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Intertissue regulation of carnitine palmitoyltransferase I (CPTI): Mitochondrial membrane properties and gene expression in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Carnitine palmitoyltransferase (CPT) I is regulated by several genetic and non-genetic factors including allosteric inhibition, mitochondrial membrane composition and/or fluidity and transcriptional regulation of enzyme content. To determine the intrinsic differences in these regulating factors that may result in differences between tissues in fatty acid oxidation ability, mitochondria were isolated from red, white and heart muscles and liver tissue from rainbow trout. Maximal activity (V_{max}) for β-oxidation enzymes and citrate synthase per mg tissue protein as well as CPT I in isolated mitochondria followed a pattern across tissues of red muscle>heart>white muscle>liver suggesting both quantitative and qualitative differences in mitochondria. CPT I inhibition showed a similar pattern with the highest malonyl-CoA concentration to inhibit activity by 50% (IC_{50}) found in red muscle while liver had the lowest. Tissue malonyl-CoA content was highest in white muscle with no differences between the other tissues. Interestingly, the gene expression profiles did not follow the same pattern as the tissue enzyme activity. CPT I mRNA expression was greatest in heart>red muscle>white muscle>liver. In contrast, PPARα mRNA was greatest in the liver>red muscle>heart>white muscle. There were no significant differences in the mRNA expression of PPARβ between tissues. As well, no significant differences were found in the mitochondrial membrane composition between tissues, however, there was a tendency for red muscle to exhibit higher proportions of PUFAs as well as a decreased PC:PE ratio, both of which would indicate increased membrane fluidity. In fact, there were significant correlations between IC_{50} of CPT I for malonyl-CoA and indicators of membrane fluidity across tissues. This supports the notion that sensitivity of CPT I to its allosteric regulator could be modulated by changes in mitochondrial membrane composition and/or fluidity.

1. Introduction

The regulation of lipid metabolism is complex, and is currently an extensive area of research. Although there is keen interest into pathology aspects of lipid metabolism, there are still many unanswered questions regarding the normal regulation of cellular lipid oxidation. In general terms, it has been recognized that mitochondrial fatty acid oxidation is regulated by both genetic and various other non-genetic cellular mechanisms that can affect fat entry into, and oxidation by mitochondria (for review see [1]). For instance, a key enzyme in mitochondrial β-oxidation, carnitine palmitoyltransferase (CPT) I, is transcriptionally regulated in mammals, but this enzyme also experiences allosteric modulation [2], is sensitive to changes in cellular pH [3], and possibly undergoes covalent modulation [4]. Currently it is unclear how these regulatory mechanisms affect CPT I differently and if regulation is similar across tissues with varying rates of fatty acid oxidation or even if these mechanisms are evolutionarily conserved.

CPT I is located on the inner side of the outer mitochondrial membrane and catalyses the conversion of acyl-CoA to fatty acyl-carnitine [5]. Fatty acyl-carnitine can then be transferred into the mitochondrial matrix by carnitine–acyl-carnitine translocase (CAT) and converted back to fatty acyl-CoA by the enzyme CPT II. Only acyl-carnitines can be transferred across the inner mitochondrial membrane, thus, CPT I is thought to be a major regulating step in mitochondrial fat oxidation [5]. CPT I is allosterically modulated by malonyl-CoA (M-CoA), which is produced during the first step of de novo fatty acid synthesis by acetyl-CoA carboxylase [6]. This is a simple mechanism that prevents the oxidation of newly synthesized fats in the liver [6]. In the muscle, however, where fatty acid synthesis rates are low, M-CoA is thought to be present solely as a regulator of CPT I [7].

Due to regulatory role of CPT I, changes in its abundance, activity, or substrate kinetics, as well as the concentration of M-CoA can lead to changes in overall rates of fatty acid oxidation. In humans and other mammals, there are two isoforms of the CPT I enzyme, encoded for by two different genes; L-CPT I (or CPT Iα) which is dominant in the liver, and M-CPT I (or CPT I-β) which is dominant in skeletal muscle (a brain isoform has also been identified) [8]. Both α and β isoforms are expressed in the heart but at different stages of development in mammals [9]. McGarry and Brown have shown that in rats, these two isoforms vary greatly in their kinetics. They have also shown that the two isoforms differ significantly in their sensitivity to M-CoA with M-

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CPT I being approximately 100 times more sensitive than the liver isoform [7].

CPT I has not been studied as extensively in non-mammalian vertebrates. For example, there is limited information on CPT I activity or gene expression in different tissues from fish. In rainbow trout CPT I has been found to be expressed in various tissues including liver and muscle, however, it has been suggested that this species expresses only one CPT I gene which corresponds to the mammalian liver-type CPT I [10]. To our knowledge, there has neither been a systematic determination of the sensitivity of CPT I for M-CoA across tissues in rainbow trout (*Oncorhynchus mykiss*), nor has the relative expression levels of CPT I been determined between various tissues. The mRNA expression of CPT I is thought to be influenced by the transcription factor peroxisome proliferator-activated receptor (PPAR), as it contains a PPAR response element (PPRE) [11] at least in mammals. Hence, it will be important to determine the relative expression of PPARs across tissues.

Aside from allosteric modulation and changes in gene expression, mitochondrial membrane composition and fluidity have been proposed to play a role in CPT I regulation [12]. Since CPT I is located in the outer mitochondrial membrane changes in the properties of the surrounding membrane can affect enzyme activity and kinetics. Maintaining a particular state of fluidity in membranes is necessary for several important cellular functions including ion transport and protein function, and is the basis for homeoviscous adaptation [13]. Under changing environmental conditions, such as a decrease in temperature, ectothermic animals selectively incorporate long chain polyunsaturated fatty acids into their membranes to combat the effects of temperature on changes in membrane order. As well, other modifications such as the ratio between phospholipid classes phosphatidylcholine (PC) and phophatidylethanolamine (PE) can also affect fluidity of the membrane due to their differential effects on membrane order [14].

In vitro studies using rat liver mitochondria have demonstrated that changing the fluidity of the mitochondrial membrane does affect the activity of CPT I as well as its sensitivity to M-CoA [12]. This has also been confirmed through in vivo studies using 48-h fasted rats and streptozotocin-induced diabetic rats, both of which show decreases in M-CoA sensitivity paralleled by increases in membrane fluidity as measured by DPH fluorescence anisotropy [15].

Little is known about these mechanisms in non-mammalian vertebrates. Fish, in particular naturally experience temperature and diet induced changes in membrane composition and fluidity [13]. As well, fish offer many advantages for the study of muscle fibre-specific and tissue-specific regulation of lipid metabolism since they have anatomically separated 'red' and 'white' muscle masses. Previously, clear fibre type differences have been shown in muscle mitochondrial profiles of rainbow trout, including membrane fluidity [16] but the effect on CPTI kinetics have not been determined.

