Identification of a novel malonyl-CoA IC$_{50}$ for CPT-I: implications for predicting in vivo fatty acid oxidation rates

Brennan K. SMITH*1, Christopher G. R. PERRY*2, Timothy R. KOVES*†, David C. WRIGHT*†, Jeffrey C. SMITH‡, P. Darrell NEUFER§, Deborah M. MUOIO† and Graham P. HOLLOWAY*†

*Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, ON, Canada, †Sarah W. Stedman Nutrition and Metabolism Center, Departments of Medicine and Pharmacology and Cancer Biology, Duke University, Durham, NC 27704, U.S.A., ‡Department of Chemistry, Carleton University, Ottawa, ON, Canada, and §East Carolina Diabetes and Obesity Institute, Departments of Physiology and Kinesiology, East Carolina University, Greenville, NC 27834, U.S.A.

INTRODUCTION

The regulation of LCFA (long-chain fatty acid) oxidation is a central field of study in the context of exercise and metabolic disease. The rate-limiting step in LCFA oxidation has been attributed mainly to its transport across the mitochondrial outer membrane via CPT-I (carnitine palmitoyltransferase I) [1, 2]. Carnitine-dependent transport of LCFA into the mitochondrion was first conceptualized in the 1960s and strong evidence has now accumulated to indicate that CPT-I has an obligatory role in LCFA oxidation [3–5].

All previous work investigating the regulatory mechanisms surrounding CPT-I have been performed in isolated mitochondria. Although isolated mitochondria are a standard tool for certain measurements (i.e. P/O ratios), several physiologically relevant mitochondrial characteristics are altered during the isolation procedure [6–8]. Additionally, the removal of the cytoskeleton during the isolation procedure may alter the inherent regulatory mechanisms associated with LCFA oxidation as the cytoskeleton has previously been shown to influence CPT-I activity [9, 10] and other mitochondrial regulatory processes [11, 12]. In contrast with isolated mitochondria, PMFs (permeabilized muscle fibres) maintain the inherent cytoskeletal architecture and mitochondrial morphology while the sarcolemma is selectively permeabilized [13, 14]. This approach enables in situ investigations into mitochondrial physiology as substrates/chemicals added to the medium have direct access to the mitochondria within their native state. In addition, the provision of a myosin ATPase inhibitor (blebbistatin) to the PMF preparation enables the analysis of mitochondrial parameters under more physiological conditions (i.e. 37°C), and better represents the in vivo situation accordingly [15, 16].

In skeletal muscle, M-CoA (malonyl-CoA) inhibits CPT-I activity, and therefore the content of M-CoA is considered an important regulator of skeletal muscle LCFA oxidation [2, 17]. However, a number of discrepancies currently exist within the literature surrounding M-CoA inhibition kinetics of CPT-I. First, the reported concentration of M-CoA required to inhibit CPT-I activity by 50% (IC$_{50}$) in isolated mitochondria (~0.025–0.49 μM) [2, 18, 19] is lower than the resting M-CoA content [2, 20, 21], suggesting that CPT-I activity and rates of LCFA oxidation should be negligible at rest. This is inconsistent with the well-characterized reliance on LCFA oxidation at rest in vivo [22, 23]. Secondly, during exercise, a decrease in M-CoA content is thought to ‘release the brake’ on CPT-I and increase LCFA transport into the mitochondria for subsequent oxidation [24, 25]. However, previous studies in humans have reported unchanged [26, 27] or negligible decreases [28] in skeletal muscle M-CoA concentrations during exercise despite pronounced increases in LCFA oxidation. In addition, the role of M-CoA in regulating mitochondrial LCFA entry in Type 2 diabetes has shown disparate findings as M-CoA levels are elevated in the skeletal muscle of Type 2 diabetic humans [29] and rats [ZDF (Zucker diabetic fatty) rats] [30, 31], yet LCFA entry into the mitochondria is increased in both species [32, 33]. Of potential importance, during exercise and in Type 2 diabetes, LCFA-CoA levels within skeletal muscle are increased [34–36] and LCFA-CoA levels have been previously shown to decrease the effectiveness of M-CoA inhibition on CPT-I [37, 38]. Therefore any change in

