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Identification of a novel malonyl-CoA IC_{50} for CPT-1: implications for predicting *in vivo* fatty acid oxidation rates

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Synopsis

Published values regarding the sensitivity (IC_{50}) of carnitine palmitoyl transferase I (CPT-I) to malonyl-CoA (M-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 varying palmitoyl-CoA (P-CoA) concentrations in both isolated mitochondria and permeabilized muscle fibres (PMF). PMF have an 18-fold higher IC_{50} (0.61 vs 0.034 μM) in the presence of 25 μM P-CoA and a 13-fold higher IC_{50} (6.3 vs 0.49 μM) in the presence of 150 μM P-CoA compared to isolated mitochondria. M-CoA inhibition kinetics determined in PMF predicts that CPT-I activity is inhibited by 33% in resting muscle compared to >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 PMF appears to provide a M-CoA IC_{50} that better reflects the predicted *in vivo* rates of fatty acid oxidation. These findings also demonstrate 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 II diabetes.

Keywords

skeletal muscle; carnitine palmitoyl transferase-I; malonyl-CoA; palmitoyl-CoA; isolated mitochondria; permeabilized fibres

Introduction

The regulation of long chain fatty acid (LCFA) 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 carnitine palmitoyltransferase I (CPT-I) [1,2]. Carnitine-dependent transport of LCFA into the

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mitochondria 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 to isolated mitochondria, permeabilized skeletal muscle fibres (PMF) maintain the inherent cytoskeletal architecture and mitochondrial morphology while the sarcolemma is selectively permeabilzed [13,14]. This approach enables *in situ* investigations into mitochondrial physiology as substrates/chemicals added to the media 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, malonyl-coenzyme A (M-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. Firstly, the reported concentration of M-CoA required to inhibit CPT-I activity 50% (IC_{50}) in isolated mitochondria (~0.025-0.49 μM) [2,18,19] is lower than 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 vrvo* [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 II diabetes has shown disparate findings as M-CoA levels are elevated in the skeletal muscle of type II diabetic humans [29] and rats (ZDF rats) [30,31] yet LCFA entry into the mitochondria is increased in both species [32,33]. Of potential importance, during exercise and in type II 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 LCFA-CoA content can influence CPT-I activity independent of changes in M-CoA content.

Therefore, to address the controversies surrounding M-CoA inhibition kinetics of CPT-I, we aimed to determine the sensitivity of CPT-I to M-CoA in isolated mitochondria and in PMF under varying concentrations of palmitoyl-CoA (P-CoA, a LCFA-CoA moiety). We report that PMF have a 13 tol8-fold higher IC_{50} 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

Ten-week-old female Sprague-Dawley rats (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. Malonyl-CoA decarboxylase knockout ($mcd^{-/-}$) mice [39] were bred onsite at Duke University. All facets of this study were approved by the University of

Guelph Animal Care Committee and the Duke University Institutional Animal Care 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 permeabilized fibres

The preparation of PMF was adopted from prior publications [15,40], 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 containing, CaK_2EGTA (2.77 mM), K_2EGTA (7.23 mM), Na_2ATP (5.77 mM), $MgCl_2$*6H20 (6.56 mM), Na_2Phosphocreatine (15 mM), Imidazole (20 mM), Dithiothreitol (0.5 mM) and MES (50 mM). Following separation, fibre bundles were placed in BIOPS containing 50μg/ml saponin, agitated for 30min and then washed in respiration buffer (MIR05) containing EGTA (0.5 mM), MgC12*6H20 (3 mM), K-lactobionate (60 mM), Taurine (20 mM), KH_2P0_4 (10 mM), HEPES (20 mM), Sucrose (110 mM) and fatty acid free BSA (1 g/L). Fibres were left in cold MIR05 until respiration analysis.

Permeabilized muscle fibre respiration

Mitochondrial respiration was measured in PMF by high-resolution respirometry (Oroboros Oxygraph-2 k, Innsbruck, Austria) 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, MIR05, 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, 150 and 200 μM) were titrated into the chambers. To measure M-CoA sensitivity, respiration was determined in the presence of 150 μM and 25 μM P-CoA, and various concentrations of M-CoA (0.25, 0.5, 1, 2, 10, 25 and 50 μM) were subsequently titrated into the respiration chambers. Additionally, the impact of a fixed M-CoA concentration (7 μM) on respiration supported by varying P-CoA concentrations (25, 50, 75, and 100 μM) was performed. Separate fibres were used to determine respiratory control ratios in the presence of malate (2 mM) + glutamate (10 mM) +/- 5 mM ADP in the presence and absence of exogenous cytochrome c (10 μM) and oligomycin (2 μg/ml). P-CoA and palmitoylcarnitine were made up in water whereas the palmitate was made up in MIR05 supplemented with 10% BSA representing a 6.6:1 palmitate: BSA ratio. Optimization experiments of the various substrates have previously been done to ensure high quality, consistent experiments.