The purpose of this study was to evaluate cross-tissue variation in the capacity for fatty acid oxidation and to identify potential genetic and non-genetic factors by which it is regulated in rainbow trout (*Oncorhynchus mykiss*). More specifically, we sought to determine; 1) the non-genetic regulation of CPT I across tissues by examining differences in V_{max}, M-CoA sensitivity, M-CoA concentrations, and mitochondrial membrane composition, and 2) differences in constitutive transcription for CPT I and transcription factors important for the expression of genes for fat oxidizing enzymes.

2. Materials and methods

2.1. Experimental fish

Rainbow trout, *Oncorhynchus mykiss*, (~500 g) were obtained from a local hatchery (Humber Springs, Orangeville, ON) and kept in 500 l tanks with circulating water at 12 °C on a commercial fish diet, Profishent Classic Floating Trout Grower (Martin Mills, Elmira, ON).

2.2. Mitochondrial isolation

Fish were killed by a blow to the head followed by severing of the spinal cord. Mitochondria were isolated from red muscle (RM), white muscle (WM), heart (H) and liver (L) from rainbow trout according to Suarez and Hochachka [17] and Moyes et al. [18]. Each tissue was immediately excised (WM ~ 15 g, RM ~ 4 g, whole H and whole L) and placed in mitochondrial isolation buffer (MIB) consisting of (in mM) 140 KCl, 10 EDTA, 5 $MgCl_2$, 20 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 0.5% BSA (pH 7.0) for RM, WM and H, and 250 sucrose, 1 EDTA, 20 HEPES and 0.5% BSA (pH 7.4) for L, on ice. Tissues were diced, washed twice with fresh chilled MIB, and then homogenized 3 times, first using a wide clearance Teflon pestle on a chilled glass homogenizer, then 3 times with a narrow clearance Teflon homogenizer to lyse cells. Homogenates were centrifuged at 800 ×g for 10 min at 4 °C. For RM, H and L the supernatant was spun at 9000 ×g for 10 min at 4 °C. WM supernatant was first strained through 2 layers of cheesecloth and then spun at 800 ×g for 10 min at 4 °C, strained again through 4 layers of cheesecloth and spun again at 9000 ×g for 10 min at 4 °C. Supernatants for all tissues were then discarded and pellets were resuspended in a small volume of the appropriate MIB lacking BSA. The resuspended homogenate was colleted into a 15 ml centrifuge tube and spun again at 9000 ×g for 10 min at 4 °C. The supernatant was discarded and the mitochondrial pellet was resuspended in an appropriate volume of MIB lacking BSA and kept on ice.

2.3. Enzyme and protein assays

All assays (except CPT I radioisotope assay) were performed in triplicate at room temperature using a Spectramax Plus 384 and clear 96-well flat bottom assay plates and data was analysed using Softmax Pro 4.7.1 software (Molecular Devices, Sunnyvale, CA).

2.3.1. CPTI assay (isotope)

Radioactive CPT I assays followed McGarry et al. [19] for mammals and modified to use the assay conditions of Rodnick and Sidell [20] to obtain CPT I V_{max} and IC_{50}. The assay buffer (pH 7.0) contained (in mM) 20 HEPES, 40 KCl, 1 EGTA, 220 sucrose, 0.1 DTT, 0.04 palmitoyl-CoA, 1 carnitine and 1.3 mg/ml BSA. 1 μCi/sample of L-[*methyl*-^{3}H] carnitine hydrochloride (specific activity 82.0 Ci/mmol) (Amersham Biosciences, Quebec) was added and 70 μl of the assay mixture was placed in 1.5 ml Eppendorf tubes and incubated with 10 μl of 0.5–500 mM malonyl-CoA or water in place of mitochondria for blanks or in place of malonyl-CoA and maximum activity. Tubes sat for 5 min at room temperature. The reaction was started by the addition of 20 μl of mitochondria diluted 5× in MIB (~2 mg/ml), and incubated at room temperature for 8 min. The reaction was stopped by the addition of 60 μl of 1 M HCl. The palmitoyl-[^{3}H]-carnitine was collected according to Starritt et al. [21]. 20 μl of the assay mixture with L-[*methyl*-^{3}H]carnitine hydrochloride was also counted in duplicate for determination of individual specific activity. Background counts were determined from a blank sample containing aqueous counting scintillation. The decays per minute (DPM) were read for 5 min per sample on a Tricarb 2900 TR Liquid Scintillation Analyzer (PerkinElmer) using QuantaSmart 1.31 (Packard Instrument Company) analysis software.

2.3.2. CPTI assay (spec)

MIB was used as the assay buffer and contained 0.1 mM 5,5′ Dithiobis (2-nitrobenzoic acid) (DTNB), 0.1 mM palmitoyl-CoA and 5.0 mM L-carnitine (omitted from control) ± 50 μM malonyl-CoA (pH 7.0 for RM, WM, and H; pH 7.4 for L) to determine V_{max}. The reaction was started with 10 μl of mitochondria (~10 mg/ml). Solutions were mixed and DTNB absorbance read at 412 nm for 5 min. The IC_{50} was also determined using this method; however, the sensitivity was lower than the isotope method and thus yielded much higher IC_{50} values for all tissues (data not shown).

Frozen tissue samples were powdered using a mortar and pestle chilled with liquid N_2 to determine whole tissue maximum enzyme activity under saturating conditions. Powdered tissue (50–100 mg) was homogenized in 20 volumes of an enzyme extraction buffer (20 mM HEPES, 1 mM EDTA and 0.1% Triton at pH 7.4) using a glass on glass homogenizer and enzyme assays were performed on this crude homogenate.

2.3.3. Long-chain acyl-CoA dehydrogenase (LCAD)

LCAD was assayed according to Davidson and Schulz [22]. The assay contained (in mM) 100 potassium phosphate buffer (pH 7.6) with 0.028 2,6-dichlorophenolindophenol (DCPIP), 0.65 phenazine methosulfate (PMS), 0.2 *N*-ethylmaleimide and 0.45 KCN. The reaction was started with the addition of 0.1 mM palmitoyl-CoA and DCPIP absorbance was monitored at 600 nm. Control wells lacking palmitoyl-CoA were assayed to correct for background hydrolase activity.

2.3.4. β-hydroxy-acyl-CoA dehydrogenase (HOAD)

The assay followed McClelland et al. [23] and contained (in mM) 50 imidazole (pH 7.4), 0.1 acetoacetyl-CoA, 0.15 NADH and 0.1% Triton 100-X and NADH absorbance was monitored at 340 nm. Controls lacking substrate were used to correct for background activity.

2.3.5. CPT II

The assay followed McClelland et al. [23] and contained (in mM) 20 Tris buffer (pH 8.0), 0.1 DTNB and 5 L-carnitine. The reaction was started with the addition of 0.1 mM palmitoyl-CoA and DTNB absorbance was monitored at 412 nm. Control wells lacking carnitine were assayed to correct for thiolase activity.