Published values regarding the sensitivity (IC$_{50}$) of CPT-I (carnitine palmitoyltransferase I) to M-CoA (malonyl-CoA) inhibition in isolated mitochondria are inconsistent with predicted in vivo rates of fatty acid oxidation. Therefore we have re-examined M-CoA inhibition kinetics under various P-CoA (palmitoyl-CoA) concentrations in both isolated mitochondria and PMFs (permeabilized muscle fibres). PMFs have an 18-fold higher IC$_{50}$ (0.61 compared with 0.034 μM) in the presence of 25 μM P-CoA and a 13-fold higher IC$_{50}$ (6.3 compared with 0.49 μM) in the presence of 150 μM P-CoA compared with isolated mitochondria. M-CoA inhibition kinetics determined in PMFs predicts that CPT-I activity is inhibited by 33% in resting muscle compared with >95% in isolated mitochondria. Additionally, the ability of M-CoA to inhibit CPT-I appears to be dependent on P-CoA concentration, as the relative inhibitory capacity of M-CoA is decreased with increasing P-CoA concentrations. Altogether, the use of PMFs appears to provide an M-CoA IC$_{50}$ that better reflects the predicted in vivo rates of fatty acid oxidation. These findings also demonstrate that the ratio of [P-CoA]/[M-CoA] is critical for regulating CPT-I activity and may partially rectify the in vivo disconnect between M-CoA content and CPT-I flux within the context of exercise and Type 2 diabetes.

Key words: carnitine palmitoyltransferase I (CPT-I), isolated mitochondrion, malonyl-CoA, palmitoyl-CoA, permeabilized muscle fibre, skeletal muscle.
LCFA-CoA content may influence CPT-I activity, independent of changes in M-CoA content.

Therefore, to address the controversies surrounding M-CoA inhibition kinetics of CPT-I, in the present study we aimed to determine the sensitivity of CPT-I to M-CoA in isolated mitochondria and in PMFs under various concentrations of P-CoA (palmitoyl-CoA, a LCFA-CoA moiety). We report that PMFs have a 13–18-fold higher IC₅₀ than isolated mitochondria, and that the ability of M-CoA to inhibit CPT-I is dependent on the concentration of P-CoA in both preparations.

EXPERIMENTAL

Animals

Sprague–Dawley rats (10-week-old, 274 ± 8 g) were bred on-site at the University of Guelph, and housed in a climate control facility on a 12 h light/dark cycle and provided rat chow and water ad libitum. All facets of the present study were approved by the University of Guelph Animal Care Committee and conform to the guide for the care and use of laboratory animals published by the US National Institutes of Health. The red gastrocnemius muscle was used for all experiments.

Preparation of PMFs

The preparation of PMFs was carried out as described previously [15,40], and as we have previously reported [41]. Following dissection of red gastrocnemius (n = 6), fibre bundles (~2 mg) were separated with fine forceps under a binocular dissecting microscope in BIOPS buffer (2.77 mM Ca₉EGTA, 7.23 mM K₉EGTA, 5.77 mM Na₃ATP, 6.56 mM MgCl₂·6H₂O, 15 mM sodium phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM Mes). Following separation, fibre bundles were placed in BIOPS buffer containing 50 μg/ml saponin, agitated for 30 min and then washed in respiration buffer (MIRO5) (0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM monopotassium phosphate, 20 mM Hepes, 110 mM sucrose and 1 g/l fatty-acid-free BSA). Fibres were left in ice-cold MIRO5 until analysis of respiration.

PMF respiration

Mitochondrial respiration was measured in PMFs by high-resolution respirometry (Oroboros Oxygraph-2 k) at 37°C and room air-saturated oxygen tension in the presence of 25 μM blebbistatin to ensure PMF relaxation. Separate fibres from the same animal were used to determine (in duplicate) the kinetic properties of P-CoA-supported respiration and the sensitivity of P-CoA respiration to M-CoA inhibition. To measure P-CoA-supported respiration, MIRO5, 5 mM ADP, 2 mM malate and 2 mM L-carnitine were used as the respiration medium. Once a baseline respiration was determined, various concentrations of P-CoA (25, 50, 75, 100 and 200 μM) were added to each chamber. To measure M-CoA sensitivity, respiration was determined in the presence of 150 and 250 μM P-CoA. Once a baseline respiration was determined, various concentrations of M-CoA (0.03, 0.05, 0.1 and 0.25 μM) were added to the chambers. Quality-control experiments were also performed with isolated mitochondria to determine respiratory control ratios and mitochondrial coupling (ADP/O). In brief, state IV respiration was determined in the presence of 10 μM pyruvate + 5 mM malate and state III respiration was initiated with the addition of 100 μM ADP. Maximal state III respiration was determined in the presence of 2 mM ADP.

