Does skeletal muscle carnitine availability influence fuel selection during exercise?

Francis B. Stephens[1]

[1]Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, United Kingdom

Corresponding author:
Dr Francis B. Stephens,
University of Exeter,
Department of Sport and Heath Sciences,
St Luke's Campus,
Heavitree Road,
Exeter,
EX1 2LU,
United Kingdom

Email: f.b.stephens@exeter.ac.uk
Tel: +44 (0)1392 722157

Short Title: Skeletal muscle carnitine and fuel metabolism

Keywords: Exercise, Skeletal muscle, Fat metabolism, Carbohydrate metabolism, Carntitine

Abstract

Fat and carbohydrate are the major fuel sources utilised for oxidative, mitochondrial adenosine triphosphate (ATP) resynthesis during human skeletal muscle contraction. The relative contribution of these two substrates to ATP resynthesis and total energy expenditure during exercise can vary substantially, and is predominantly determined by fuel availability and exercise intensity and duration. For example, the increased ATP demand that occurs with an increase in exercise intensity is met by increases in both fat and carbohydrate oxidation up to an intensity of around 60 to 70% of maximal oxygen consumption (VO_2max). When exercise intensity increases beyond this workload skeletal muscle carbohydrate utilisation is accelerated, which results in a reduction and inhibition of the relative and absolute contribution of fat oxidation to total energy expenditure. However, the precise mechanisms regulating muscle fuel selection and underpinning the decline in fat oxidation remain unclear. This brief review will primarily address the theory that a carbohydrate flux mediated reduction in the availability of muscle carnitine to the mitochondrial enzyme carnitine palmitoyltransferase 1 (CPT1), a rate limiting step in mitochondrial fat translocation, is a key mechanism for the decline in fat oxidation during high intensity exercise. This is discussed in relation to recent work in this area investigating fuel metabolism at various exercise intensities and taking advantage of the discovery that skeletal muscle carnitine content can be nutritionally increased *in vivo* in humans.

Introduction

Carbohydrate and fat are the primary fuel sources for mitochondrial ATP resynthesis in human skeletal muscle. The relative utilisation of these two substrates during exercise can vary substantially and depends strongly on substrate availability and exercise duration and intensity. Fat constitutes the largest energy reserve in the body and in terms of the amount available it is not limiting to endurance exercise performance. However, fat exhibits a relatively low maximal rate of oxidation *in vivo*, which begins to measurably decline at an exercise intensity of around 60 to 70% of maximal oxygen consumption (VO_2max), above which muscle glycogen becomes the major fuel supporting ATP resynthesis[1,2,3]. The muscle glycogen stores are limited and it has been well established that muscle glycogen depletion coincides with fatigue during very prolonged high intensity endurance exercise[4]. As fatigue can be postponed by increasing pre-exercise muscle glycogen content[4], it is thought that augmenting the rate of fat oxidation during endurance exercise could delay glycogen depletion and improve prolonged endurance exercise performance. Indeed, it has long been known that enhanced fat oxidation is one of the main muscle adaptations to endurance exercise training[5]. However, the mechanisms that regulate the relative contributions of carbohydrate and fat oxidation in skeletal muscle during exercise have not been clearly elucidated. Several potential regulatory points (Figure 1) could influence the decline in the rate of fat oxidation in skeletal muscle during high intensity endurance exercise, including: 1) regulation of lipolysis within adipose tissue and intramuscular triacylglycerol (IMTG) pools; 2) transport and delivery of circulating free fatty acids from adipose tissue stores to working skeletal muscle; 3) transport of fatty acids across the muscle cell membrane and within the cell cytosol to the outer mitochondrial membrane; 4) transport of fatty acid across the outer mitochondrial membrane via carnitine palmitoyltransferase 1 (CPT1), the rate limiting step in fatty acid oxidation, and 5) intramitochondrial enzymatic reactions distal to CPT1. The present review will primarily address the theory that a carbohydrate flux mediated reduction in the availability of muscle carnitine, the major substrate for CPT1, is a key mechanism for the decline in fat oxidation during high intensity exercise. This is discussed in relation to recent work in this area taking advantage of the discovery that skeletal muscle carnitine content can be nutritionally increased *in vivo* in humans and have a significant impact upon fuel selection during endurance exercise.