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Table 1
Forward (F) and reverse (R) primers used for real-time PCR analysis of mRNA expression in trout

Gene	Primer
EF1α	F — 5′ CAT TGA CAA GAG AAC CAT TGA 3′
	R — 5′ CCT TCA GCT TGT CCA GCA C 3′
CPT I	F — 5′ GCC GCA AAC TAG AGA GAG GA 3′
	R — 5′ CCC GTA GTA CAG CCA CAC CT 3′
PPARα	F — 5′ CCA AGT TCA GTT TGC CAT GA 3′
	R — 5′ ATT GGG GAA GAG GAA GGT GT 3′
PPARβ	F — 5′ CTG GAG CTG GAT GAC AGT GA 3′
	R — 5′ GTC AGC CAT CTT GTT GAG CA 3′

2.3.6. Citrate synthase (CS)

CS assays followed McClelland et al. [23]. Whole tissue homogenates were frozen and thawed 3× using liquid N_2 and kept on ice until further use. As well, an aliquot of the 5× diluted isolated mitochondria used for the CPT I assay was diluted a further 5× using the enzyme extraction buffer (see above) and frozen and thawed 3 times using liquid N_2 and kept on ice. Isolated intact mitochondria in MIB were also assayed for CS. These three homogenates were used to determine the amount of intact mitochondria versus ruptured mitochondria in our CPTI preparations and to extrapolate CPT I enzyme activities to the tissue level. The CS assay buffer consisted of (in mM) 20 TRIS (pH 8.0), 0.1 DTNB and 0.3 acetyl-CoA. The reaction was started by the addition of 0.5 oxaloacetate and absorbance was monitored at 412 nm. Control wells lacking acetyl-CoA were assayed to correct for hydrolase activity.

2.3.7. Protein content

Protein concentrations were determined by the Bradford method [24] using a commercial kit (Bio-Rad).

2.3.8. Malonyl-CoA (M-CoA) content

M-CoA concentrations were determined using a modified method from Richards et al. [25]. Briefly frozen tissue samples were powdered using a liquid N_2 cooled mortar and pestle, lyophilized for 24 h and kept at −80 °C until analysis. 50 mg of lyophilized tissue was homogenized at 4 °C for 20 s using a Teflon pestle in 200 µl of 0.5 M perchloric acid with 50 µM DTE and 10 mg/ml propionyl-CoA used as an internal standard. Homogenized samples were centrifuged at 20,000 ×g for 10 min at 4 °C and 200 µl of the supernatant was transferred and adjusted to pH 3 using 4 M NaOH while being vortexed. The sample was transferred to an autosampler vial containing 20 µl of MOPS (pH 6.8) and the final pH was determined (always less than 5). Autosampler vials were placed in a Waters 717 Plus autosampler (Waters, Missisauga, ON) at room temperature and M-CoA was separated using reverse-phase HPLC based on a method from Demoz et al. [26]. For RM, WM and L, 200 µl of the sample was injected onto a Zorbax ODS Rx C-18 column (25 cm × 0.46 mm) (Agilent Technologies, Mississauga, ON). The elution gradient was created using a Waters Model 510 pump controller. Mobile phase A was 100 mM sodium phosphate and 75 mM sodium acetate in deionized water (pH 4.6). Mobile phase B was the same as A except that it contained 30% CH_3CN. The elution gradient was as follows: 0 min, 90% A; 17 min, 50% A; and 17.6 min, 90% A. Baseline conditions were established after 5 min of 90% A. The flow rate was 1.5 ml/min and absorbance measurements were made at 254 nm on a Lambda Max 481 LC spectrophotometer (Waters, Mississauga, ON). For heart, the procedure was the same except that an extra column was added to the loop and the flow adjusted to 1 ml/min to separate peaks co-eluting with the M-CoA peak. Peaks were manually identified by comparisons to known M-CoA standards and quantified using the internal standard (propionyl-CoA).

2.4. mRNA quantification by real-time PCR

Total RNA from each tissue was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. Total RNA concentrations were quantified by UV spectrophotometry at 260 nm and diluted to 0.5 µg/µl. cDNA was synthesized using 1 µg of DNase (Invitrogen, Carlsbad, CA) treated RNA and SuperScript RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA). SYBR green (Bio-Rad, Mississauga, ON) with ROX as a reference dye was used for quantitative real-time PCR in 25 µl reactions using a Stratagene Mx3000P real-time PCR system. Each reaction contained 12.5 µl SYBR green mix, 1 µl each of forward and reverse primer (5 µM), 5.5 µl of DNase/RNase-free water and 5 µl of 5× diluted cDNA. Primers were designed using a CPT I sequence from rainbow trout liver [10] (see Table 1 for specific primer sequences). The thermal program included 3 min at 95, 40 cycles of 95 for 15 s, 60 for 30 s and 72 for 30 s. A no-template control and dissociation curve was performed to ensure only one PCR product and stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1-α, which did not change between tissues. Primers were designed using Primer3 software [27].

2.5. Analysis of mitochondrial membrane phospholipid composition

Mitochondrial total lipid was extracted and phospholipids were analysed according to Gillis and Ballantyne [28], based on a modified protocol from Bligh and Dyer [29]. Mitochondrial lipid extracts were dried and resuspended in 25 µl chloroform:methanol (2:1) and spotted along with a standard phospholipid mix (sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cardiolipin (CL)) (Sigma, Oakville, ON) onto silica gel 60 pre-coated 250 µM thick plates (Fisher Scientific, Ottawa, ON) for thin layer chromatography. The solvent system used to separate the phospholipids was chloroform: methanol:acetic acid:water (50:37.5:3.5:2, by volume). Once the solvent had run to within 5 cm of the top of the plate, they were removed, air dried, then sprayed with a saturated solution of 2,7-dichlorofluorescein and allowed to sit in a tank containing 25% ammonium hydroxide for 5 min. Plates were viewed under UV light and individual phospholipid fractions (SPH, PC, PS, PI, PE, CL) were scraped into individual Kimex tubes for saponification and methylation. Two ml of 6% sulphuric acid in methanol and 10 µl heptadecanoic acid as an internal standard (0.6 mg/ml, C17:0) were added to each fraction and incubated for 2 h at 80 °C. The samples were allowed to cool for 10 min and 1 ml of water and 2 ml of petroleum ether were added and the tubes vortexed. Samples were spun at 2000 rpm for 6 min and the top phase containing the fatty acid methyl ester (FAME) fraction was removed into a new tube and dried under N_2. The FAMEs were resuspended in petroleum ether and transferred into autosampler vials for gas chromatograph analysis. 1 µl of each sample was injected using a 7683B series automatic injection system (Agilent Technologies) onto a Hewlett–Packard 6890N series gas chromatograph (GLC) (Agilent Technologies) equipped with either an Innowax or a DB-23 (J&W Scientific) 30-m fused silica capillary column (Supelco, Bellefonte, PA) at 250 °C and followed the following temperature profile: initial oven temperature was kept at 160 °C for 4 min, ramped up to 220 °C at 2 °C/min, held at 220 °C for 16 min, ramped up to 240 °C at 10 °C/min and held for 2 min. Post-run was 130 °C for 6 min. The flow was 1.8 ml/min and the velocity through the column was 37 cm/s. GLC retention times were verified using two standards, PUFA No. 3 from menhaden oil and fatty acid methyl esters mix C4–C24 (Supelco, Bellefonte, PA). Identified fatty acids in each phospholipid class were compared to the known internal standard concentration and then summed for each sample. The percent contribution of each fatty acid was determined by adding the concentration of that fatty acid from each phospholipid class and then dividing by the total concentration of all fatty acids in all phospholipid classes for that sample. The percent contribution of each phospholipid