Carnitine palmitoyl transferase-I activity

CPT-I activity was determined as described by McGarry *et al* [2] with minor modifications as we have previously reported [19,42]. CPT-I activity is defined as the rate at which tritiated palmitoylcarnitine is formed from tritiated L-carnitine following the addition of isolated mitochondria to the reaction medium. The assay buffer consisted of 117 mM Tns-HCl, 0.28 mM reduced glutathione, 4.4 mM ATP, 2.22 mM KCN, 0.1 mM rotenone, 0.5% BSA, 5 mM L-carnitine and 1 μCi tritiated L-carnitine (L-[^{3}H]carnitine Amersham Bioscience, Buckinghamshire, England). Briefly, the assay was conducted at 37°C and initiated by the addition of 10 μl of mitochondrial suspension (final protein content of 5 μg/reaction) to 10 μl of varying P-CoA concentrations (18.75, 37.5, 75, 150, and 200 μM) and 80 μl of a standard reaction medium. The reaction containing 150 μM P-CoA was also carried out in the presence of various M-CoA concentrations (0.5, 1, 2, 10, 25 and 50 μM). The reaction was stopped after 6 min with the addition of ice-cold HCL. Palmitoyl-[^{3}H] carnitine was extracted in water-saturated butanol in a process involving three washes with

distilled water and subsequent re-centrifugation steps to separate the butanol phase, in which the radioactivity was counted.

Isolated mitochondrial respiration

Differential centrifugation was used to isolate mitochondria from the red gastrocnemius muscle as we have previously published [41,43]. To measure M-CoA sensitivity, MIR05, 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, 0.25 μM) were titrated into 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 mM pyruvate+5 mM malate and state III respiration was initiated with the addition of 100 μM ADP. Maximal state HI respiration was determined in the presence of 2 mM ADP.

One phase exponential decacy predictive equation

One phase exponential decacy is defined as: $Y = Y0\text{-}Plateau*e^{(-K*X)} + Plateau$ Where:

Y = respiration rate

Y0 = respiration rate without exogenous M-CoA

Plateau = where any subsequent addition of M-CoA has no effect on respiration

K = rate constant

X = M-CoA concentration

Plateau was constrained to zero (Graphpad Prism 5).

Statistics

Michaelis-Menten kinetics and IC_{50} values for M-CoA were determined by plotting data points in GraphPad Prism 5 software following the subtraction of baseline values. The IC_{50} is defined as the concentration of M-CoA where CPT-I activity and/or P-CoA supported respiration were reduced by half. Unpaired 2-tailed t-tests were used to compare isolated mitochondria and permeabilized fibres P-CoA Km and M-CoA IC_{50}. Statistical significance was accepted with a p value ≤ 0.05.

Results and Discussion

The current manuscript evaluated M-CoA inhibition kinetics (IC_{50}) of CPT-I in isolated mitochondria and PMF. The integrity of the isolated mitochondria was ensured by determining pyruvate/malate respiratory control ratio measurements (5.4±0.4 nmol O_2/mg/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; data not shown). Combined, 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 current study.

CPT-I remains rate-limiting for fatty-acid supported respiration in permeabilzed muscle fibres

We first determined that CPT-I represented a rate-limiting step in state 3 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, palmitoyl carnitine supported respiration, which does not require CPT-I, displayed a marked increase in sensitivity (Km = 23 μM). Altogether, these data indicate that similar to isolated mitochondria, CPT-I is rate limiting for lipid-supported respiration in PMF.

Use of permeabilzed muscle fibres yields a higher IC_{50} for malonyl-CoA

Prior to performing experiments to elucidate the M-CoA inhibitory kinetics on CPT-I in PMF, we determined the kinetic response of isolated mitochondria and PMF 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 utilizing a CPT-I activity assay as classically performed [2]. The sensitivities of PMF and isolated mitochondria to the substrate P-CoA were similar as the Km values were 80 μM and 75 μM, respectively (Figure 2A and B). In addition, the Lineweaver-Burk plots located as insets in Figures 2A and B, also displayed similar slopes in isolated mitochondria and permeabilized fibres. These data suggest that a diffusion limitation does not exist within PMF 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 PMF. 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 IC_{50} value observed in PMF. Altogether, these data support the use of PMF to assess M-CoA sensitivity.