Insert Figure 1

Integration of fat and carbohydrate oxidation during exercise

There is increasing evidence to suggest that fat oxidation during exercise is directly regulated by the rate of flux though the mitochondrial pyruvate dehydrogenase enzyme complex (PDC), a rate limiting step in skeletal muscle carbohydrate oxidation. Increasing exercise intensity, from moderate to high, or increasing carbohydrate availability during exercise, increases glycolysis and flux through the PDC with a corresponding decrease in the rate of fat oxidation[1,2,6]. A likely site of the regulation of fat oxidation by carbohydrate flux is CPT1. Increasing glucose availability at rest, via a hyperglycaemic-hyperinsulinaemic clamp, decreases long-chain fatty acid ([^{13}C]oleate) oxidation with no effect on medium-chain fatty acid ([^{13}C]octanoate) oxidation[7]. This would suggest an inhibition of fat oxidation at the level of CPT1 as medium-chain fatty acids cross the mitochondrial membrane to be oxidized independently of CPT1. The same effect of carbohydrate was seen during moderate intensity exercise at 50% VO_2max, where hyperglycaemia (induced as a result of pre-exercise ingestion of glucose) increased glycolytic flux and reduced long-chain fatty acid ([1-^{13}C]palmitate) oxidation[8]. Again, medium-chain [1-^{13}C]octanoate oxidation was unaffected, suggesting that carbohydrate metabolism can also directly limit fat oxidation at the level of CPT1 during exercise. It should be noted that these responses might have been mediated, at least in part, via an insulin-induced inhibition of adipose tissue lipolysis, resulting in decreased plasma free fatty acid (FFA) availability. However, in the study of Sidossis and Wolfe[7], normal plasma FFA was maintained constant and muscle long-chain acyl-CoA levels did not change, suggesting FFA availability, uptake, and cytosolic transport was not limiting. Furthermore, restoring plasma FFA concentration during the exercise trial, via lipid and heparin infusion, did not fully restore fat oxidation rates, again suggesting that the inhibitory effect of increased carbohydrate availability resides at the level of oxidation, rather than FFA availability[9]. In support of this theory, increasing fat oxidation rates 2-fold during exercise at 65% VO_2peak, due to different pre-exercise muscle glycogen content, has no effect on muscle FFA uptake rates or FAT/CD36 (the protein responsible for FFA uptake into skeletal muscle) protein levels[6].

A glance at CPT1 regulation during exercise

Several intracellular mechanisms leading to the down regulation of long-chain fatty acid oxidation at the level of CPT1 during exercise with high glycolytic flux have been proposed, including inhibition of CPT1 by malonyl-CoA, acylcarnitine, or acidosis, and reduced substrate availability of carnitine or long-chain fatty acyl-CoA. The following section will focus on mechanisms that have been investigated *in vivo* and in humans.

Malonyl-CoA is a potent inhibitor of CPT1 activity *in vitro* and is a likely candidate as the intracellular regulator of the rate of long-chain fatty acid oxidation human skeletal muscle. Indeed, in resting human skeletal muscle, changes in malonyl-CoA concentration have occurred with opposite changes in fat oxidation[10, 11]. Skeletal muscle malonyl-CoA concentration is regulated by AMP-activated protein kinase (AMPK) and by the cytosolic concentration of citrate, which inactivate and activate the enzyme responsible for malonyl-CoA synthesis from acetyl-CoA (acetyl-CoA carboxylase; ACC), respectively[12]. Thus, increased muscle concentrations of acetyl-CoA and citrate, due to increased glycolytic flux, would increase muscle malonyl-CoA concentration (via an increase in ACC activity) and, therefore, inhibit long-chain fatty acid oxidation via CPT1. In support of this theory, a significant negative correlation was obtained between muscle malonyl-CoA content and fat oxidation rates in healthy middle-aged men during a two-step euglycaemic-hyperinsulinaemic clamp[10]. Furthermore, hyperglycaemia with hyperinsulinaemia increased resting skeletal muscle malonyl-CoA content 3-fold in healthy humans, resulting in a functional decrease in CPT1 activity, as evidenced by an 80% inhibition of leg [^{13}C]oleate oxidation, with no change in [^{13}C]octanoate oxidation[11]. However, it appears that muscle malonyl-CoA concentration may not regulate long-chain fatty acid oxidation during exercise. Roepstorff *et al*[6] demonstrated that, despite a 122% increase in fat oxidation rates (due to depleted pre-exercise muscle glycogen content) during exercise at 65% $\dot{V}O_2$peak, there were no differences in muscle malonyl-CoA content compared to control. These findings are also supported by other human exercise studies where no association between malonyl-CoA content and fat oxidation rates were obtained in skeletal muscle during prolonged moderate-intensity exercise[13] or during graded-intensity exercise[14,15]. Furthermore, α_2-AMPK activity

more than doubled with an associated 6-fold increase in ACCβ (muscle isoform) phosphorylation and significant decrease in malonyl-CoA concentration following both prolonged and high intensity exercise, where fat oxidation paradoxically increased and decreased, respectively[6,15]. Taken together, these findings would suggest that malonyl-CoA does not play a major role in the regulation of long-chain fatty acid oxidation in human skeletal muscle during exercise.

Carnitine is the principal substrate for CPT1, suggesting that it may play a role in the regulation of fat oxidation. Irving Fritz and colleagues first established that mitochondria in a variety of tissues are impermeable to fatty acyl-CoA, but not to fatty acylcarnitine, and that carnitine and CPT1 are essential for the translocation of long-chain fatty acids into skeletal muscle mitochondria for β-oxidation[16]. Since these discoveries it has been established that CPT1, situated within the outer mitochondrial membrane, catalyses the reversible esterification of carnitine with long-chain acyl-CoA to form long-chain acylcarnitine. Cytosolic acylcarnitine is then transported into the mitochondrial matrix in a simultaneous 1:1 exchange with intramitochondrial free carnitine via the carnitine acylcarnitine translocase (CACT), which is situated within the mitochondrial inner membrane (Figure 1). Once inside the mitochondrial matrix, acylcarnitine is transesterified back to free carnitine and long-chain acyl-CoA in a reaction catalysed by carnitine palmitoyltransferase 2 (CPT2), which is situated on the matrix side of the inner mitochondrial membrane. The intramitochondrial long-chain acyl-CoA is then oxidised and cleaved by the β-oxidation pathway[17]. The significance of this "carnitine cycle" to fat oxidation during exercise is highlighted in patients with carnitine, CPT2, or CACT deficiency (CPT1 deficiency appears to be incompatible with life) who typically experience severely reduced rates of fat oxidation during prolonged exercise along with muscle pain and weakness, hypoglycaemia, and hypoketosis. CPT1 is considered to be the rate-limiting enzyme for long-chain fatty acid entry into the mitochondria and oxidation, but carnitine is classically not thought to be rate limiting to CPT1. Indeed, the concentration of carnitine in skeletal muscle is around 5 mM intracellular water[18] and far in excess of the *in vitro* Michaelis-Menten constant (K_m) of muscle CPT1 for carnitine of approximately 0.5 mM[19]. However, the enzymes of the carnitine cycle co-immunoprecipitate and are mainly located in the specialised contact sites between