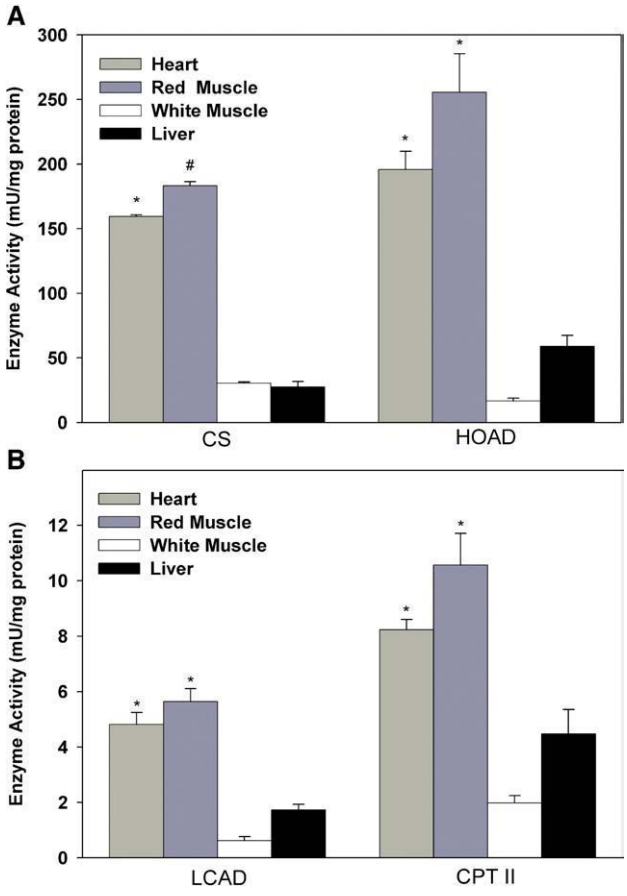

Fig. 1. The apparent V_{max} (in mU per mg total protein) for (A) citrate synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (HOAD), and (B) long chain acyl-CoA dehydrogenase (LCAD) and carnitine palmitoyltransferase (CPT) II in white muscle, heart, red muscle and liver. Values are means ± SE for 5 animals. Different symbols denote significance between tissues for each enzyme, $p<0.05$, similar symbols indicate no significant difference between tissues for each enzyme, $p>0.05$.

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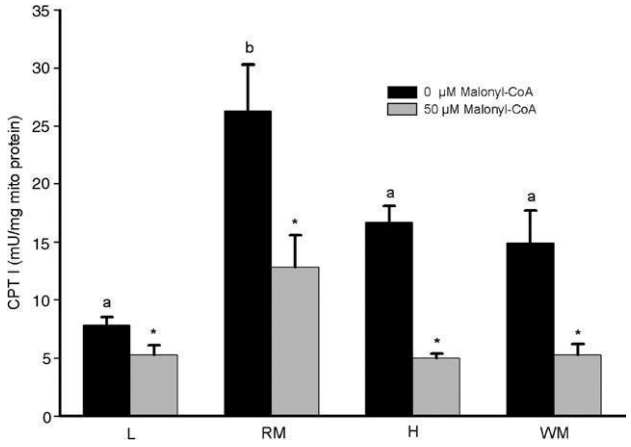

Fig. 2. CPT activity (mU per mg mitochondrial protein) in liver (L), red muscle (RM), heart (H) and white muscle (WM). Coloured bars, 0 μM M-CoA; black bars, 50 μM M-CoA. Values are means±SE for 4 animals. Different letters denote significance, $p<0.05$.

class was determined by adding the total fatty acid concentration in each individual phospholipid class and dividing by the total concentration of all fatty acids in all phospholipid classes for that sample.

2.6. Statistical analysis

All statistical analyses were performed using SigmaStat (Systat Software Inc., San Jose, CA). One-way ANOVA and Tukey's tests were used to test for significance between tissues. Correlational analyses were performed using a linear regression. Significance level was set at $p<0.05$.

3. Results

3.1. Cross-tissue variation in enzymatic activities

The apparent V_{max} of citrate synthase (CS), long chain acyl-CoA dehydrogenase (LCAD), carnitine palmitoyltransferase (CPT) II and β-hydroxy acyl-CoA dehydrogenase (HOAD) from whole tissue homogenates were assayed as indicators of mitochondrial content and fatty acid oxidation potential. All four enzymes demonstrate the same pattern of activity across tissues; red muscle having the highest activity, followed by heart, then liver, and then white muscle (Fig. 1A–B). Red muscle CS was significantly higher than all other tissues ($p<0.001$), as well, heart CS was significantly higher than liver and white muscle ($p<0.001$). Red muscle and heart LCAD and HOAD were both significantly higher than liver and white muscle ($p<0.001$) but not significantly different from each other. Red muscle ($p<0.001$) and heart CPT II were significantly higher than liver ($p=0.013$) and white muscle ($p<0.001$). There were no significant differences between liver and white muscle for all of the enzymes measured.

Table 2
The concentration of malonyl-CoA (μM) to reduce the activity of malonyl-CoA-sensitive carnitine palmitoyltransferase (CPT) I activity by 50% (IC_{50}) as determined by isotope assay

	Tissue			
	Liver	Red muscle	Heart	White muscle
IC_{50}	0.079±0.037†	0.55±0.06*	0.40±0.10*	0.37±0.09*†

Values are means±SE. Liver and white muscle, $n=5$; heart, $n=4$; red muscle, $n=3$. Results that share a symbol are not significantly different.

