Skeletal muscle AMPK is not activated during 2 h of moderate intensity exercise at \( \sim 65\% \dot{V}_{O_2}\text{peak} \) in endurance trained men

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**Key points**
- AMP-activated protein kinase (AMPK) is considered a major regulator of skeletal muscle metabolism during exercise.
- However, we previously showed that, although AMPK activity increases by 8–10-fold during \( \sim 120 \) min of exercise at \( \sim 65\% \dot{V}_{O_2}\text{peak} \) in untrained individuals, there is no increase in these individuals after only 10 days of exercise training (longitudinal study).
- In a cross-sectional study, we show that there is also a lack of activation of skeletal muscle AMPK during 120 min of cycling exercise at 65\% \( \dot{V}_{O_2}\text{peak} \) in endurance-trained individuals.
- These findings indicate that AMPK is not an important regulator of exercise metabolism during 120 min of exercise at 65\% \( \dot{V}_{O_2}\text{peak} \) in endurance trained men.
- It is important that more energy is directed towards examining other potential regulators of exercise metabolism.

**Abstract**
AMP-activated protein kinase (AMPK) is considered a major regulator of skeletal muscle metabolism during exercise. Indeed, AMPK is activated during exercise and activation of AMPK by 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) increases skeletal muscle glucose uptake and fat oxidation. However, we have previously shown that, although AMPK activity increases by 8–10-fold during \( \sim 120 \) min of exercise at \( \sim 65\% \dot{V}_{O_2}\text{peak} \) in untrained individuals, there is no increase in these individuals after only 10 days of exercise training (longitudinal study). In a cross-sectional study, we examined whether there is also a lack of activation of skeletal muscle AMPK during 120 min of cycling exercise at 65\% \( \dot{V}_{O_2}\text{peak} \) in endurance-trained individuals. Eleven untrained (UT; \( \dot{V}_{O_2}\text{peak} = 37.9 \pm 5.6 \text{ ml.kg}^{-1} \text{ min}^{-1} \) )
Introduction

The signalling events that regulate skeletal muscle exercise metabolism have not been fully elucidated (Richter & Hargreaves, 2013). There is evidence for feed forward (calcium activated CaMK) (Wright et al. 2004; Wright et al. 2005; Jensen et al. 2007; Witzczak et al. 2010) and feedback (AMP-activated protein kinase; AMPK) (Hayashi et al. 1998; Mu et al. 2001; Lee-Young et al. 2009; Abbott et al. 2011) regulation being involved, as well as nitric oxide (Balan & Nadler, 1997; Roberts et al. 1997; Bradley et al. 1999; Inyard et al. 2007; Ross et al. 2007; Merry et al. 2010a) and reactive oxygen species production (Toyoda et al. 2004; Sandstrom et al. 2006; Merry et al. 2010a), which are increased during contraction (Roberts et al. 1999; Reid & Durham, 2002; Linden et al. 2011). There is also some evidence that cytoskeletal forces during contraction may signal glucose uptake via Rac1 (Sylow et al. 2013; Sylow et al. 2015).

Skeletal muscle AMPK activity increases during exercise in rodents (Winder & Hardie, 1996; Lee-Young et al. 2009) and humans (Chen et al. 2000; Fujiji et al. 2000; Wojtaszewski et al. 2000; Musi et al. 2001; Mortensen et al. 2013) and, given that activation of AMPK by 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) increases skeletal muscle glucose uptake and fat oxidation (Merrill et al. 1997; Hayashi et al. 2000; Jorgensen et al. 2004), it has been assumed that activation of AMPK during exercise increase fat and glucose metabolism in humans. However, a number of studies have shown dissociations between activation of AMPK and glucose uptake during muscle contraction in rodents (Jorgensen et al. 2004; Fujiji et al. 2005; Kjobstad et al. 2017) and during exercise in humans (Wojtaszewski et al. 2000; McConell et al. 2005; Mortensen et al. 2013). In addition, several studies have shown that fat oxidation increases normally during contraction (Jeppesen et al. 2011) and during exercise (Jeppesen et al. 2013) in AMPK dominant negative/kinase dead mice.