Therefore, we next investigated the kinetic properties of M-CoA inhibition of oxygen consumption in isolated mitochondria and PMF in the presence of resting concentrations of P-CoA (25 μM) [34]. Maximal inhibition by M-CoA was similar between methodologies (-50%; Figure 3A, B), however this occurred at vastly different M-CoA concentrations (∼10-fold higher in PMF). In these highly controlled conditions, and in the presence of a physiologically relevant P-CoA concentration, the IC_{50} for M-CoA was 0.034 μM and 0.61 μM in isolated mitochondria and PMF, respectively (Figure 3A and B), suggesting 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 current study are virtually identical to previous reports examining M-CoA inhibition of CPT-I activity [2,2,38,44] 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 PMF, as the IC_{50} were 0.49 μM and 6.30 μM in isolated mitochondria and PMF, respectively (Figure 4A and B). However, compared to the IC_{50} values found using resting concentrations of P-CoA, these exercise values represent 14-fold and 10-fold increases in isolated mitochondria and PMF, respectively.

Altogether, these data indicate that the sensitivity for M-CoA inhibition is vastly lower in PMF compared to isolated mitochondria, as the IC_{50} is higher in PMF in the presence of both resting (18-fold) and exercise (13-fold) P-CoA concentrations.

Role of palmitoyl-CoA in regulating malonyl-CoA inhibition kinetics

The pronounced difference in M-CoA IC_{50} values in the presence of higher P-CoA concentrations in both isolated mitochondria and PMF (Figures 3 and 4) led us to further examine the notion that P-CoA concentrations can alter M-CoA sensitivity in PMF. 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 PMF and isolated mitochondria, we decided to re-examine this concept in PMF. This was done by determining the ability of 7 μM M-CoA to inhibit P-CoA respiration at varying (25-100 μM) concentrations of P-CoA in PMF [34]. With this approach, M-CoA inhibited respiration -63% in the presence of 25μM P-CoA, but only -26% in the presence of 100 μM P-CoA (Figure 5A and B). These data clearly show that increasing P-CoA concentration attenuates M-CoA inhibition of CPT-I suggesting that the ratio of M-CoAP-CoA is important in determining the overall catalytic activity of CPT-I. This implies when LCFA-CoA levels are elevated, such as during exercise or in type II 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 present data and others [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 II diabetes mitochondrial matrix fatty acid oversupply exists in the presence of elevated M-CoA concentrations [29-33,46]. In the context of the present data and others [2,37,38], this disconnect may be explained by the increase in LCFA-CoA levels observed in type II 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 II diabetes.

Perspectives and significance

Predicted physiological M-CoA inhibition of fatty acid oxidation

As previously documented [2,18,19,38,44], the IC_{50} 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 our results into physiological context, we applied the data from our “at rest” M-CoA inhibition curves derived from isolated mitochondria and PMF (Figure 3A and B) to a one phase exponential decay equation to predict the % 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 [48]. As seen in Table 1, inhibition of LCFA oxidation is predicted to be >95% in isolated mitochondria at rest. However, in PMF, 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 PMF compared to 44% in isolated mitochondria (Table 1).

Altogether, these data suggest that in an *in situ* model the predicted % inhibition of fatty acid oxidation appears to better reflect the predicted *in vivo* state compared to 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 previously suggested that the β-isoform 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 recently characterized [52]. Therefore, it is possible that the use of total cellular content of M-CoA (0.7 μM) as we have done is not reflective of the *in vivo* exposure of CPT-I to M-CoA. While 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 PMF may help explain the differences between methodologies. 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 (Kd) 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 favorable [37]. These experiments [37] were done in the presence of maximal P-CoA and therefore the 10-fold increase in the Kd 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

Previous literature displays ∼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 our present results 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 the M-CoA kinetic properties are evolutionary conserved.

Potential explanations for differences between methodologies

It is currently unknown why PMF have a higher M-CoA IC_{50} value compared to isolated mitochondria. However, isolating mitochondria from skeletal muscle requires homogenization followed by a series of differential centrifugation steps, a process that likely exerts substantial sheer 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 PMF and not in isolated mitochondria [20,55,56].