Total CPT activity was assayed in isolated mitochondrial preparations by spectrophotometry. Red muscle CPT had the highest activity at 26.3±4.0 nmol min^{-1} mg mito $protein^{-1}$, which was significantly higher than liver (7.8±0.7 nmol·min^{-1} mg mito $protein^{-1}$) ($p<0.001$) and white muscle (14.9±2.7 nmol min^{-1} mg mito $protein^{-1}$, $p=0.028$), but not significantly different from heart CPT activity (16.7±1.4 nmol min^{-1} mg mito $protein^{-1}$, Fig. 2). In the presence of high levels of an inhibitor (50 μM M-CoA), CPT activity declined in all tissues. In liver, CPT was inhibited by 32% whereas in red, white, and heart muscles CPT was inhibited by 51%, 70% and 65%, respectively (Fig. 2). This M-CoA inhibitable activity can be considered as the maximal activity of CPTI.

3.2. Malonyl-CoA inhibition of CPT I

The concentration of malonyl-CoA required to inhibit 50% of the malonyl-CoA sensitive activity (IC_{50}) of CPT I was determined for each tissue using a range of M-CoA concentrations from 0.05 μM–50 μM. Inhibition curves are shown in Fig. 3 (A–D) and the average IC_{50} for each tissue in Table 2. Liver had an IC_{50} of 0.079±0.037 μM which is significantly

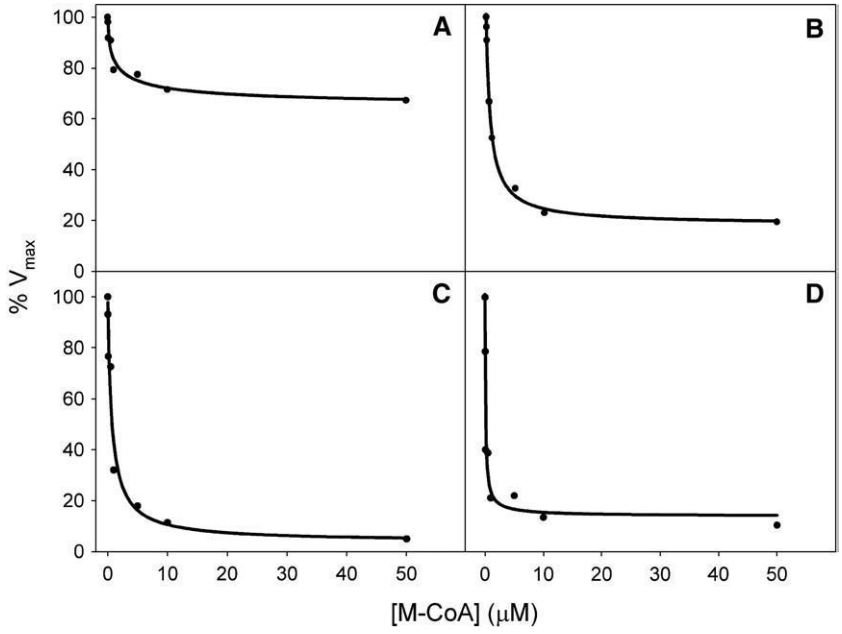

Fig. 3. Inhibition curves determined using a radioisotope assay for carnitine palmitoyltransferase (CPT)I activity in isolated mitochondrial preparation with increasing malonyl-CoA (M-CoA) concentration in (A) red muscle, (B) heart, (C) white muscle and (D) liver. M-CoA concentrations ranged from 0.05 μM to 50 μM. Liver and white muscle, $n=5$; heart, $n=4$; red muscle, $n=3$.

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Table 3
Malonyl-CoA (M-CoA) content across tissues

	Tissue			
	Liver	Red muscle	Heart	White muscle
[M-CoA] (nmol g^{-1} wet tissue)	0.014±0.004	0.030±0.004	0.013±0.002	0.196±0.027*
[M-CoA] (nmol g^{-1} wet tissue)/U citrate synthase activity	0.0005±1.8×10^{-4}†	0.0001±2.5×10^{-5}	0.00008±1.5×10^{-5}	0.006±8.8×10^{-4}*

Values are means±S.E. for 4 animals.
Results that share a symbol are not significantly different.

lower than red muscle (0.55±0.06 μM) and heart (0.40±0.10 μM, $p=0.008$ and $p=0.05$, respectively), but not white muscle (0.37±0.09 μM, $p>0.05$).

3.3. Malonyl-CoA content

Tissue M-CoA content varied between tissues with WM having significantly higher amounts than all other tissues ($p<0.001$) (Table 3). There were no significant differences between RM, H, and L. Using citrate synthase as a marker of mitochondrial density of the tissues, we show the relationship between M-CoA content and mitochondrial content for each tissue (Table 3). WM ($p<0.001$) and L ($p=0.49$) had significantly higher M-CoA per mitochondria than RM and H which were not significantly different from each other.

3.4. Gene expression profiles

The constitutive mRNA expression of CPT I was very low in liver compared to other muscle tissues (Fig. 4). Liver expression of CPT I is significantly lower than heart expression ($p=0.007$), however, not significantly different from red and white muscles ($p>0.05$). PPARα, a major transcription factor involved in expression of CPT I in mammals, followed an unexpected pattern. Although CPT I expression was lowest in the liver, the expression of PPARα tended to be higher than all other tissues, however only significantly different from white muscle (Fig. 4). In contrast, no significant differences were seen between tissues in the relative expression of PPARβ mRNA.

3.5. Mitochondrial membrane composition profiles

Membranes were dominated mainly by the saturated fatty acid C16:0, comprising 34%, 31%, 33%, and 32% in liver, red muscle, heart and white muscle, respectively and polyunsaturated fatty acid (PUFA) C22:6n3, comprising 6%, 28%, 15%, and 10% respectively. There are quite large differences between tissues in the concentration of C22:6n3, where liver has a very low amount while red muscle is quite high, however due to high variability this was not a statistically significant difference (Table 4). Other fatty acids that made a substantial contribution to the membrane composition include C18:0, C18:1 and C20:5n3.

The percentage of each phospholipid class in each tissue is presented in Table 5. As expected, levels of PC and PE were dominant in all tissues with CL also contributing a large percentage of overall phospholipid content. There are no major changes in the average percentages of the various types of fatty acids or in the unsaturation index (UI) in the different phospholipid classes between tissues. However, there are considerable differences, although not significant, in the relative proportions of each phospholipid class between tissues (Table 5). Most notably, the concentrations of PC and PE vary between tissues. The liver has a higher proportion of PC (53%) compared to red muscle (44%), heart (49%) and white muscle (42%). PE also varies between tissues, with the highest proportion being in red muscle (36%). The ratio of PC:PE is relatively low in red muscle (1.23±0.09) but not significantly different from other tissues (liver 2.07±0.32, heart 2.06±0.32, white muscle 2.02±0.31). While there are no significant differences between the average values of the classes of phospholipids between tissues, when presented in relation to CPT I inhibition, there are significant correlations (see below). Sphingomyelin was found in trace amounts indicating low contamination with other cellular membranes (data not shown).

