g* for 5 min at room temperature. A 200 μl aliquot of the butanol layer was added to 10 ml liquid scintillation fluid (Ecolite MP Biomedicals, LLC, Solon, OH, USA). ^{14}C was counted using an LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton CA, USA). Maximal GPAT activity was measured in triplicate in warm- and cold-acclimated animals ($N = 6$). Background activity was measured in reaction mixtures lacking palmitoleoyl-CoA and subtracted from activity in the presence of substrate. Specific activity of GPAT was calculated as nmol G3P acylated per min per mg protein (nmol min^{-1} mg^{-1}).

2.3. Gene expression

RNA was isolated from 10 to 30 mg of pectoral adductor muscle and liver tissue from 6 to 8 individuals harvested at each time point during cold and warm acclimation (described above) using the RNeasy Fibrous Tissue Mini-kit (Qiagen, Valencia, CA, USA) as described previously (Orczewska et al., 2010). RNA levels were quantified by measuring the absorbance at 260 nm with a NanoDrop® (ND-1000) spectrophotometer. RNA integrity was verified by mixing 2 μl RNA with loading buffer (5% glycerol, 0.04% bromophenol blue, 0.1 mM EDTA, pH 8.0) and separating it on a 2% agarose gel. Total RNA was reverse transcribed to complementary DNA (cDNA) using TaqMan® reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) as described previously (Orczewska et al., 2010). Briefly, cDNA was synthesized in a 10 μl reaction containing 5.5 mM $MgCl_2$, 2.5 μM random hexamers, 2 mM deoxynucleotides (dNTPs), 4 U of RNase inhibitor, 37.5 U reverse transcriptase and 200 ng RNA. Reactions were performed using an iCycler (Bio-Rad Laboratories) programmed at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min.

Transcript levels of CDS1, CDS2, GPAT1, GPAT2, and AGPAT2 were measured in pectoral adductor muscle and liver tissue using quantitative real-time PCR (qRT-PCR) as described previously (Orczewska et al., 2010). Briefly, gene-specific primers were designed using sequence information obtained from Ensembl (www.ensembl.org) and the software Primer Express (Applied Biosystems) with at least one primer from each pair spanning a splice site to ensure that genomic DNA was not amplified (Table 1). Primers were synthesized commercially (Invitrogen, Carlsbad, CA, USA). Transcript levels were measured using an ABI 7900HT sequence detection system (Applied Biosystems) in triplicate. 20 μl reaction mixtures contained 5 ng cDNA, 10 μl Power SYBR® Green Master Mix (Applied Biosystems) and 0.3 μM of each forward and reverse primer, with the exception that only 1 ng of cDNA was