Further evidence that questions a role for AMPK in glucose and fat metabolism during exercise is that substantial increases in glucose uptake and fat oxidation are seen during low intensity exercise (40–45% $V_{O_2\text{peak}}$) in humans even though AMPK signalling is not increased at such intensities (Wojtaszewski et al. 2002; Chen et al. 2003). In addition, in a longitudinal study, we showed in previously untrained men that 10 days of exercise training abolishes the ~10-fold increase in AMPK α2 activity during exercise at 65% $V_{O_2\text{peak}}$ despite fat oxidation being higher and glucose disposal, although attenuated, still being substantially increased during exercise (McConell et al. 2005). We found no relationship between AMPK activation (and AMPK αThr172 phosphorylation and ACCβ phosphorylation) and muscle glycogen use, glucose uptake and fat oxidation during exercise (McConell et al. 2005). There is no doubt that AMPK activation during exercise is important for post-exercise adaptations (Winder et al. 2000; McGee et al. 2003), although these findings imply that AMPK activation during moderate intensity exercise is not necessary for normal increases in glucose uptake and fat oxidation during exercise.

It can be argued that there is less activation of AMPK after short-term exercise training because there is less of an energy deficit in skeletal muscle during exercise. Indeed, we found this to be the case in humans in our short-term training study reporting no activation of AMPK during exercise at 65% of pretraining $V_{O_2\text{peak}}$ (McConell et al. 2005). The exercise after training was conducted at the pre-exercise training workload; as such, the exercise was at a little lower relative workload after training (McConell et al. 2005). However, it has subsequently been shown in humans that, after 12 weeks of exercise training, there is less activation of skeletal muscle AMPK when the post training exercise was conducted at the same relative intensity of 65% $V_{O_2\text{peak}}$ as the pre-training exercise (Mortensen et al. 2013).

Another way of examining the effect of exercise training on AMPK activation is to conduct a cross-sectional study...
comparing endurance trained with untrained individuals exercising at the same relative intensity. Nielsen et al. (2003) found similar increases in AMPK activation during 20 min of exercise at 80% $\dot{V}_{O_2\text{peak}}$ in trained compared with untrained individuals. This was surprising because they and others have found less of a skeletal muscle energy imbalance and therefore less of an increase in skeletal muscle AMP and ADP at 80% $\dot{V}_{O_2\text{peak}}$ in trained compared with untrained men (Baldwin et al. 1999; Nielsen et al. 2003). It is not known whether skeletal muscle AMPK activity would increase during prolonged moderate intensity exercise in well trained individuals. This is important to determine because, if AMPK does not increase during such exercise, this would imply that AMPK is not important for the regulation of exercise metabolism during such exercise.

Therefore, the present study aimed to examine whether long-term endurance exercise trained individuals have an increase in skeletal muscle AMPK during 120 min of exercise at 65% $\dot{V}_{O_2\text{peak}}$. Based on our findings of no activation at this workload in previously untrained individuals after 10 days of exercise training (McConnell et al. 2005), we hypothesized that skeletal muscle AMPK would not be activated during 120 min of exercise at 65% $\dot{V}_{O_2\text{peak}}$ in endurance trained men.

**Methods**

**Ethical approval**

The present study was approved by the Human Research Ethics Committee of the University of Melbourne (Study number 040090) and conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

**Subjects**

Seven endurance-trained cyclists and triathletes (26 ± 2 years; 72 ± 4 kg; $\dot{V}_{O_2\text{peak}} = 4.4 ± 0.35$ L min$^{-1}$, mean ± SD) and eleven healthy but otherwise untrained (23 ± 3 years; 69 ± 9 kg; $\dot{V}_{O_2\text{peak}} = 2.6 ± 0.4$ L min$^{-1}$, mean ± SD) non-smoker males participated in the study (Table 1). Trained participants cycled on average 300 ± 100 km week$^{-1}$, whereas untrained participants undertook no regular exercise.

**Experimental design**

Participants were required to attend the laboratory on three separate occasions. The first visit involved a peak pulmonary oxygen consumption test during cycling ($\dot{V}_{O_2\text{peak}}$), followed 2–3 days later by a 30 min familiarisation ride at a workload calculated from the $\dot{V}_{O_2\text{peak}}$ test to be ~65% $\dot{V}_{O_2\text{peak}}$ to confirm the power output for the experimental trials. Approximately 1 week later, participants returned to the laboratory for an exercise trial, which involved cycling for 120 min at ~ 65% $\dot{V}_{O_2\text{peak}}$ (untrained; 100 ± 21 W; trained 190 ± 15) (Table 2).