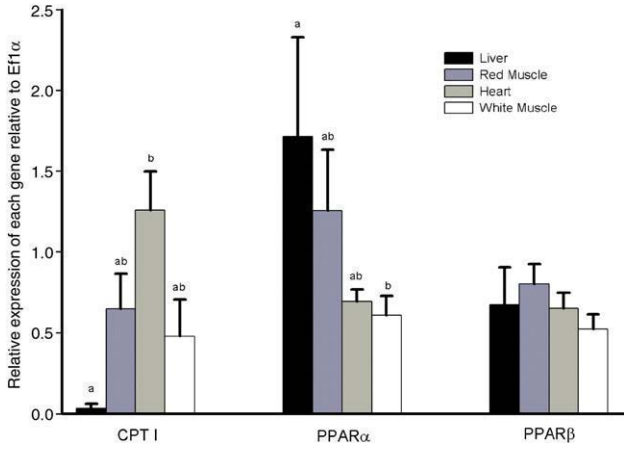

Fig. 4. Real-time PCR gene expression profiles in liver, red muscle, heart and white muscle for carnitine palmitoyltransferase (CPT) I, peroxisome proliferator-activated receptor (PPAR)α and PPARβ. Values are expressed relative to EF1α. Values are means± SE. $n=4$ for each tissue. Different letters denote significance within group, $p<0.05$.

Table 4
Total average mol% contributions of individual fatty acids (FA) to total FA from the mitochondrial membrane phospholipids across tissues*

Fatty acid	Liver	Red muscle	Heart	White muscle
C14:0	2.5±0.5	3.8±1.3	4.3±1.7	5.1±1.4
C16:0	34.2±2.0	30.6±3.0	32.9±4.4	32.1 ± 0.7
C16:1	5.5±1.2	6.2±2.5	7.6±3.2	9.6±2.6
C18:0	8.0±2.3	7.8±1.3	8.5±0.9	7.4±0.3
C18:1n9	5.1±3.0	3.5±1.3	10.7±2.1	6.6±2.0
C18:1n7	10.8±2.1	8.0±3.7	6.5±2.6	7.1±1.6
C18:2n6	8.5±0.8	6.3±0.8	5.0±1.2	5.4±1.2
C18:3n3	0.6±0.1	0.6±0.1	n.d.	0.7±0.1
C18:4n3	0.6±0.3	n.d.	1.1±0.6	n.d.
C20:1n9	3.5±0.7	3.8±0.9	2.3±0.8	1.7±0.3
C20:4n6	2.7±0.5	1.5±0.2	2.4±0.5	1.2±0.2
C20:5n3	10.9±3.6	2.7±1.9	1.6±1.0	7.3±2.6
C22:5n3	1.9±1.4	3.4±0.8	4.8±2.0	7.5±4.0
C22:6n3	5.8±4.8	21.5±12.3	11.6±6.2	10.4±8.3
Saturates	44.9±3.6	42.2±4.4	45.8±3.8	44.6±0.7
Monounsaturates	25.1±1.9	21.5±7.3	27.1±5.3	25.1±3.7
Polyunsaturates	29.9±1.8	36.3±11.0	27.0±3.1	30.3±4.4
η-3 polyunsaturates	18.6±1.4	28.4±10.8	19.6±3.8	23.6±4.4
η-6 polyunsaturates	11.3±0.8	7.8±0.7	7.5±1.3	6.6±1.0
η-3/η-6	1.7±0.1	3.6±1.3	3.1±0.9	3.9±1.2
UI†	148.9±9.6	202.6±59.8	154.4±20.3	164.9±22.8

Values are means±S.E., $n=4$.
*Values are means±SE; n.d. = not detectable.
†Unsaturation index = $\Sigma m_i \cdot n_i$; where m_i is the mole percentage and n_i is the number of C–C double bonds in the fatty acid "i".

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Table 5
Mol% of mitochondrial membrane phospholipid classes across tissues*

Phospholipid	Liver	Red muscle	Heart	White muscle
Phosphatidylcholine	52.7±2.5	43.7±3.5	49.0±7.7	41.9±2.1
Phosphatidylserine	2.5±0.6	4.6±1.7	6.5±2.8	6.2±2.3
Phosphatidylinositol	8.0±3.0	5.7±2.7	7.7±2.9	13.3±4.4
Phosphatidylethanolamine	27.2±4.3	35.7±2.5	23.9±1.2	22.4±4.3
Cardiolipin	9.5±1.1	13.9±2.8	13.0±2.2	16.0±2.0
PC:PE†	2.1±0.3	1.2±0.1	2.1±0.3	2.0±0.3

Values are means±S.E., $n=4$.
*Values are means±SE.
†PC = phosphatidylcholine, PE = phosphatidylethanolamine.

3.6. Relationship between IC_{50} and mitochondrial membrane composition

Significant correlations can be drawn from the current CPT I inhibition data and the mitochondrial membrane composition. There is a negative correlation between IC_{50} based on isotope determination and PC:PE ratio, indicating that as the PC:PE ratio increases, IC_{50} decreases, thus, increasing the sensitivity to M-CoA ($p=0.002$) (Fig. 5). Moreover, a correlation exists between IC_{50} and the percent of docosahexanoic acid (DHA, C22:6n3) in the membrane ($p=0.021$, Fig. 5). As the %DHA increases the IC_{50} increases, thus, decreasing the sensitivity to M-CoA.

4. Discussion

The regulation of CPT I and fat oxidation occurs by both genetic and non-genetic mechanisms. We found intrinsic differences in sensitivity of CPT I to the allosteric inhibitor M-CoA. Across tissues differences exist in 1) M-CoA content, 2) membrane composition, and 3) in constitutive mRNA expression for genes involved in the fat oxidation pathway. In general the apparent V_{max} for enzymes involved in mitochondrial fat oxidation followed the pattern red muscle>heart>liver>white muscle on a per gram tissue basis. Maximal activity of CPT I measured in isolated mitochondria followed a similar pattern of red muscle>heart>white muscle>liver suggesting that both mitochondrial quantity and quality play a role in tissue fat oxidative capacity. This pattern can be partially explained by tissue differences in mRNA expression pattern of heart>red muscle>white muscle>liver for CPT I. In contrast, the expression pattern of transcription factors PPARα and β did not correspond to enzyme patterns across tissues. Tissue malonyl-CoA content was highest in white muscle than other tissues and there were no differences between the other tissues. Amongst the factors that appear to be involved in non-genetic regulation of fat oxidation mitochondrial membrane fluidity has received little attention. Here we find significant correlations between key indices of membrane fluidity (%DHA and PC:PE) and sensitivity (IC_{50}) of CPT I to its allosteric regulator M-CoA.