outer- and inner-mitochondrial membranes in order to allow metabolic channelling[20]. Thus, it is likely that the intramitochondrial content of free carnitine determines carnitine availability to CPT1 and, as this is around 10% of the whole muscle carnitine pool[21], that *in vivo* carnitine availability could be rate limiting to the CPT1 reaction. Indeed, during conditions of high glycolytic flux, such as during exercise at a high intensity or with elevated carbohydrate availability, muscle free carnitine availability is reduced[2,6].

Free carnitine availability is reduced during high intensity exercise

The reduction in free carnitine availability observed with high glycolytic flux first became apparent in 1966 when Childress and colleagues recognised that the blowfly flight muscle, which does not oxidise fatty acids during flight, was rich in carnitine and carnitine acetyltransferase (CAT) [22]. During the initial phase of flight, acetyl-CoA from glycolysis was generated faster than its utilisation by the TCA cycle, with a corresponding 4-fold increase in acetylcarnitine. Childress suggested that carnitine accepted the excess acetyl groups, via the action of mitochondrial carnitine acetyltransferase, in order to maintain a viable pool of free coenzyme A to permit the continuation of pyruvate oxidation (Figure 1). Subsequent work from Harris and colleagues in humans and thoroughbred horses demonstrated that following a few minutes of intense, near maximal exercise, muscle free carnitine content was reduced from approximately 80% of the total muscle carnitine pool at rest (muscle total carnitine content is approximately 20 mmol·(kg dry muscle)$^{-1}$ in humans) to around 20%, with almost all of the reduction being attributed to formation of acetylcarnitine[23,24,25]. However, there was no change in muscle acetylcarnitine content following a few minutes of exercise at lower intensities[26,27]. Constantin-Teodosiu and colleagues[28] also demonstrated a rapid increase in acetylcarnitine formation in humans from 3 to 13 mmol·(kg dm)$^{-1}$ following 3 min of intense exercise at 75% VO_2max. Furthermore, over the next hour of exercise, acetylcarnitine gradually increased to 16 mmol·(kg dm)$^{-1}$, suggesting that exercise duration, as well as intensity, contributes to acetylcarnitine accumulation, albeit to a lesser degree. If a second bout of exercise is performed[29], or if exercise is continued for several hours[30], then acetyl-CoA availability may actually be less than the demands of the TCA cycle and the stockpiled acetyl groups can be used as a fuel source via the

reverse CAT reaction (Figure 1). Indeed, Seiler *et al*[31] recently observed an inability to use stockpiled acetylcarnitine following exercise in an incomplete CAT knockout mouse, i.e. the incomplete reverse CAT reaction resulted in a decline in acetyl-CoA. However, this did not warrant their conclusion "this observation is consistent with the premise that during intense exercise CAT catalyzes net flux in the direction of acetyl-CoA", which is certainly not consistent with the aforementioned studies in humans that observed a net accumulation of acetylcarnitine during intense exercise.

Research by van Loon *et al*[2] demonstrated that a 35% decrease in the rate of long-chain fatty oxidation that occurred at an exercise intensity above 70% VO_2max, measured using a [U-^{13}C]palmitate tracer, was paralleled by a 65% decrease in skeletal muscle free carnitine content. In support of these findings, Roepstorff *et al*[6] demonstrated a 2.5-fold decrease in the rate of fat oxidation, compared to control, during moderate intensity exercise (65% VO_2max) when free carnitine availability was reduced to 50% (12 mmol·(kg dm)$^{-1}$) of the total carnitine store, as a result of pyruvate, and thereby acetyl-CoA, production being increased due to elevated pre-exercise muscle glycogen stores. Also, during bicycle exercise at 75% VO_2max to exhaustion, both muscle free carnitine content and fat oxidation rates were markedly higher with low pre-exercise muscle glycogen content compared to control[32]. Taken together, the above studies suggest a relationship between skeletal muscle free carnitine availability (and therefore carbohydrate metabolism) and the regulation of long-chain fatty acid oxidation rates, via CPT1, in human skeletal muscle during exercise.

Is reduced free carnitine availability related to insulin resistance?