The presence of M-CoA binding proteins in PMF may also explain the discrepancy in IC_{50} values between methodologies. In support of this proposal, the presence of liver M-CoA binding proteins has been previously documented [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 PMF as M-CoA inhibition was attenuated at all concentrations <10 μM in comparison to isolated mitochondria but inhibition was similar to isolated mitochondria at higher M-CoA concentrations (25 and 50 μM). Therefore, the current data 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, we provide evidence that the IC_{50} value for M-CoA in PMF 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 PMF 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-1. Additionally, P-CoA levels can alter M-CoA inhibition kinetics of CPT-I which may explain some of the previous literature discrepancies. Lastly, within the context of exercise and type II diabetes, the impact of altering P-CoA concentrations may address the disconnect between M-CoA levels and CPT-I flux previously observed.

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Abbreviations

CPT-I	carnitine palmitoyl transferase-I
P-CoA	palmitoyl-CoA
M-CoA	malonyl-CoA
PmFB	permeabilzed muscle fibres
IC_{50}	concentration of inhibitor that decreases enzyme activity by 50%
Km	concentration of substrate that stimulates enzyme activity by 50%
LCFA	long chain fatty acids

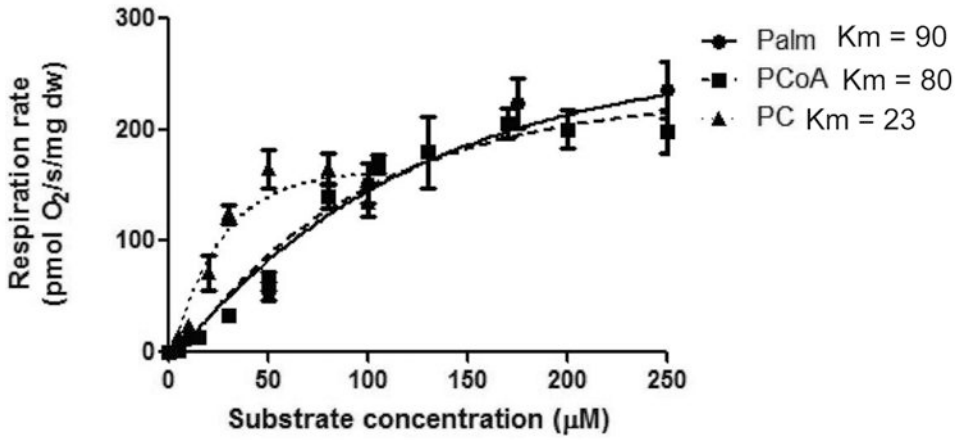

Figure 1.

Palmitate, palmitoyl-CoA (P-CoA) and palmitoyl carnitine (PC) respiration kinetics in permeabilized muscle fibres. Palmitate was titrated in the presence of malate (2mM), ADP (5mM), Co-enzyme A (1mM) and L-carnitine (2mM). P-CoA was titrated in the presence of malate (2mM), ADP (5mM) and L-carnitine (2mM). PC was titrated in the presence of malate (2mM) and ADP (5mM). All respiration chambers contained 25 μM blebbistatin. All R^2 values are above 0.98. P-CoA was inhibitory on respiration at ~220 μM and PC was inhibitory on respiration at 100 μM. N=6 and each independent experiment was performed in duplicate, and data is expressed as mean ± SEM.

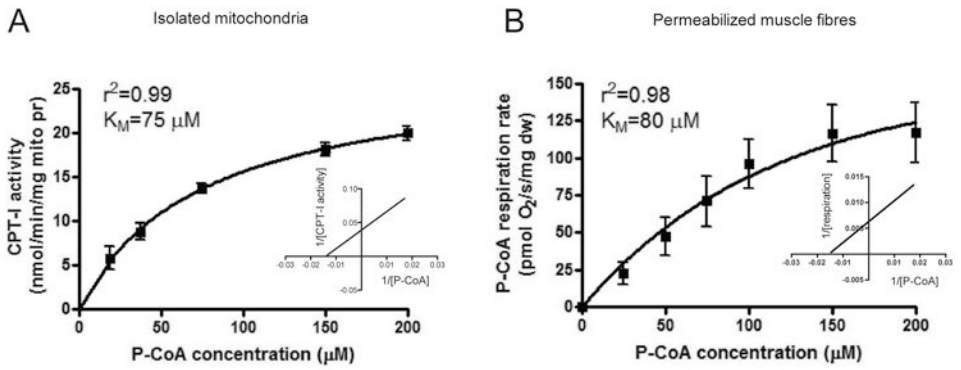

Figure 2.