Isolated mitochondrial respiration

Differential centrifugation was used to isolate mitochondria from the red gastrocnemius muscle as described previously [41,43]. To measure M-CoA sensitivity, MIRO5 buffer containing 800 μM ADP, 2 mM malate and 750 μM L-carnitine was used as the respiration medium and respiration was stimulated with 25 μM P-CoA. Once a baseline respiration was determined, various concentrations of M-CoA (0.03, 0.05, 0.1 and 0.25 μM) were added to the chambers. Quality-control experiments were also performed with isolated mitochondria to determine respiratory control ratios and mitochondrial coupling (ADP/O). In brief, state IV respiration was determined in the presence of 10 μM pyruvate + 5 mM malate and state III respiration was initiated with the addition of 100 μM ADP. Maximal state III respiration was determined in the presence of 2 mM ADP.

One-phase exponential decay predictive equation

One-phase exponential decay is defined as:

\[ Y = Y_0 - \text{Plateau} \times e^{-K \times X} + \text{Plateau} \]

where \( Y \) is the respiration rate, \( Y_0 \) is the respiration rate without exogenous M-CoA, Plateau is where any subsequent addition of M-CoA has no effect on respiration, \( K \) is the rate constant and \( X \) is the M-CoA concentration. Plateau was constrained to zero (Graphpad Prism 5).

Statistics

Michaelis–Menten kinetics and IC₅₀ values for M-CoA were determined by plotting data points in GraphPad Prism 5 software following the subtraction of baseline values. The IC₅₀ is defined as the concentration of M-CoA where CPT-I activity and/or P-CoA-supported respiration were reduced by half. Unpaired two-tailed Student’s t tests were used to compare the P-CoA \( K_m \) and M-CoA IC₅₀ values of isolated mitochondria and PMFs. Statistical significance was accepted with a \( P \leq 0.05 \).
and P-CoA (increase in sensitivity (μmol O2/mg per min) and appropriate P/O ratios (2.9–6.30)) was ensured by determining pyruvate/ADP respiratory control ratio was 9.5 ± 0.2. In addition, the Lineweaver–Burk plots (insets in Figure 2) also displayed similar slopes in isolated mitochondria and PMFs. These data suggest that a diffusion limitation does not exist within PMFs for saturating fatty acid concentrations, and validate the use of identical P-CoA concentrations between methodologies in our subsequent experiments. Additionally, we performed experiments without saponin in PMFs. In these experiments, although the respiration rates were ~30–50% lower, M-CoA sensitivity was again not different with or without saponification, indicating that the saponin-permeabilization step does not affect the IC50 value observed in PMFs. Taken together, these data support the use of PMFs to assess M-CoA sensitivity.

Therefore we next investigated the kinetic properties of M-CoA inhibition of oxygen consumption in isolated mitochondria and PMFs in the presence of resting concentrations of P-CoA (25 μM) [34]. Maximal inhibition by M-CoA was similar between methodologies (~50%; Figure 3); however, this occurred at vastly different M-CoA concentrations (~10-fold higher in PMFs). In these highly controlled conditions, and in the presence of a physiologically relevant P-CoA concentration, the IC50 for M-CoA was 0.034 μM and 0.61 μM in isolated mitochondria and PMFs respectively (Figure 3), suggesting that M-CoA inhibition of CPT-I is considerably attenuated in an in situ model. In addition, the kinetics of M-CoA inhibition on P-CoA-supported respiration in isolated mitochondria in the present study are virtually identical with previous reports examining M-CoA inhibition of CPT-I activity [2,28,44]. Further suggesting that CPT-I is rate-limiting for fatty acid oxidation under these experimental conditions.

