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Characterization of mitochondrial glycerol-3-phosphate acyltransferase in notothenioid fishes

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ABSTRACT

Hearts of Antarctic icefishes (suborder Notothenioidei, family Channichthyidae) have higher densities of mitochondria, and mitochondria have higher densities of phospholipids, compared to red-blooded notothenioids. Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the rate-limiting step in glycerolipid biosynthesis. There are four isoforms of GPAT in vertebrates; GPAT1 and GPAT2 are localized to the outer mitochondrial membrane, whereas GPAT3 and GPAT4 are localized to the endoplasmic reticulum membrane. We hypothesized that transcript levels of GPAT1 and/or GPAT2 would mirror densities of mitochondrial phospholipids and be higher in the icefish *Chaenocephalus aceratus* compared to the red-blooded species *Notothenia coriiceps*. Transcript levels of GPAT1 were quantified in heart ventricles and liver using qRT-PCR. Additionally, GPAT1 cDNA was sequenced in the Antarctic notothenioids, *C. aceratus* and *N. coriiceps*, and in the sub-Antarctic notothenioid, *Eleginops maclovinus*, to identify amino acid substitutions that may maintain GPAT1 function at cold temperature. Transcript levels of GPAT1 were higher in liver compared to heart ventricles but were not significantly different between the two species. In contrast, transcripts of GPAT2 were only detected in ventricle where they were 6.6-fold higher in *C. aceratus* compared to *N. coriiceps*. These data suggest GPAT1 may be more important for synthesizing triacylglycerol, whereas GPAT2 may regulate synthesis of phospholipids. GPAT1 amino acid sequences are highly conserved among the three notothenioids with 97.9–98.7% identity. Four amino acid substitutions within the cytosolic region of Antarctic notothenioid GPAT1 may maintain conformational changes necessary for binding and catalysis at cold temperature.

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1. Introduction

Antarctic notothenioid fishes are excellent model organisms for studying adaptations to life at cold temperature. Notothenioids are the dominant suborder of fishes in the Southern Ocean, where seawater temperatures have been <5 °C for approximately 12 MY and now range between −1.9 °C and −1.7 °C (Eastman, 1993). While the majority of notothenioids are endemic to the Southern Ocean, 21% of the species reside north of the Antarctic Polar Front, offshore of New Zealand, South America, Australia, and northern peri-Antarctic Islands (Eastman, 1993). Antarctic notothenioids possess an array of adaptations to life at cold temperature including cold-stable microtubules and lens crystalline proteins (Detrich and Overton, 1988; Kiss et al., 2004), membranes enriched in polyunsaturated fatty acids (Logue et al., 2000), cold-adapted metabolic enzymes (Fields and Somero, 1998), and high mitochondrial densities in some tissues (Johnston et al., 1998).

Elevations in mitochondrial density offset the depressive effects of cold temperature on the catalytic rate of enzymes and increase the diffusion rate of metabolites by minimizing diffusion distances (Sidell, 1998). Aerobic muscles of the hemoglobinless Channichthyidae family of Antarctic notothenioids have remarkably high mitochondrial densities. In heart ventricles, mitochondria displace between 20% and 37% of cell volume and in oxidative pectoral adductor muscle, between 39% and 51% of fiber volume (Archer and Johnston, 1991; Feller et al., 1985; O'Brien and Sidell, 2000; O'Brien et al., 2003). High mitochondrial densities in icefish oxidative muscles do not enhance aerobic metabolic capacity but rather, likely compensate for the lack of hemoglobin (Hb) and myoglobin (Mb) and enhance oxygen storage and diffusion

Abbreviations: DAG, diacylglycerol; dN/dS, rate of nonsynonymous-to-synonymous amino acid substitutions; EF-1α, elongation factor-1 alpha; G3P, glycerol 3-phosphate; GPAT, glycerol 3-phosphate acyltransferase; HKG, housekeeping gene; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; NRF-1, nuclear respiratory factor -1; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PGC-1, peroxisome proliferator-activated receptor gamma coactivator -1; TAG, triacylglycerol; TBP, TATA-binding protein; 18S, 18S ribosomal RNA.