It would be remiss not to mention a recent growing body of research in the area of skeletal muscle carnitine metabolism, particularly related to skeletal muscle insulin resistance. Insulin resistance is characterized by a 'metabolic inflexibility' in the reciprocal relationship between fatty acid and glucose oxidation within skeletal muscle[33]. In keeping with observation in the same incomplete CAT knockout model by Muoio *et al*[34], a recent article of Lindboom and colleagues[35] asserts that "the transformation of excessive acetyl-CoA into acetylcarnitine is important to maintain metabolic flexibility", and that "compromised capacity to generate acetylcarnitine,

either due to reduced CAT activity or low carnitine concentration, may reduce pyruvate dehydrogenase (PDH) activity, hence reducing oxidative degradation of glucose". Contrary to this opinion, biochemically determined human muscle acetylcarnitine content is reduced under insulin-stimulated, high glucose conditions[36], and increased in the insulin resistant state[37,38]. What's more, as mentioned above, compelling evidence has demonstrated a positive, constant linear relationship exists between muscle acetyl-CoA formation and acetylcarnitine accumulation over low to maximum rates of mitochondrial flux[22, 24, 27, 39]. Lindeboom *et al*[35] themselves report maximal *ex vivo* CAT activity in participants with type 2 diabetes (T2D) of 52.6 nmol/mg protein/min, which is far in excess of PDC flux rates even during high intensity exercise (15 nmol/mg protein/min)[27]. This clearly demonstrates that CAT is not limiting the equilibrium between acetyl-CoA and acetylcarnitine, making it highly unlikely that acetylcarnitine accumulation could be compromised for reasons other than rates of acetyl-CoA formation from PDH or β-oxidation reactions. Illustrated another way, if CAT activity was indeed limiting in insulin resistant individuals, then mitochondrial ATP production would be markedly suppressed, as the rapid sequestering of the micromolar concentrations of mitochondrial free CoA as acetyl-CoA would immediately compromise PDH and TCA cycle flux. This clearly doesn't happen, rather it's likely that group differences in *ex vivo* maximal CAT activity and *in vivo* mitochondrial function reported by Lindboom *et al*[35] would be dissipated if corrected for mitochondrial content, particularly given the apparent impairment of muscle mitochondrial function in patients with T2D is accounted for by differences in mitochondrial content[40]. Finally, the resting muscle acetylcarnitine content of participants with T2D in Lindboom et al. was 12% of the measured total carnitine pool, suggesting that free carnitine was 88% of the total muscle carnitine pool and making it highly improbable that free carnitine availability was limiting to PDH flux and acetylcarnitine formation.

Can skeletal muscle carnitine content be increased?

Further insight into whether free carnitine availability is limiting to CPT1 and the rate of fatty acid oxidation can be provided by increasing skeletal muscle carnitine content. However, the majority of the pertinent studies in healthy humans to date have failed to increase skeletal muscle carnitine content via oral or intravenous L-carnitine administration[39]. For example, neither feeding L-carnitine daily for up 3 months[41],

279 nor intravenously infusing L-carnitine for up to 5 hours[42], had an effect on muscle
280 total carnitine content, or indeed net uptake of carnitine across the leg[43] or arm[44]
281 (Table 1). Furthermore, feeding 2-5 $g \cdot d^{-1}$ of L-carnitine for 1 week to 3 months prior
282 to a bout of exercise, had no effect on perceived exertion, exercise performance,
283 VO_2max, or markers of muscle substrate metabolism such as RER, VO_2, blood
284 lactate, leg FFA turnover, and post exercise muscle glycogen content[39]. What was
285 apparent from the earlier carnitine supplementation studies was that muscle carnitine
286 content was either not measured or, if it was, not increased. This is likely explained
287 by the finding that carnitine is transported into skeletal muscle against a considerable
288 concentration gradient (>100 fold) that is tightly regulated and saturated under normal
289 conditions, and so it is unlikely that simply increasing plasma carnitine availability
290 *per se* will increase muscle carnitine transport and storage[39]. Indeed, recent human
291 and porcine studies of arterio-venous carnitine fluxes confirmed that net muscle
292 carnitine uptake/efflux is negligible under fasting conditions[44, 45], with systemic
293 concentrations of carnitine and acylcarnitines largely governed by gut absorption,
294 hepatic release and renal filtration[45]. However, insulin appears to stimulate skeletal
295 muscle carnitine transport, and intravenously infusing L-carnitine in the presence of
296 high circulating insulin (>50 $mU \cdot l^{-1}$) can increase muscle carnitine content by
297 15%[36,42, 46]. Furthermore, ingesting relatively large quantities of carbohydrate in a
298 beverage (>80 g) can stimulate insulin release to a sufficient degree to increase whole
299 body carnitine retention when combined with 3 g of carnitine feeding and, if
300 continued for up to 6 months (80 g carbohydrate + 1.36 g L-carnitine twice daily) can
301 increase the muscle store by 20% compared to carbohydrate feeding alone[47]. Indeed,
302 ingesting 80 g of carbohydrate facilitated a positive net arterio-venous carnitine
303 balance across the forearm[44] which, assuming the majority of the measured plasma
304 carnitine extraction across the forearm occurred into skeletal muscle, equated to a
305 whole body muscle accumulation of 390 µmol and aligned well with the 370 µmol
306 (60 mg) carnitine retention predicted from differences in urinary carnitine excretion in
307 previous studies[48]. Extended to a chronic feeding scenario, this would equate to a
308 daily increase in muscle carnitine content of 50 $\mu mol \cdot (kg\ dm)^{-1}$, which would
309 augment muscle total carnitine content stores (~20 $mmol \cdot (kg\ dm)^{-1}$) by 22% over 12
310 weeks. Again, this extrapolation is in good agreement with the 21% increase in
311 muscle carnitine content reported by Stephens *et al*[49]. However, such a large

carbohydrate load *per se* (160 g·d^{-1}) will likely affect metabolism and alter body composition[49] and so investigation into alternative oral insulinogenic formulations that can stimulate muscle carnitine accumulation using lower carbohydrate loads is warranted. For example, whey protein has previously been fed with carbohydrate to promote insulin-mediated muscle creatine retention[50] and, unlike carbohydrate, prolonged protein supplementation is less likely to influence body fat content[51]. Unpublished data from our lab would suggest that daily feeding of 3 g of L-carnitine in combination with a beverage containing 44 g of carbohydrate and 13 g of protein results in around a 20% increase in the muscle carnitine pool over 24 weeks (Chee *et al*, unpublished results).