We next determined the kinetic properties of M-CoA inhibition in the presence of exercise concentrations of P-CoA (150 μM) [34]. We found that the sensitivity to M-CoA remained substantially lower in PMFs, as the IC50 values were 0.49 μM and 6.30 μM in isolated mitochondria and PMFs respectively (Figure 4). However, compared with the IC50 values found using resting concentrations of P-CoA, these exercise values represent 14-fold and 10-fold increases in isolated mitochondria and PMFs respectively.

Taken together, these data indicate that the sensitivity for M-CoA inhibition is vastly lower in PMFs compared with isolated mitochondria, as the IC50 is higher in PMFs in the presence of both resting (18-fold) and exercise (13-fold) P-CoA concentrations.

Role for P-CoA in regulating M-CoA inhibition kinetics

The pronounced difference in M-CoA IC50 values in the presence of higher P-CoA concentrations in both isolated mitochondria and PMFs (Figures 3 and 4) led us to further examine the idea that P-CoA concentrations can alter M-CoA sensitivity in PMFs. Previous work in isolated mitochondria has highlighted the interaction between M-CoA and P-CoA [2,37,38] but, considering the marked differences observed between PMFs and isolated mitochondria, we decided to re-examine this concept in PMFs. This was done by determining the ability of 7 μM M-CoA to inhibit P-CoA respiration at various (25–100 μM) concentrations of P-CoA in PMFs [34]. With this approach, M-CoA inhibited respiration ~63% in the presence of 25 μM P-CoA, but only ~26% in the presence of 100 μM P-CoA (Figure 5). These

RESULTS AND DISCUSSION

The present study evaluated M-CoA inhibition kinetics (IC50) of CPT-I in isolated mitochondria and PMFs. The integrity of the isolated mitochondria was ensured by determining pyruvate/malate respiratory control ratio measurements (5.4 ± 0.4 nmol O2/mg per min) and appropriate P/O ratios (2.9 ± 0.1). In the PMF preparation, the malate (2 mM) + glutamate (10 mM) + 5 mM ADP respiratory control ratio was 9.5 ± 0.2. In addition, the exogenous provision of cytochrome c during ADP-supported respiration did not increase respiration >10%, and the addition of oligomycin fully prevented P-CoA respiration (indicating coupled respiration; results not shown). Taken together, these quality-control experiments indicate that the isolated mitochondria and the mitochondria within the PMF preparation were fully coupled and intact and therefore appropriate for the present study.

CPT-I remains rate-limiting for fatty-acid-supported respiration in PMFs

We first determined that CPT-I represented a rate-limiting step in state III mitochondrial fatty-acid-supported respiration within the PMF preparation. This was accomplished by examining the kinetics of various lipids species proximal and distal to CPT-I. The sensitivity of the PMF preparation to palmitate (Km = 90 μM) and P-CoA (Km = 80 μM), substrates that require CPT-I, were very similar (Figure 1). In contrast, palmitoylcarnitine-supported respiration, which does not require CPT-I, displayed a marked increase in sensitivity (Km = 23 μM). Taken together, these data indicate that, similar to isolated mitochondria, CPT-I is rate-limiting for lipid-supported respiration in PMFs.

Use of PMFs yields a higher IC50 for M-CoA

Before performing experiments to elucidate the M-CoA inhibitory kinetics on CPT-I in PMFs, we determined the kinetic response of isolated mitochondria and PMFs to titrations of P-CoA, a substrate for CPT-I. High P-CoA concentrations (>40 μM) prevent respiration in isolated mitochondria and therefore we determined the sensitivity of isolated mitochondria to P-CoA using a CPT-I activity assay as classically performed [2]. The sensitivities of PMFs and isolated mitochondria to the substrate P-CoA were similar as the Ks values were 80 μM and 75 μM respectively (Figure 5). In addition, the Lineweaver–Burk plots (insets in Figure 2) also displayed similar slopes in isolated mitochondria and PMFs. These data suggest that a diffusion limitation does not exist within PMFs for saturating fatty acid concentrations, and validate the use of identical P-CoA concentrations between methodologies in our subsequent experiments. Additionally, we performed experiments without saponin in PMFs. In these experiments, although the respiration rates were ~30–50% lower, M-CoA sensitivity was again not different with or without saponification, indicating that the saponin-permeabilization step does not affect the IC50 value observed in PMFs. Taken together, these data support the use of PMFs to assess M-CoA sensitivity.