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(Sidell and O'Brien, 2006). The lipid-rich mitochondria in icefish hearts facilitate oxygen diffusion because oxygen is approximately four times more soluble in phospholipids than in water (Battino et al., 1968; Smotkin et al., 1991), and oxygen concentrates within the hydrophobic core of the phospholipid bilayer, increasing oxygen storage capacity (Smotkin et al., 1991). The two most abundant mitochondrial phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are 1.3- to 1.4- fold higher per g mitochondrial protein in hearts of the icefish *Chaenocephalus aceratus* compared to the red-blooded species *Notothenia coriiceps* (O'Brien and Mueller, 2010). The overall increase in mitochondrial phospholipids may be attributable to an upregulation in the glycerolipid synthesis pathway, yet it is unknown how the synthesis of phospholipids is integrated into mitochondrial biogenesis in either fish or mammals (O'Brien, 2011). The enzyme glycerol-3-phosphate acyltransferase (GPAT), which catalyzes the initial and rate-limiting step in phospholipid and triacylglycerol (TAG) biosynthesis (Wendel et al., 2009), may regulate mitochondrial phospholipid density and contribute to differences in mitochondrial densities between red- and white-blooded notothenioids. Consistent with this, a recent study from our lab has provided evidence that GPAT may regulate mitochondrial membrane biosynthesis in threespine stickleback in response to cold acclimation (Keenan et al., 2016).

GPAT catalyzes the acylation of glycerol-3-phosphate at the *sn*-1 position, synthesizing lysophosphatidic acid (LPA). LPA acyltransferase (LPAAT) then acylates the *sn*-2 position, forming phosphatidic acid (PA), which is then hydrolyzed to diacylglycerol (DAG). PA is the precursor for the synthesis of phosphatidylinositol (PI), phosphatidylglycerol (PG), and cardiolipin (CL), whereas DAG is the precursor for synthesizing TAG, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) (Coleman and Lee, 2004). In vertebrates, there are at least four GPAT isoforms (GPAT1–4) differing in their subcellular location, sensitivity to the sulfhydryl reagent N-ethylmaleimide (NEM), and fatty acyl-CoA preference (Coleman and Lee, 2004; Gimeno and Cao, 2008). GPAT1 and GPAT2 are associated with the outer mitochondrial membrane, and in humans have molecular masses of 94 kDa and 88 kDa, respectively (Coleman and Lee, 2004). GPAT3 and GPAT4 are localized to the endoplasmic reticulum (ER) membrane, and in humans have molecular masses of 50 kDa and 52 kDa, respectively (Cao et al., 2006; Nagle et al., 2008).

Activity and expression of each isoform vary with tissue type. In most tissues, the activity of microsomal GPAT isoforms accounts for 90% of the total GPAT activity, but in liver activities of the microsomal and mitochondrial isoforms are nearly equivalent (Coleman and Lee, 2004). Activity of the mitochondrial isoforms is higher in liver and adipose compared to heart, although protein levels are higher in heart (Lewin et al., 2001). This discrepancy is likely due to assaying activity of the enzyme in the presence of NEM, and thus only accounting for activity of GPAT1, which is NEM sensitive (Lewin et al., 2001; Lewin et al., 2004). The functional differences among the four GPAT isoforms remain unclear. Most studies to date have focused on the function of GPAT1, which changes in response to diet in a fashion consistent with its role in mediating TAG biosynthesis, but GPAT1 null mice also display alterations in phospholipid composition, suggesting a role in phospholipid synthesis as well (Hammond et al., 2002; Lewin et al., 2008).