Insert Table 1 here

Does increasing muscle carnitine content reduce the inhibition of fat oxidation during high intensity exercise?

Consistent with the hypothesis that free carnitine availability is limiting to CPT1 flux and fat oxidation, the increase in muscle total carnitine content in the study of Wall *et al*[47] equated to an 80% increase in free carnitine availability during 30 minutes of exercise at 50% VO_2max compared to control and resulted in a 55% reduction in muscle glycogen utilisation. Furthermore, this was accompanied by a 30% reduction in PDC activation status during exercise, suggesting that a carnitine mediated increase in fat derived acetyl-CoA inhibited PDC and muscle carbohydrate oxidation. Put another way, L-carnitine supplementation increased muscle free carnitine concentration during exercise at 50% VO_2max from 4.4 to 5.9 mmol·l^{-1} intracellular water, equating to an intra-mitochondrial concentration of 0.44 and 0.59 mmol·l^{-1}, which is below and above the K_m of CPT1 for carnitine, respectively[19]. On the other hand, during 30 min of exercise at 80% VO_2max with increased muscle total carnitine content the same apparent effects on fat oxidation were not observed[47]. In contrast, there was greater PDC activation (40%) and flux (16% greater acetylcarnitine), resulting in markedly reduced muscle lactate accumulation in the face of similar rates of glycogenolysis compared to control. This suggested that free carnitine availability was limiting to PDC flux during high intensity exercise and that increasing muscle carnitine content resulted in a greater acetyl-CoA buffering capacity and better

345 matching of glycolytic to PDC flux. The study by Wall *et al*[47] also demonstrated a
346 remarkable improvement in exercise performance in all participants (11% mean
347 increase) during a 30-minute cycle ergometer time trial in the carnitine loaded state.
348 This is consistent with animal studies reporting a delay in fatigue development by
349 25% during electrical stimulation in rat soleus muscle strips incubated in carnitine *in*
350 *vitro*[52]. Whether these improvements in endurance performance are due to glycogen
351 sparing as a result of a carnitine mediated increase in fat oxidation, or the reduced
352 reliance on non-oxidative ATP production from carbohydrate oxidation (increase
353 acetyl group buffering and reduced lactate accumulation) requires further
354 investigation, but due to the high intensity nature of the time trial the latter would
355 appear more likely. Nevertheless, the long-held belief that carnitine supplementation
356 can improve endurance performance via augmenting its role in fat oxidation should be
357 revised to place more emphasis on the major role that carnitine plays in carbohydrate
358 metabolism during exercise.
359
360 **Does increasing muscle carnitine content affect muscle metabolism during very**
361 **high intensity exercise?**
362 During very high intensity exercise (>100% VO_2max), where there is a negligible
363 contribution of fat oxidation to energy expenditure, there is a plateau in muscle
364 acetyl-group accumulation that is associated with a greatly accelerated rate of lactate
365 production[39,53]. This increasing proportion of glycolytic flux that is directed towards
366 lactate formation is often cited as a key component in the development of fatigue
367 during very intense exercise[54]. Under these conditions it is unclear whether free
368 carnitine availability is limiting to PDC flux and mitochondrial ATP resynthesis, or
369 simply that TCA cycle flux and PDC flux (i.e. mitochondrial capacity) are maximal.
370 This is most evident during repeated bouts of very high intensity exercise, where
371 acetylcarnitine accumulation during the first bout of exercise prevents further acetyl
372 group buffering during subsequent bouts due to reduced free carnitine availability,
373 which results in an increased reliance on phosphocreatine degradation (indicative of a
374 declining contribution from mitochondrial ATP delivery) and ultimately fatigue[55].
375 Thus, if carnitine availability is indeed limiting to PDC flux during intense, repeated-
376 bout exercise, it is plausible that increasing skeletal muscle carnitine availability as
377 in[47] during a prolonged period of high intensity interval (i.e. repeated bout) training
378 (HIIT) could influence the adaptations to this type of training. However, we have

recently demonstrated that although increasing skeletal muscle free carnitine content via 24 weeks of twice daily L-carnitine (1.36 g) and carbohydrate (80 g) feeding increased the capacity to buffer excess acetyl groups during a single 3 min bout of high-intensity exercise at 100% VO_2max (as evidenced by a reduced muscle phosphocreatine degradation), it did not further influence skeletal muscle metabolism during a repeated bout, nor improve training-induced changes in muscle metabolism, VO_2max, or work output over 24 weeks of HIIT[55]. This would suggest that either acetylcarnitine formation, or PDC flux, is not limiting to mitochondrial ATP delivery during repeated bouts of exercise of this duration and intensity, or that the adaptations to HIIT outweighed any benefit of increasing free carnitine availability. Indeed, following 24 weeks of HIIT non-mitochondrial ATP production and acetylcarnitine accumulation during a second bout of exercise were blunted to such an extent that they were lower than the pre-training first bout, suggesting a better matching of PDC and TCA cycle flux during repeated bouts of exercise and a lower dependence on mitochondrial acetyl-group buffering.