Carnitine palmitoyltransferase I (CPT-I) activity in isolated mitochondria (A) and oxygen consumption in permeabilized muscle fibres (B) in the presence of various palmitoyl-CoA (P-CoA) concentrations. The Km 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, which are located as an inset in each graph. The slopes for these graphs were 0.41 and 0.39 for isolated mitochondria and permeabilized fibres, respectively. N=4 in isolated mitochondria, and N=6 in permeabilized fibres. Each independent experiment was performed in duplicate, and data is expressed as mean ±SEM.

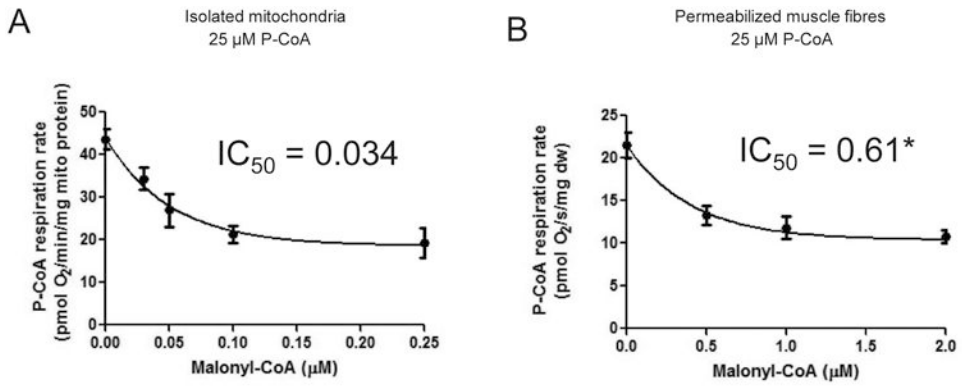

Figure 3.

Malonyl-CoA inhibition kinetics in isolated mitochondria (A) and in permeabilized muscle fibres (B) in the presence of 25 µM palmitoyl-CoA The IC_{50} values shown represent those determined for the mean regression line. Permeabilzed fibres displayed an ∼18-fold higher IC_{50} compared to isolated mitochondria. N=4 in isolated mitochondria and N=4 in permeabilized fibres. Each independent experiment was performed in duplicate and data is expressed as mean ± SEM. * significantly different from isolated mitochondria.

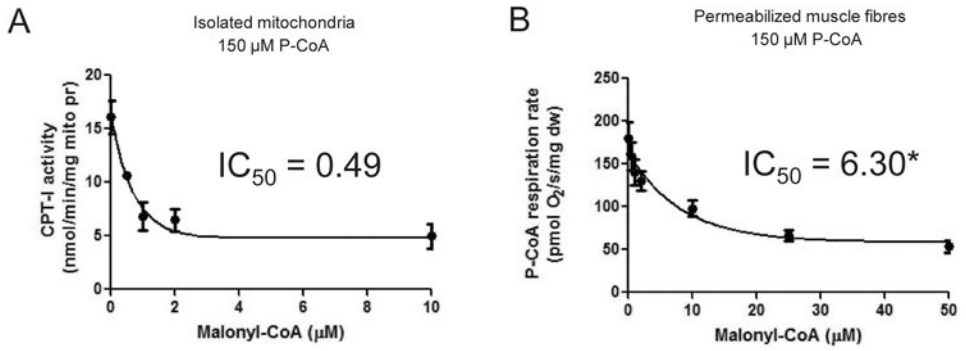

Figure 4.

Malonyl-CoA inhibition kinetics in isolated mitochondria (A) and in permeabilized muscle fibres (B) in the presence of 150 μM palmitoyl-CoA The IC_{50} values shown represent those determined for the mean regression line. Permeabilzed muscle fibres display a ∼13-fold higher IC_{50} compared to isolated mitochondria. N=4 in isolated mitochondria and N=6 in permeabilized fibres. Each independent experiment was performed in duplicate, and data is expressed as mean ± SEM. * significantly different from isolated mitochondria.