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**Figure 2**  CPT-I activity in isolated mitochondria (A) and oxygen consumption in PMFs (B) in the presence of various P-CoA concentrations

The $K_m$ values shown on the Figures represent those determined for the mean regression lines and they are not different between methodologies. These values were used to generate the Lineweaver–Burk plots for enzyme kinetics (insets). The slopes for these graphs were 0.41 and 0.39 for isolated mitochondria and PMFs respectively. $n = 4$ in isolated mitochondria, and $n = 6$ in PMFs. Each independent experiment was performed in duplicate, and data are expressed as means ± S.E.M.

**Figure 3**  M-CoA inhibition kinetics in isolated mitochondria (A) and in PMFs (B) in the presence of 25 μM P-CoA

The IC$_{50}$ values shown represent those determined for the mean regression line. PMFs displayed an ∼18-fold higher IC$_{50}$ compared with isolated mitochondria. $n = 4$ in isolated mitochondria and $n = 6$ in PMFs. Each independent experiment was performed in duplicate and data are expressed as means ± S.E.M. * indicates this is significantly different from isolated mitochondria.

data clearly show that increasing P-CoA concentration attenuates M-CoA inhibition of CPT-I, suggesting that the ratio of M-CoA/P-CoA is important in determining the overall catalytic activity of CPT-I. This implies that when LCFA-CoA levels are elevated, such as during exercise or in Type 2 diabetes, a reduction in M-CoA is not required to alter CPT-I flux. This could explain the pronounced increase in LCFA oxidation that occurs during exercise in humans in the face of little [28] or no change [26] in M-CoA levels. Additionally, a hallmark of chronic exercise training is the ability to increase fatty acid oxidation quickly at the onset of exercise, yet, following exercise training in rats, the normal exercise decline in M-CoA content is attenuated [45], suggesting additional mechanisms regulate CPT-I. In the context of the results of the present study and previous studies [2,37,38], the increase in the rate of fatty acid supply in the trained state would negate the need for M-CoA to substantially decrease in order to increase CPT-I flux and therefore fatty acid oxidation.

The interaction between P-CoA and M-CoA may also explain how, in Type 2 diabetes, mitochondrial matrix fatty acid oversupply exists in the presence of elevated M-CoA concentrations [29–33,46]. In the context of the results of the present study and previous studies [2,37,38], this disconnect may be explained by the increase in LCFA-CoA levels observed in Type 2 diabetes which would render M-CoA inhibition less effective and potentially account for the increase in CPT-I flux and resultant mitochondrial matrix fatty acid oversupply present in this disease [35,36]. Considering fatty acid oversupply to the mitochondria has been associated with impaired insulin sensitivity [33,40], LCFA-CoA levels within the context of CPT-I regulation may be an important factor to consider in Type 2 diabetes.
The IC₅₀ values shown represent those determined for the mean regression line. PMFs display a ∼13-fold higher IC₅₀ compared with isolated mitochondria. n = 4 in isolated mitochondria and n = 6 in PMFs. Each independent experiment was performed in duplicate, and data are expressed as means ± S.E.M. * indicates this is significantly different from isolated mitochondria.

Perspectives and significance

Predicted physiological M-CoA inhibition of fatty acid oxidation

As described previously [2,18,19,38,44], the IC₅₀ value determined in isolated mitochondria would suggest that LCFA oxidation is substantially inhibited at rest. Considering the respiratory quotient across a leg muscle at rest is ∼0.77–0.83 [22,23], these current values do not appear to represent the predicted in vivo situation. To put the results of the present study into physiological context, we applied the data from our ‘at rest’ M-CoA inhibition curves derived from isolated mitochondria and PMFs (Figure 3) to a one-phase exponential decay equation to predict the percentage inhibition of LCFA oxidation in the presence of resting concentrations of M-CoA (Table 1). We assumed resting M-CoA concentrations to be 0.7 μM as previously documented in two independent studies using HPLC-MS [47] and HPLC-MS/MS (tandem MS) [48]. As seen in Table 1, inhibition of LCFA oxidation is predicted to be >95% in isolated mitochondria at rest. However, in PMFs, the predicted inhibition of LCFA oxidation is ∼33%, which appears to better reflect the in vivo state, as reported respiratory quotients across a leg muscle [22,23] indicates that fatty acid oxidation would contribute ∼56–77% of the necessary energy at rest. In addition, independent of potential minor reductions in M-CoA content, the increase in P-CoA content (150 μM) within muscle during exercise attenuates M-CoA inhibition of LCFA oxidation to a predicted 2.1% in PMFs compared with 44% in isolated mitochondria (Table 1).