Conclusion

In conclusion, it appears that skeletal muscle carnitine availability influences fuel selection during exercise. Carnitine availability appears limiting to the CPT1 reaction, such that during high intensity exercise a carbohydrate flux mediated decline in free carnitine is paralleled by a decline in the rate of fat oxidation. Indeed, nutritionally increasing skeletal muscle carnitine content increases fat oxidation during low intensity exercise. However, increasing carnitine availability during high intensity exercise does not offset the decline in fat oxidation, but facilitates a better matching of carbohydrate flux by buffering excess acetyl groups from the PDC reaction. This, the long-held belief that carnitine supplementation can improve endurance performance via augmenting its role in fat oxidation should be revised to place more emphasis on the major role that carnitine plays in carbohydrate metabolism during exercise, particularly as increasing muscle carnitine content can improve high intensity exercise performance and most athletes compete at high exercise intensities. However, caution should be taken as nutritionally increasing carnitine content appears to have little effect during repeated bouts of exercise at very high intensities or adaptation to HIIT training, likely because oxidative metabolism is already maximal.

Acknowledgements

I would like to acknowledge all of my co-authors of the manuscripts referenced in the present review.

Financial support

There was no financial support for the production of this review.

Conflicts of interest

Francis Stephens is a scientific advisor to Beachbody Inc., USA.

References

1. Romijn JA, Coyle EF, Sidossis LS *et al.* (1993) Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* **265**, E380-91.

2. van Loon LJ, Greenhaff PL, Constantin-Teodosiu D *et al.* (2001) The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* **536**, 295-304.

3. Achten J, Gleeson M, Jeukendrup AE (2002) Determination of the exercise intensity that elicits maximal fat oxidation. *Med Sci Sports Exerc* **34**, 92-7.

4. Bergstrom J, Hermansen L, Hultman E *et al.* (1967) Diet, muscle glycogen and physical performance. *Acta Physiol Scand* **71**, 140-50.

5. Johnson RH, Walton JL, Krebs HA *et al.* (1969) Metabolic fuels during and after severe exercise in athletes and non-athletes. *Lancet* **2**, 452-455.

6. Roepstorff C, Halberg N, Hillig T *et al.* (2005) Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. *Am J Physiol* **288**, E133-142.

7. Sidossis LS & Wolfe RR (1996) Glucose and insulin-induced inhibition of fatty acid oxidation: the glucose-fatty acid cycle reversed. *Am J Physiol* **270**, E733-8.

8. Coyle EF, Jeukendrup AE, Wagenmakers AJ *et al.* (1997) Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. *Am J Physiol* **273**, E268-275.

9. Romijn JA, Coyle EF, Sidossis LS *et al.* (1995) Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J Appl Physiol* **79**, 1939-1945.

10. Bavenholm PN, Pigon J, Saha AK *et al.* (2000) Fatty acid oxidation and the regulation of malonyl-CoA in human muscle. *Diabetes* **49**, 1078-1083.

11. Rasmussen BB, Holmback UC, Volpi E *et al.* (2002) Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. *J Clin Invest* **10**, 1687-1693.

12. Saha AK, Kurowski TG, Ruderman NB (1995) A malonyl-CoA fuel-sensing mechanism in muscle: effects of insulin, glucose, and denervation. *Am J Physiol* **269**, E283-289.

13. Odland LM, Heigenhauser GJ, Lopaschuk GD *et al.* (1996) Human skeletal muscle malonyl-CoA at rest and during prolonged submaximal exercise. *Am J Physiol* **270**, E541-544.

14. Odland LM, Heigenhauser GJ, Wong D *et al.* (1998) Effects of increased fat availability on fat-carbohydrate interaction during prolonged exercise in men. *Am J Physiol* **274**, R894-902.

15. Dean D, Daugaard JR, Young ME *et al.* (2000) Exercise diminishes the activity of acetyl-CoA carboxylase in human muscle. *Diabetes* **49**, 1295-1300.

16. Fritz IB & Marquis NR (1965) The role of acylcarnitine esters and carnitine palmityltransferase in the transport of fatty acyl groups across mitochondrial membranes. *Proc Natl Acad Sci* **54**, 1226-1233.

17. McGarry JD & Brown NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem* **244**, 1-14.

18. Cederblad G, Lindstedt S, Lundholm K (1974) Concentration of carnitine in human muscle tissue. *Clin Chim Acta* **53**, 311-321.

19. McGarry JD, Mills SE, Long CS *et al.* (1983) Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem J* **214**, 21-28.

20. Fraser F & Zammit VA (1998) Enrichment of carnitine palmitoyltransferases I and II in the contact sites of rat liver mitochondria. *Biochem J* **329**, 225-229.

21. Idell-Wenger JA, Grotyohann LW *et al.* (1978) Coenzyme A and carnitine distribution in normal and ischemic hearts. *J Biol Chem* **253**, 4310-4318.

22. Childress CC & Sacktor B (1966) Pyruvate oxidation and the permeability of mitochondria from blowfly flight muscle. *Science* **154**, 268-270.

23. Foster CV & Harris RC (1992) Total carnitine content of the middle gluteal muscle of thoroughbred horses: normal values, variability and effect of acute exercise. *Equine Vet J* **24**, 52-7.

