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It's well and truly time to stop stating that AMPK regulates glucose uptake and fat oxidation *during* exercise

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Running title: Does AMPK really regulate exercise metabolism?

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AMPK and exercise

In the late 1990s it was shown *in vivo* that skeletal muscle AMP-activated protein kinase (AMPK) activity is increased during exercise whilst pharmacological activation of AMPK could also increase skeletal muscle glucose uptake and free fatty acid (FFA) oxidation *in vivo* (22, 37). Taken together, these findings provided the impetus for the multitude of subsequent studies that have been undertaken to elucidate the role of AMPK in skeletal muscle metabolism. AMPK is a αβγ heterotrimer comprised of two α, two β, and three γ subunits (8). The α-subunits contain the catalytic domain responsible for activation which, during contraction, occurs via an increase in cellular stress due to increases in AMP_{free} and ADP_{free} levels. The increase in AMP_{free} allosterically activates AMPK, makes AMPK more susceptible to phosphorylation by upstream AMPK kinase(s), and the increase in AMP_{free} and ADP_{free} antagonizes the effect of protein phosphatases on AMPK, reducing the likelihood of dephosphorylation of AMPK (8, 40). AMPK is activated during moderate or harder exercise in humans (3, 6, 39) and there is evidence that AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC)-β which is then thought to increase fat oxidation (37). However, AMPK cannot simultaneously act as a primary regulator of both glucose uptake and FFA oxidation during exercise because there are situations (e.g. increases in exercise intensity, pre- vs. post-training exercise, high vs. low carbohydrate diets) whereby increases in glucose uptake are accompanied by simultaneous decreases in fat oxidation, and vice-versa. Indeed, below I will discuss evidence that AMPK actually does not regulate either glucose uptake or fat oxidation during exercise.

AMPK is not required for skeletal muscle glucose uptake during exercise

Support for a role of AMPK in the regulation of skeletal muscle glucose uptake were initially derived from pharmacological studies whereby the cell permeable agent 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMP mimetic, was shown to increase AMPK activity, Glut-4 translocation and glucose uptake (16, 22). However, it is important to note that whilst AICAR and contraction both activate AMPK and increase glucose uptake, this appears to be mediated via different pathways. While skeletal muscle glucose uptake in response to hypoxia/AICAR appears to be highly dependent on AMPK (7, 13, 25), as explained below there is evidence that this is not the case for contraction-stimulated glucose uptake. Thus, attempting to compare between AICAR, hypoxia, and contraction studies in relation to AMPK and glucose uptake can be problematic.

Although skeletal muscle AMPK α2 activity activation and glucose uptake go hand in hand with increases in exercise intensity in humans (2) and rats (27), one cannot simply assume that this indicates a cause and effect relationship. Indeed, most human and rodent studies have found clear dissociations between skeletal muscle AMPK activation and glucose uptake. Already in 2002 Wojtaszewski et al. (38) found increases in leg glucose uptake during the first hour of cycling at ~45% VO_{2peak}, yet muscle AMPK α2 activity and AMPKαThr172 phosphorylation only significantly increased at the end of exercise (~3.5 hours). In addition, glucose uptake at the end of exercise was actually lower than earlier in exercise (38). Also, whole-body glucose uptake and skeletal muscle AMPK

activity increase 8-10 fold during prolonged cycling exercise at ~66% VO_2 peak in untrained humans, but after just 10 days of exercise training there is no measurable increase in AMPK activity at all during the same absolute intensity of exercise in these individuals (19). The exercise training did attenuate the increase in glucose uptake during prolonged exercise, but it was still substantial, so if AMPK was playing a role one would expect some increase in skeletal muscle AMPK activity but none occurred (19). Similarly, there is no activation of skeletal muscle AMPK during 120 min of exercise at ~65% VO_2 peak in well trained endurance athletes (McConell et al. in review), despite substantial glucose uptake at this intensity in well trained individuals (34). It should be noted though that there may have been activation of some subunits of AMPK that were missed when only total AMPK activity is measured. Indeed, although increases in AMPK activity that occur before exercise training are totally nonexistent during exercise at the same relative intensity (~65% VO_2 peak) after 12 weeks of exercise training, there is still 20% of the pre-training increase in the AMPKα2β2γ3 trimer of AMPK during such exercise (24).

Whole-body deletion of AMPK α1 or α2 fails to inhibit contraction-stimulated glucose uptake *in vitro* (13), whilst muscle glucose uptake during *ex vivo* contraction is either normal or only partially reduced in mice expressing a dominant-negative AMPK α2 isoform (7, 23, 25, 35)). When interpreting these findings one should consider the potential confounding effects of knocking-out or suppressing AMPK or LKB1, such as alterations and/or compensation of enzymes upstream and/or downstream. For example, knocking-out AMPK α2 results in compensatory increases in AMPK α1 expression in

mouse skeletal muscle (15), whilst skeletal muscle of the AMPK α2 dominant-negative mouse has attenuated force generation *ex vivo* (7, 25). Importantly, muscle-specific AMPKα1α2 double knockout (mdKO) mice, which have only 3-4% of normal AMPK α2 activity and no activation of AMPK during exercise, have normal increases in skeletal muscle glucose uptake during exercise at the same relative intensity (14).