We hypothesized that if GPAT1 and GPAT2 are involved in synthesizing mitochondrial membranes, then transcript levels of these genes would be higher in heart ventricles of the icefish *C. aceratus,* with mitochondrial densities of 36.53 ± 2.08% compared to the red-blooded species *N. coriiceps*, with mitochondrial densities of 18.18 ± 0.69% (O'Brien and Sidell, 2000; Urschel and O'Brien, 2008). We also predicted that mRNA levels of both genes would be higher in liver than heart, consistent with expression levels in mammals. Transcript levels of GPAT1 and GPAT2 were quantified in liver and ventricle tissue of *N. coriiceps* and *C. aceratus* using real-time quantitative PCR. GPAT1 cDNA was sequenced in three notothenioid species: *C. aceratus, N. coriiceps*, and the sub-Antarctic species, *Eleginops maclovinus*, which inhabits warmer coastal waters around the Falkland Islands and South America and diverged prior to the isolation of Antarctica and formation of the Antarctic circumpolar current (Eastman, 1993), to provide insight to the evolution of a mitochondrial transmembrane protein in fishes inhabiting a chronically cold environment.

2. Material and methods

2.1. Tissue collection

Notothenia coriiceps (Richardson, 1844) and *Chaenocephalus aceratus* (Lönnberg, 1905) were captured in Dallmann Bay (64°10′S, 62°35′W) and off the southwestern shore of Low Island (63°24′S, 62°10′W) using an otter trawl and baited pots deployed from the ARSV *Laurence M. Gould*. Animals were maintained in circulating seawater tanks on board the ship and then transferred to circulating seawater tanks at the U.S. Antarctic Research Station, Palmer Station where they were maintained at 0 ± 0.5 °C. Fish were euthanized by either a sharp blow to the head or an overdose of tricaine methanesulfonate (MS-222) (1:7500 in seawater) followed by spinal cord transection. Heart ventricles and livers were excised immediately, frozen in liquid nitrogen, and stored at −80 °C. Heart ventricles of the sub-Antarctic notothenioid species, *E. maclovinus* (Cuvier), captured in 2004, were provided by Drs. B. Sidell and C. Cheng. Animals used for measuring mRNA abundance were collected in austral fall and winter of 2013 and 2015. Animals used for sequencing GPAT1 cDNA were collected in austral fall and winter of 2007 and 2009. All procedures were approved by the University of Alaska Fairbanks Institutional Animal Care Committee (134774-8).

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol except that RNA was treated with DNase I twice, once for 25 min and second time for 20 min. Concentration and purity of RNA were determined spectrophotometrically with a Nanodrop ND-1000 spectrophotometer (ThermoScientific Fisher, Pittsburgh, PA, USA). All samples had 260 nm-to-230 nm ratios of 1.8–2.3 and 260 nm-to-280 nm ratios of 2.0–2.2. Integrity of RNA was determined by separating RNA on a 2% agarose gel stained with ethidium bromide. RNA was stored at −80 °C until further use. First-strand complimentary DNA (cDNA) was synthesized using Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Each 50 μl reaction volume contained 1 mg RNA, 1× RT buffer, 3 mM $MgCl_2$, 2 mM dNTPs, 2.5 μM random hexamers, 20 U RNase inhibitor, and 37.5 U reverse transcriptase. cDNA was stored at −20 °C.

2.3. Sequencing GPAT1 and GPAT2 cDNA

Degenerate primers were designed using CODEHOP (http://blocks.fhcrc.org/codehop.html) and based on amino acid sequences from four fish species for GPAT1 (*Gasterosteus aculeatus*, Ensembl accession no. **ENSGACP00000008124**; *Dicentrarchus labrax*, GenBank accession no. **CBN81479.1**; *Oreochromis niloticus*, GenBank accession no. **XP 005471367.1**; and *Takifugu rubripes*, GenBank accession no. **XP 011620187.1**) and three species for GPAT2 (*G. aculeatus*, Ensembl accession no. **ENSGACT00000007807.1**; *T. rubripes*, GenBank accession no. **XP 011614214.1**; *O. niloticus*, GenBank accession no. **XP 003451146.2**) (Table 1). These primers were used to amplify 502 bp of GPAT1 and 1250 bp of GPAT2 with PCR in a reaction mixture containing 3 μl of cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.4 mM dNTP's, 1 μM forward and reverse primers, and 2.5 U Taq polymerase (Promega, Madison, WI, USA) and using a touchdown protocol with an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). A forward gene-specific primer (GSP) for GPAT1 was then designed based on the GPAT1 sequence in *N. coriiceps* and paired with a degenerate

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