24. Harris RC, Foster CV, Hultman E (1987) Acetylcarnitine formation during intense muscular contraction in humans. *J Appl Physiol* **63**, 440-442.

25. Carlin JI, Harris RC, Cederblad G *et al.* (1990) Association between muscle acetyl-CoA and acetylcarnitine levels in the exercising horse. *J Appl Physiol* **69**, 42-45.

26. Sahlin K (1990) Muscle carnitine metabolism during incremental dynamic exercise in humans. *Acta Physiol Scand* **138**, 259-262.

27. Constantin-Teodosiu D, Carlin JI *et al.* (1991) Acetyl group accumulation and pyruvate dehydrogenase activity in human muscle during incremental exercise. *Acta Physiol Scand* **143**, 367-372.

28. Constantin-Teodosiu D, Cederblad G *et al.* (1992) PDC activity and acetyl group accumulation in skeletal muscle during prolonged exercise. *J Appl Physiol* **73**, 2403-2407.

29. Campbell-O'Sullivan SP, Constantin-Teodosiu D, Peirce N *et al*. (2002) Low intensity exercise in humans accelerates mitochondrial ATP production and pulmonary oxygen kinetics during subsequent more intense exercise. *J Physiol* **538**, 931-939.

30. Watt MJ, Heigenhauser GJ, Dyck DJ *et al*. (2002) Intramuscular triacylglycerol, glycogen and acetyl group metabolism during 4 h of moderate exercise in man. *J Physiol* **541**, 969-978.

31. Seiler SE, Koves TR, Gooding JR *et al*. (2015) Carnitine acetyltransferase mitigates metabolic inertia and muscle fatigue during exercise. *Cell Metab* **22**, 65-76.

32. Putman CT, Spriet LL, Hultman E *et al.* (1993) Pyruvate dehydrogenase activity and acetyl group accumulation during exercise after different diets. *Am J Physiol* **265**, E752-760.

33. Kelley DE & Mandarino LJ (2000) Fuel selection in human skeletal muscle in insulin resistance: a re-examination. *Diabetes* **49**, 677-683.

34. Muoio DM, Noland RC, Kovalik JP *et al.* (2012) Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. *Cell Metab* **15**, 764–777.

35. Lindeboom L Nabuurs CI, Hoeks J *et al*. (2014) Long–echo time MR spectroscopy for skeletal muscle acetylcarnitine detection. *Clin Invest* **124**, 4915-25.

36. Stephens FB, Constantin-Teodosiu D, Laithwaite D *et al.* (2006) Skeletal muscle carnitine accumulation alters fuel metabolism in resting human

skeletal muscle. *J Clin Endocrinol Metab* **91**, 5013-5018.

37. Constantin-Teodosiu D, Constantin D, Stephens FB *et al.* (2012) The role of FOXO and PPAR transcription factors in diet-mediated inhibition of PDC activation and carbohydrate oxidation during exercise in humans and the role of pharmacological activation of PDC in overriding these changes. *Diabetes* **61**, 1017-1024.

38. Tsintzas K, Chokkalingam K, Jewell K et al. (2007) Elevated free fatty acids attenuate the insulin-induced suppression of PDK4 gene expression in human skeletal muscle: potential role of intramuscular long-chain acyl-coenzyme A. *J Clin Endocrinol Metab* **92**, 3967-72.

39. Stephens FB, Constantin-Teodosiu D & Greenhaff PL (2007) New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. *J Physiol* **581**, 431-44.

40. Boushel R, Gnaiger E, Schjerling P *et al.* (2007) Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. *Diabetologia* **50**, 790-796.

41. Wächter S, Vogt M, Kreis R *et al.* (2002) Long-term administration of L-carnitine to humans: effect on skeletal muscle carnitine content and physical performance. *Clin Chim Acta* **318**, 51-61.

42. Stephens FB, Constantin-Teodosiu D, Laithwaite D *et al.* (2006a) Insulin stimulates L-carnitine accumulation in human skeletal muscle. *FASEB J* **20**, 377-379.

43. Soop M, Bjorkman O, Cederblad G *et al.* (1988) Influence of carnitine supplementation on muscle substrate and carnitine metabolism during exercise. *J Appl Physiol* **64**, 2394-2399.

588 44. Shannon CE, Nixon AV, Greenhaff PL *et al.* (2016) Protein ingestion acutely
589 inhibits insulin-stimulated muscle carnitine uptake in healthy young men. *Am*
590 *J Clin Nutr* **103**, 276-82.
591
592 45. Schooneman MG, Ten Have GA, Van Vlies N *et al.* (2015) Transorgan fluxes
593 in a porcine model reveal a central role for liver in acylcarnitine metabolism.
594 *Am J Physiol Endocrinol Metab* **209**, E256-64.
595
596 46. Stephens FB, Constantin-Teodosiu D, Laithwaite D *et al.* (2007) A threshold
597 exists or the stimulatory effect of insulin on plasma L-carnitine clearance in
598 humans. *Am J Physiol Endocrinol Metab* **292**, E637-E641.
599
600 47. Wall BT, Stephens FB, Constantin-Teodosiu D *et al.* (2011) Chronic oral
601 ingestion of L-carnitine and carbohydrate increases muscle carnitine content
602 and alters muscle fuel metabolism during exercise in humans. *J Physiol* **589**,
603 963-973.
604
605 48. Stephens FB, Evans CE, Constantin-Teodosiu D *et al.* (2007b) Carbohydrate
606 ingestion augments L-carnitine retention in humans. *J Appl Physiol* **102**, 1065-
607 70.
608
609 49. Stephens FB, Wall BT, Marimuthu K *et al.* (2013) Skeletal muscle carnitine
610 loading increases energy expenditure, modulates fuel metabolism gene
611 networks and prevents body fat accumulation in humans. *J Physiol* **591**, 4655-
612 66.
613
614 50. Steenge GR, Simpson EJ, Greenhaff PL (2000) Protein- and carbohydrate-
615 induced augmentation of whole body creatine retention in humans. *J Appl*
616 *Physiol* **89**, 1165-71.
617
618 51. Cermak NM, Res PT, de Groot LC *et al.* (2012) Protein supplementation
619 augments the adaptive response of skeletal muscle to resistance-type exercise
620 training: a meta-analysis. *Am J Clin Nutr* **96**, 1454-64.
621