Some findings over the years have provided small indications that AMPK may play a role in glucose uptake with contraction in rodents but it is important to consider that these situations are often non physiological. For example, it was shown that glucose uptake was lower during ex vivo twitch contractions in AMPKα1 KO mouse muscle than wild type mouse muscle and it was suggested that this may indicate that AMPKα1 plays a role in glucose uptake during short-duration low intensity exercise (11). But AMPKα1 is usually not activated during short-duration low-moderate intensity exercise (40-70% VO_2 max) (6, 11, 26, 38, 39).

A recent transgenic mouse study by Kjobsted et al. (14) convincingly showed that AMPK is not required for glucose uptake during contraction/exercise but rather is important for metabolism after exercise. Using muscle specific AMPKα1α2 double KO and inducible muscle specific AMPKα1α2 double KO mice, that have extremely low AMPK activity, they showed that glucose uptake was normal during ex vivo and in situ contraction. They also mentioned in the discussion ("unpublished observations") that glucose uptake is normal in these mice during exercise (14). The authors said "We cannot ultimately rule out that AMPK may be necessary for regulating muscle glucose uptake during longer

periods of exercise (>30 min) and contraction (>10 min) where the myocellular stress may be further elevated" (14). However, as mentioned above, dissociations between glucose uptake and AMPK activation have been observed in humans during exercise lasting up to 3.5 hours (19, 38).

An important point made by the authors of the recent rodent study (14) was that of the 10 studies conducted previously that found impaired glucose uptake with contractions in AMPK-deficient mouse muscle, 8 of these actually examined the glucose uptake after, rather than during the contractions. As we pointed out previously in regards to a similar situation in studies examining the role of nitric oxide in glucose uptake with contraction (18), such study designs more investigate the effect of the intervention on glucose uptake after, than during, exercise.

Therefore, it is clear from both mouse and human studies that AMPK is not required for glucose uptake during exercise. It is not possible however to discount it altogether however because there is likely redundancy in signalling to skeletal muscle glucose uptake during exercise.

AMPK is not required for skeletal muscle fat oxidation during exercise

In skeletal muscle, malonyl-CoA is believed to be a major factor involved in the regulation of FFA oxidation due to its inhibitory effects on carnitine palmitoyltranferase (CPT) 1, the main enzyme controlling fatty acid flux into the mitochondria for oxidation. Malonyl-CoA is synthesized by ACC-β, and skeletal muscle contraction and AMPK activation phosphorylates ACC-β at a serine residue (212 in rodents, 221 in humans) in skeletal muscle (2, 8, 31), and *in vitro* this phenomenon inhibits ACC-β (37). This has led

to the assumption that the activation of AMPK during exercise increases FFA oxidation due to AMPK phosphorylating and inhibiting ACC-β and thus reducing malonyl-CoA and removing the inhibition of CPT1. However, as with glucose uptake, many dissociations have been observed between AMPK activation, ACC-β phosphorylation, ACC-β activity and FFA oxidation during contraction and exercise.

Low intensity exercise performed for ~3.5 hours in humans progressively increases skeletal muscle net FFA uptake and fat oxidation, despite the fact that ACC-β phosphorylation is only increased at 1hr, and AMPK activity and is only elevated at the end of exercise (38). Similarly, low intensity contraction increases FFA oxidation in rat skeletal muscle despite no alterations in AMPK or ACC-β activity (32). It has also been shown in humans that increasing exercise from moderate- to high-intensity results in a suppression of fat oxidation, but AMPK activity and ACC-β phosphorylation both increase (2) and ACC activity decreases (4). Furthermore, 10 days of exercise training in previously untrained individuals augments fat oxidation during prolonged exercise, yet skeletal muscle AMPK activity was not increased at all and ACC-β phosphorylation only increased slightly unlike larger increases during exercise before training (19).

AMPK α2 DN mice, despite having very low AMPK activity, have normal increases in FAT/CD36 translocation to the plasma membrane (12), palmitate uptake (12) and fat oxidation (5) during contraction/exercise. Surprisingly, they also have normal increases in ACCβ phosphorylation (5) and reductions in malonyl CoA (5) during contraction. It has also been shown in perfused rat hindlimbs that fat uptake increases within one minute

of in situ contractions but AMPK is not activated until later (12). A study that did find a reduction in fat oxidation during ex vivo contraction of muscle specific AMPK a1α2 KO mice also found that these mice had reduced CD36 and FABPpm, important for fatty acid uptake and oxidation, which likely accounted for these findings (15). Finally, there is evidence from skeletal muscle ACCβ Ser 212 knock in mice that ACCβ is important for AICAR-activated fatty acid oxidation at rest (30), but ACCβ is not required for fatty acid oxidation during ex vivo contraction or during treadmill exercise (29). Therefore, transgenic models support the human findings that there are dissociations between AMPK and fat oxidation during contractions and exercise.

Conclusions

In conclusion, although activation of AMPK during exercise appears to be critical for adaptations after exercise (10, 14, 17, 21, 28), AMPK activation is not necessary for increases in glucose uptake or fat oxidation during exercise. In 2005 George Brooks wrote "Now, with the results of McConell *et al.* (19). ….. it is time to reassess the relevance of the AMPK signalling pathway for the regulation of metabolism in working human muscle" (1). Despite this, 14 years later most papers related to AMPK still state in the introduction that AMPK regulates glucose uptake and fat oxidation during exercise. Although redundancy likely exists, it really is time to move on and look more closely at other possible regulators of glucose uptake and fat oxidation during exercise such as nitric oxide (20, 33), Rac1 (33, 36) and ROS production (9, 33).

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