4.1. Enzyme activity

Patterns of enzyme V_{max} across tissues are useful in revealing differences in fatty acid oxidation capacity and also stoichiometry of various enzymes in this pathway. Red muscle and heart have distinctly higher activities for CS, LCAD, CPT II and HOAD on a per gram protein basis when compared to white muscle and liver. CS is commonly used as marker of mitochondrial density [30], thus, it is clear that red muscle and heart have higher mitochondrial densities which leads to increased activities of mitochondrial fatty acid oxidation enzymes and the potential for higher rates of fat oxidation. Further to this, the activity of CPT I, thought to be the regulating enzyme of fat entry into the mitochondria, also exhibits tissue differences (but on a per mg mitochondrial protein basis). This suggests that mitochondria are not equivalent and that there are tissue-specific qualitative differences in fat oxidative ability per unit mitochondria. Similar patterns have been observed by others for CPT I activity per g tissue in rainbow trout, with red muscle and heart having the highest activity followed by liver and then white muscle [10,31]. However, our data suggests that liver CPT I has significantly lower activity compared to red muscle and that heart and white muscle have an intermediate activity level (Fig. 2). This data shows that there are stoichiometric changes in enzyme content with differences in fatty acid oxidation capacity across tissues and that this occurs through quantitative differences in mitochondrial density and qualitative differences in β-oxidation ability of each mitochondrial unit.

4.2. Allosteric regulation of CPT I

M-CoA reduced CPT I activity by allosteric inhibition in all tissues, however, to varying extents (Figs. 2 and 3A–D). At high levels of M-CoA liver, red, white and heart muscles were inhibited by 32%, 51%, 70% and 65%, respectively. Residual activity in each tissue may be due to activity of CPT II which is M-CoA insensitive and can be expressed if membranes are damaged during mitochondrial preparation. The mitochondrial preparations used in these experiments were between 60–85% intact depending on the tissue (based on CS measurements). However this cannot explain all of the M-CoA insensitive activity since liver mitochondrial preparations were 85% intact but had the highest residual activity (Fig. 3). Alternatively, it may be due to a M-CoA insensitive splice variants of CPT I as seen in rat red muscle [32]. We determined the IC_{50} for M-CoA sensitive moiety of CPT I in all tissues and have found that the liver ($IC_{50}=0.079$ μM) is significantly more sensitive to M-CoA than red muscle ($IC_{50}=0.55$ μM). In contrast, in

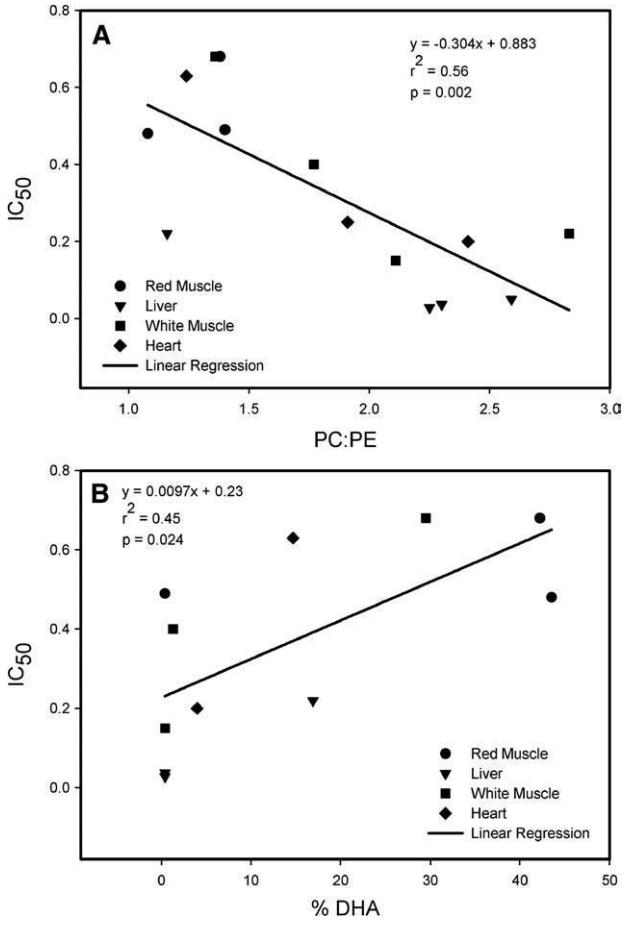

Fig. 5. The relationship between mitochondrial membrane composition and sensitivity of carnitine palmitoyltransferase (CPT) I to malonyl-CoA (IC50) across tissues. (A) Negative correlation between the PC:PE ratio and IC_{50}. Liver, $n=4$; red muscle, $n=3$; heart, $n=3$; white muscle, $n=4$. (B) Positive correlation between the % of docosahexanoic acid (DHA) and IC_{50}. Liver, $n=3$; red muscle, $n=3$; heart, $n=2$; white muscle, $n=3$.

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mammals, CPT I is 100 times more sensitive to M-CoA in muscle than in liver [7]. In fish, it appears that the opposite is true, liver CPT I is approximately 10 times more sensitive than muscle CPT I to M-CoA. However, this is a small difference compared to that seen between tissues in mammals.

The M-CoA content varied across tissues but was only significantly different in white muscle compared to the other 3 tissues. White muscle M-CoA content (0.19 $nmolg^{-1}$ wet weight) is similar to the only other data published on any tissue rainbow trout [25]. When the M-CoA content is expressed per unit citrate synthase, a mitochondrial density marker, it suggests that white muscle and liver have significantly higher M-CoA concentrations per unit mitochondria. Although these tissues also had the lowest CPT I activities per mg mitochondria this relatively high inhibitor concentration most likely contributes to low in vivo fat oxidation capacities.

Interestingly, our data indicate no changes in M-CoA between red muscle, heart and liver, which can be interpreted in a number of ways. It would be expected that liver would have higher M-CoA content than other tissues because it is the primary site of fat synthesis. On the other hand, we have shown here that liver CPT I is significantly more sensitive to M-CoA than other tissues. It is possible that in trout hepatocyte M-CoA concentrations are kept low to prevent total inhibition of CPT I and maintain fatty acid oxidation. In fact resting levels of M-CoA in all tissues were well below the IC_{50} (Table 2, 3).

4.3. Gene expression profile

CPT I has been found to be expressed in many tissues of rainbow trout [10]. However, the relative expression levels across tissues have not been previously determined in this species. Using real-time PCR we show that CPT I expression was very low in the liver compared to heart, but not significantly different from red and white muscles (Fig. 4). The low expression of CPT I in liver corresponds to its low activity (Fig. 2). Unlike mammals which express two different CPT I isoforms, trout are thought to express a single isoform and phylogenetic analysis has shown it to be similar to mammalian CPT I-α [10]. However, it is possible that our measurements reflect the expression of an uncharacterized muscle isoform which is expressed at low levels in liver. Moreover, the differences in CPT I kinetics between tissues shown here, are very suggestive of second isoform in trout. This would not be surprising since salmonids have gone through several genome duplications and possibly have retained 50–75% of the loci as duplicates [33]. However, intertissue differences in enzyme milieu could be a nongenomic explanation for the intertissue kinetic differences (see below).