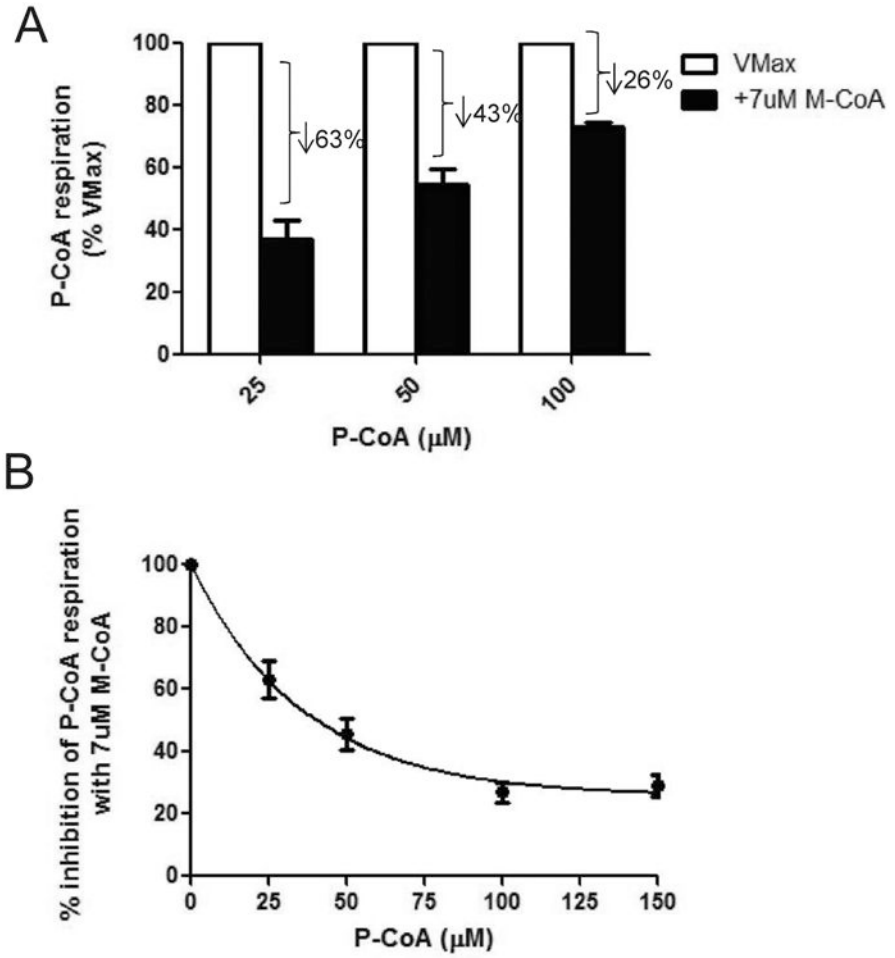

Figure 5.

Palmitoyl-CoA (P-CoA) respiration in permeabilzed muscle fibres in the presence of 7 μM malonyl-CoA (M-CoA). Respiration of varying concentrations of P-CoA was inhibited by the addition of 7 μM. At each increased concentration of P-CoA, inhibition by M-CoA was decreased. N=4 and each independent experiment was performed in duplicate. Data is expressed as mean ± SEM.

Table 1

Predicted *in vivo* fatty acid oxidation inhibition

Method	P-CoA concentration	Rate constant (K)	M-CoA concentration	Predicated rate of fat oxidation	Predicted % inhibition
Isolated mitochondria	25 μM	4.076	0.7 μM	2.20 nmol/min/mg mito pr	>95%
PMF	25 μM	0.03707	0.7 μM	14.1 pmol/s/mg dw	33%
Isolated mitochondria	150 μM	1.417	0.7 μM	8.98 nmol/min/mg mito pr	44%
PMF	150 μM	0.03026	0.7 μM	150 pmol/s/mg dw	2.1%

The rate constant (K) used in the one phase exponential decay equation was derived from Graphpad Prism 5. The malonyl-CoA concentration was adopted from two previous publications [47,48].

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

Method	Species	P-CoA μM used	IC_{50}	Reference
Isolated mitochondria: CPT-I activity	Rat	50	0.023	[38]
	Human	50	0.025	[2]
	Rat	50	0.034	[2]
	Rat	50	0.041	[44]
	Rat	40	0.07	[58]
	Human	300	0.490	[18]
	Human	300	1.600	[19]
	Rat	150	0.43	Present study
Isolated mitochondria: respiration	Rat	25	0.034	Present study
Permeabilzed muscle fibres: respiration	Rat	25	0.61	Present study
	Rat	150	6.3	Present study