Taken together, these data suggest that in an in situ model the predicted percentage inhibition of fatty acid oxidation appears to better reflect the predicted in vivo state compared with isolated mitochondria. However, it should be acknowledged that we have not been able to account for subcellular or regional differences in M-CoA concentrations. It has been suggested previously that the β-isofrom of acetyl-CoA carboxylase located on the mitochondrial outer membrane ‘channels’ M-CoA to CPT-I [49–51] and, additionally, a mitochondrial enzyme in mammals capable of synthesizing M-CoA within the mitochondria has been characterized recently [52]. Therefore it is possible that the use of total cellular content of M-CoA (0.7 μM), as in the present study, is not reflective of the in vivo exposure of CPT-I to M-CoA. Although we acknowledge limitations pertaining to selecting the biologically relevant M-CoA concentration, these do not affect our interpretations, as these same limitations exist for both isolated mitochondrial and PMF preparations. We therefore hypothesize that the presence of the inherent cellular architecture within PMFs may help to explain the differences between methodologies.
that probably exerts substantial shear stress on the mitochondrial membranes, and therefore CPT-I [7]. This process has been shown to alter several characteristics of mitochondria, including respiration of specific substrates and susceptibility of calcium-induced opening of the permeability transition pore [6]. Current models of CPT-I propose a hairpin structure, with both N- and C-termini located in the cytosol [53], and the interaction of the N- and C-termini is essential for modulating and preserving M-CoA sensitivity [7,54]. Therefore isolating mitochondria may alter the interaction of these two cytosolic loops. However, this proposition is unlikely as current literature suggests that altering this interaction prevents M-CoA binding and therefore a challenge to this interaction would increase the IC_{50} [7,54]. Currently, no published literature exists to explain structure/function alterations in CPT-I that increase M-CoA binding/sensitivity.

Alternatively, M-CoA has been previously hypothesized to be sequestered into specific compartments within the skeletal muscle and it therefore remains possible that whatever mechanism promoting the sequestering and compartmentalization of M-CoA in skeletal muscle could still be present in PMFs and not in isolated mitochondria [20,55,56]. The presence of M-CoA-binding proteins in PMFs may also explain the discrepency in IC_{50} values between methodologies. In support of this proposal, the presence of liver M-CoA-binding proteins has been reported previously [57]. Additionally, the presence of M-CoA-binding proteins would be expected to decrease the effect of M-CoA in muscle at lower concentrations, while at supraphysiological concentrations of M-CoA these binding proteins would become saturated, and therefore CPT-I would be inhibited. This description recapitulates the observed trends in PMFs as M-CoA inhibition was attenuated at all concentrations ≤10 μM in comparison with isolated mitochondria, but inhibition was similar to isolated mitochondria at higher M-CoA concentrations (25 and 50 μM). Therefore the results of the present study supports the idea that M-CoA-binding proteins exist in muscle, although the identification of these proteins and the potential interaction with the cytoskeleton remains to be investigated.

**Conclusion**

In conclusion, in the present study we provide evidence that the IC_{50} value for M-CoA in PMFs is much higher than in isolated mitochondria and appears to better reflect *in vivo* fatty acid oxidation rates. Therefore we hypothesize that these functional differences between PMFs and isolated mitochondria underscore the important influence of mitochondrial morphology and/or the extra-mitochondrial environment in regulating LCFA oxidation via M-CoA inhibition of CPT-I. Additionally, P-CoA levels can alter M-CoA inhibition kinetics of CPT-I, which may explain some of the discrepancies in the previous literature. Lastly, within the context of exercise and Type 2 diabetes, the effect of altering...