52. Brass EP, Scarrow AM, Ruff LJ *et al.* (1993) Carnitine delays rat skeletal muscle fatigue in vitro. *J Appl Physiol* **75**, 1595-1600.

53. Harris RC & Foster CV (1990) Changes in muscle free carnitine and acetylcarnitine with increasing work intensity in the Thoroughbred horse. *Eur J Appl Physiol* **60**, 81-85.

54. Gladden LB (2004) Lactate metabolism: a new paradigm for the third millennium. *J Physiol* **558**, 5-30.

55. Shannon CE, Ghasemi R, Greenhaff PL *et al.* (2017) Increasing skeletal muscle carnitine availability does not alter the adaptations to high-intensity interval training. *Scand J Med Sci Sports*. Published online 27 March 2017. doi:10.1111/sms12885.

638 **Tables**

639

640 **Table 1.** A summary of studies that have measured muscle carnitine content or
641 balance following L-carnitine administration

Reference	Dose of L-carnitine	Combined with insulin?	Change in muscle carnitine	Effect on metabolism
Shannon *et al.*[44]	1 x 3 g orally	No	No net uptake across forearm	Not measured
Shannon *et al.*[44]	1 x 3 g orally	Ingested 1 hour before 80 g of maltodextrin	Approx. 0.25%/day increase across forearm	Not measured
Shannon *et al.*[44]	1 x 3 g orally	Ingested 1 hour before 40 g of maltodextrin + 40 g of whey protein	No net uptake across forearm	Not measured
Soop *et al.*[43]	1 x 5 g/day orally for 5 days	No	Net release of carnitine from the leg	No
Stephens *et al.*[42]	Approx. 5 g intravenously over 5 hours	6 hours intravenous infusion of insulin to achieve fasting (5 mU/l) serum insulin concentration	No change in *vastus lateralis* total carnitine content	No
Stephens *et al.*[42]	Approx. 5 g intravenously over 5 hours	6 hours intravenous infusion of insulin to	13% increase in *vastus lateralis* total carnitine	Not measured

		achieve 180 mU/l serum insulin concentration	content	
Stephens *et al.*[46]	Approx. 5 g intravenously over 5 hours	6 hours intravenous infusion of insulin to achieve >50 mU/l serum insulin concentration	10% increase in whole body plasma carnitine clearance vs. 5 mU/l serum insulin	Not measured
Stephens *et al.*[36]	Approx. 5 g intravenously over 5 hours	6 hours intravenous infusion of insulin to achieve 180 mU/l serum insulin concentration	15% increase in *vastus lateralis* total carnitine content	Inhibition of PDC and diversion of carbohydrate flux to storage as glycogen
Wächter *et al.*[41]	2 x 2 g/day orally for 3 months	No	No change in *vastus lateralis* total carnitine content	No
Stephens *et al.*[49]	2 x 1.36 g/day orally for 12 weeks	Ingested with 80 g of high molecular weight glucose polymer	20% increase in *vastus lateralis* total carnitine content	Increase in energy expenditure at 50% VO_2max.
Wall *et al.*[47]	2 x 1.36 g/day orally for 24 weeks	Ingested with 80 g of high molecular	20% increase in *vastus lateralis* total	Sparing of muscle glycogen at

		weight glucose polymer	carnitine content	50% VO_2max. Better matching of carbohydrate to TCA cycle flux at 80% VO_2max. Increase in cycling time trial performance.
Shannon *et al.*[55]	2 x 1.36 g/day orally for 24 weeks in combination with HIIT training	Ingested with 80 g of maltodextrin	30% increase in *vastus lateralis* free carnitine content vs. placebo	Increase in efficiency at VO_2max. No adaptation to HIIT training.
Chee *et al.* unpublished.	1 x 3 g/day orally for 25 weeks	Ingested with nutritional supplement containing 44 g of maltodextrin and 13 g of whey protein	20% increase in *vastus lateralis* total carnitine content	20% increase in fat oxidation at 50% VO_2max.

642

Figures and Legends

Figure 1. A schematic diagram of the roles of carnitine within the context of skeletal muscle fuel metabolism for ATP resynthesis during exercise. Carnitine's role in long-chain fatty acid (acyl group) translocation into the mitochondrial matrix, for subsequent β-oxidation is highlighted in red, whereas the role of carnitine as a buffer of excess acetyl-CoA production and as a stockpile of acetyl groups is highlighted in blue. PDC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle; CAT, carnitine acetyltransferase; CACT, carnitine acylcarnitine translocase; CPT, carnitine palmitoyltransferase.