In mammals many fatty acid oxidation genes have been shown to have PPAR response elements (PPRE) to which PPARs can bind and induce gene expression, including CPT I, CPT II and LCAD [11]. Surprisingly, the expression of PPARα across tissues was quite different from that of CPTI, with the highest, not lowest, expression in liver. The present data may be explained if in fish: 1) the PPARα gene expression is not translated to protein, 2) CPT I does not contain a PPRE, 3) this isoform performs a different role than in mammals, 4) there are more than one PPARα isoform as is the case for PPARβ in zebra fish [34], or 5) CPT I gene expression is regulated independently of PPARα as seen in rat hepatoma cells [35]. It is also possible that basal and inducible CPT expression occur by different pathways. Basal transcription of CPTI in fish might be regulated by the transcription factor SP1 as has been suggested for mammals [36], while inducible expression is controlled by the PPARs.

There were no differences in the expression of PPARβ between any of the tissues (Fig. 4). Very little is known about the roles of PPARβ, however, it has recently been shown to play a variety of roles depending on developmental stage, gender and diet [37,38]. In adult animals, PPARβ is expressed rather ubiquitously [39]. Our results show a ubiquitous and uniform expression across tissues in adult rainbow trout. These results are similar to those found in brown trout [40] and rats [41] and may suggest that in adult trout, PPARβ plays a role in homeostatic lipid metabolism but not in determining tissue differences in fat oxidation machinery.

4.4. Mitochondrial membrane composition and relation to CPT I IC_{50}

Both the fatty acid tail composition and the phospholipid head groups contribute to the overall fluidity, with increases in PUFAs (large "kinked" fatty acid tails) and PE (a membrane destabilizing phospholipid) contributing to increased fluidity [42,14]. There has been no systematic investigation of tissue-specific differences in mitochondrial membrane composition of the same animal that may be contributing to the differences in membrane fluidity or fat oxidation capacity [16]. Past interest in membrane remodelling has focused on single tissue changes during temperature acclimation that ensure proper membrane fluidity to preserve function at low temperature (for review see [14]). As well, comprehensive studies across reptiles and mammals have shown that the activity of mitochondrial membrane bound enzymes is positively correlated to the unsaturation of the mitochondrial membrane fatty acids [43].

In the present study fatty acids contributing the most to the composition of the mitochondrial membranes were C16:0, C18:0, C18:1, C20:5n3 and C22:6n3 (Table 4). This composition is similar to those observed by Kraffe et al. in their investigation of trout red muscle mitochondria [44]. Moreover, here the majority of the fatty acids were found in phospholipid classes PC and PE and to some extent in CL (Table 5). We found no significant differences in the fatty acid composition between tissues. However, red muscle tended to have higher concentrations of PUFAs (Table 4) and lower PC:PE ratios (Table 5), both of which may indicate an increase in fluidity. Leary et al. directly measured mitochondrial membrane fluidity in rainbow trout muscles and found that red muscle is more fluid when compared to white muscle and heart [16]. Recently, it has been suggested that increases in membrane fluidity may disrupt the interaction between the N- and C-termini of CPT I and be partly responsible for the decrease in the sensitivity of CPT I to M-CoA [45,46]. We investigated if there were any correlations that might explain the variation in the data and suggest a role of membrane composition in the regulation of CPT I. Significant correlations exist when the IC_{50} values are plotted against important determinants of membrane fluidity, PC:PE ratio and %DHA (Fig. 5A–B). Lower PC:PE ratio and higher %DHA may lead to increases in membrane fluidity, with changes in phospholipid headgroups (PC:PE) probably having a greater affect than changes in DHA proportions [14]. The differences, particularly in PC:PE across tissues could potentially decrease the sensitivity of CPT I to M-CoA. It should be noted that the measurements presented in this study are for both inner and outer mitochondrial membranes while CPT I only resides in the outer membrane. Direct fluidity measurements using DPH anisotropy, as opposed to membrane composition studies will be valuable in assessing actual outer mitochondrial membrane fluidity and verify our current findings.

This evidence suggests that in fish, sensitivity to M-CoA may be regulated by the fluidity of the membrane as indicated by the mitochondrial membrane composition, but currently it is unclear if this occurs by a common mechanism to that proposed for mammals [46]. Perhaps as part of homeoviscous adaptations, fish may be able to maintain suitable rates of mitochondrial fat oxidation at low environmental temperatures through this interaction between membrane fluidity and CPT I kinetics. This would help compensation for the decelerating effects of temperature on metabolism.

4.5. Fish vs. mammals

It is interesting to note the dramatic differences between rainbow trout and rat CPT I kinetics, especially in IC_{50} values. The liver mitochondria of rainbow trout tend to oxidize pyruvate at a higher rate

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then palmitoyl-carnitine [16], whereas in mammals, acyl-carnitines are preferentially oxidized over pyruvate [47]. While this may explain the difference in liver CPTI sensitivity to M-CoA, it does not explain the differences in muscle sensitivity seen between rats and fish, as lipids are an important fuel source for endurance exercise in both species [48]. In rats, the IC_{50} of CPT I for M-CoA is approximately 0.03 μM [7], whereas, in trout red muscle it is approximately 18 times higher (IC_{50} = 0.55 μM). There may be differences in skeletal muscle fibre types in rats that are being masked in the mixed muscle samples that are most conveniently elucidated by separating out the anatomically distinct fibre types in fish.

5. Conclusions

Fatty acid oxidation is regulated by both genetic and non-genetic mechanisms. There is a growing body of evidence from mammals [12,15,46,49,50] indicating that the fluidity of the mitochondrial membrane influences the sensitivity of CPT I to M-CoA. The present study suggests that this phenomenon extends across taxa. Isolated mitochondria from white, red and heart muscles of rainbow trout have been shown to exhibit dramatic differences in a variety of components such as proton leak kinetics and fluidity [16]. We have shown tissue-specific differences in a variety of factors affecting fatty acid oxidation in rainbow trout. Most dramatically, red muscle and liver show significant differences in CPT I kinetics. These kinetic differences match changes in gene expression of CPT I and tissue differences in mitochondrial membrane composition across tissues. In contrast to a previous study [10], these differences suggest the possibility of a second CPT I isoform in fish. It will now be important to determine if changing these factors through in vivo perturbations such as diet and temperature acclimation will affect these tissues in the same or different manner and if it will have effects on overall mitochondrial fatty acid oxidation. Studying the tissue-specific differences in fat oxidation and gene expression which reflect developmental differences in tissue function also provides a window into species-specific differences in abilities to use fat as a metabolic substrate.

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