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**Table 1** *Predicted in vivo fatty acid oxidation inhibition*

<table>
<thead>
<tr>
<th>Method</th>
<th>P-CoA concentration (μM)</th>
<th>Rate constant (K)</th>
<th>M-CoA concentration (μM)</th>
<th>Predicated rate of fatty acid oxidation (μmol/min per mg of mitochondrial protein)</th>
<th>Predicted percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated mitochondria</td>
<td>25</td>
<td>0.0476</td>
<td>0.7</td>
<td>2.20 nmol/min per mg of mitochondrial protein</td>
<td>&gt;95</td>
</tr>
<tr>
<td>PMFs</td>
<td>25</td>
<td>0.03707</td>
<td>0.7</td>
<td>14.1 pmol/s per mg of dry weight</td>
<td>33</td>
</tr>
<tr>
<td>Isolated mitochondria</td>
<td>150</td>
<td>1.417</td>
<td>0.7</td>
<td>8.98 nmol/min per mg of mitochondrial protein</td>
<td>44%</td>
</tr>
<tr>
<td>PMFs</td>
<td>150</td>
<td>0.03026</td>
<td>0.7</td>
<td>150 pmol/s per mg of dry weight</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table 2** *Literature comparison of skeletal muscle IC_{50} values*

<table>
<thead>
<tr>
<th>Method</th>
<th>Species</th>
<th>P-CoA concentration (μM used)</th>
<th>IC_{50}</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated mitochondria: CPT-I activity</td>
<td>Rat</td>
<td>50</td>
<td>0.023</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>50</td>
<td>0.025</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>50</td>
<td>0.034</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>50</td>
<td>0.041</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>40</td>
<td>0.07</td>
<td>[58]</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>300</td>
<td>0.490</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>150</td>
<td>0.49</td>
<td>The present study</td>
</tr>
<tr>
<td>Isolated mitochondria: respiration</td>
<td>Rat</td>
<td>25</td>
<td>0.034</td>
<td>The present study</td>
</tr>
<tr>
<td>Permeabilzed muscle fibres: respiration</td>
<td>Rat</td>
<td>25</td>
<td>0.61</td>
<td>The present study</td>
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<tr>
<td></td>
<td>Rat</td>
<td>150</td>
<td>6.3</td>
<td>The present study</td>
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</tbody>
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We also conclude that physiological increases in P-CoA may represent a key regulator of CPT-I activity. The mechanism by which P-CoA can alter M-CoA inhibition appears to be by increasing the dissociation constant (K_{d}) of M-CoA for CPT-I (~10-fold) [37]. Therefore P-CoA binding to CPT-I potentially alters the conformation of CPT-I such that M-CoA binding is less favourable [37]. These experiments [37] were performed in the presence of maximal P-CoA and therefore the 10-fold increase in the K_{d} appears to match our data with ‘maximal’ (exercise) concentrations of P-CoA, as we report a 13-fold increase in the IC_{50} in the presence of exercise P-CoA concentrations [37].

**Literature comparison of IC_{50} values in skeletal muscle**

The previous literature displays an ~70-fold range in M-CoA IC_{50} values, creating difficulty in interpreting the importance of M-CoA even within an isolated mitochondrial preparation. Table 2 compares the results of the present study in the context of previous literature and highlights the vast range of reported IC_{50} values. The apparent disparities within the previous literature appear to be explained by the concentration of P-CoA, as the highest values were all generated with ≥150 μM P-CoA (Table 2). Additionally, the IC_{50} values between rat and human skeletal muscle are similar, suggesting that the M-CoA kinetic properties are evolutionarily conserved.

Potential explanations for differences between methodologies

It is currently unknown why PMFs have a higher M-CoA IC_{50} value compared with isolated mitochondria. However, isolating mitochondria from skeletal muscle requires homogenization followed by a series of differential centrifugation steps, a process...
P-CoA concentrations may address the disconnect between M-CoA levels and CPT-I flux previously observed.

**AUTHOR CONTRIBUTION**

Brennan Smith and Graham Holloway contributed to study design, data interpretation, performed experiments and wrote the paper. Christopher Perry contributed to data interpretation, performed experiments and edited the paper. Timothy Koves and Jeffrey Smith performed experiments and edited the paper. David Wright, Darrell Neuffer and Deborah Muoio contributed to data interpretation and edited the paper before submission.

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