| Parameter | Untrained | Trained |
|-----------|-----------|---------|
| Age (years) | 23 ± 3 | 26 ± 2 |
| Weight (kg) | 69 ± 9 | 72 ± 4 |
| Height (m) | 1.75 ± 0.35 | 1.76 ± 0.39 |
| BMI (kg m$^{-2}$) | 22 ± 2.6 | 23 ± 0.76 |
| $\dot{V}_{O_2\text{peak}}$ (L min$^{-1}$) | 2.60 ± 0.4* | 4.44 ± 0.35 |
| $\dot{V}_{O_2\text{peak}}$ (ml kg$^{-1}$ min$^{-1}$) | 37.9 ± 5.6* | 61.8 ± 2.2 |

Table 1. Subject characteristics

Values are the mean ± SD, n = 11 untrained and 7 exercise trained participants, BMI, body mass index, $\dot{V}_{O_2}$, oxygen consumption. *Significantly different to corresponding trained value ($P < 0.05$).

**Dietary and exercise controls**

All participants were asked to refrain from any formal exercise for 48 h prior to the experimental trial to minimise any acute exercise training effects and to avoid drinking alcohol or consumption of caffeine for 24 h prior. To ensure the energy intake was controlled between groups, participants were supplied with a diet to consume over the 24 h prior to each experimental trial containing ~199 kJ kg$^{-1}$ consisting of ~65% of carbohydrates, ~15% proteins and ~ 20% fats. Participants were instructed to adhere to the diet but to consume water *ad libitum* and to finish the food by 10 pm the evening prior to the experimental trial to enable attending the laboratory in a fasted state.

**Exercise trials**

On the morning of the exercise trial, a 22 gauge Teflon catheter (Optiva; Ethicon Endo-Surgery, Cincinnati, OH, USA) was inserted into an antecubital forearm vein for blood sampling. The exercise protocol consisted of cycling for 120 min at 65% of $\dot{V}_{O_2\text{peak}}$. Blood was sampled 10 min prior to the commencement of exercise and then every 30 min during exercise for the measurement of plasma glucose, lactate, insulin, glycerol and free fatty acids. Expired air was collected into Douglas bags every 30 min during exercise and heart rate (Polar Favor, Oulu, Finland) was recorded every 30 min during exercise. $\dot{V}_{O_2}$ and the respiratory exchange ratio were calculated from the expired air samples. Participants received 8 ml kg$^{-1}$ body weight of water at the start of exercise, followed by a further
2 ml kg\(^{-1}\) body weight every 15 min of exercise and were fan cooled throughout the trial. At rest and after 30 and 120 min of exercise, muscle was obtained from the vastus lateralis under local anaesthesia, using the percutaneous needle biopsy technique, with suction. Muscle samples were rapidly (8–12 s from stopping exercise) frozen and stored in liquid N\(_2\) for later analysis of AMPK \(\alpha_1\) and \(\alpha2\) activity, AMPK \(\alpha\)Thr\(^{172}\) phosphorylation and ACC\(\beta\) Ser\(^{223}\) phosphorylation and muscle metabolites.

**Analytical techniques**

**Blood.** Plasma glucose, lactate (Lowry OH, 1972) and glycerol (Chernick, 1969) were determined using an enzymatic fluorometric procedure, plasma non-esterified fatty acids (NEFA) by an enzymatic colorimetric method (NEFA-C test, Wako, Osaka, Japan) and plasma insulin using a human insulin-specific radioimmunoassay kit (Linco Research, St Charles, MO, USA).

**Muscle metabolites.** A portion of each muscle sample (\(\sim 20\) mg) was freeze-dried and subsequently crushed to a powder and any visible connective tissue was removed. The extraction of muscle glycogen commenced by incubating the sample in HCl before being neutralized with NaOH and subsequently analysed for glucosyl units using an enzymatic fluorometric method (Passonneau & Lauderdale, 1974). The metabolites (ATP, CrP, Cr and lactate) were extracted firstly with precooled perchloric acid/EDTA before the addition of precooled KHCO\(_3\) to the supernatant. The metabolites were analysed in triplicate using an enzymatic fluorometric method as reported by Harris et al. (1974). PCR, Cr and ATP were normalised to the participant’s highest total creatine (Cr + CrP). The concentration of ADP (ADP\(_{\text{free}}\)) and AMP (AMP\(_{\text{free}}\)) was calculated based on the near equilibrium nature of the CK and adenylate kinase reactions, respectively. ADP\(_{\text{free}}\) was calculated from the measured ATP, Cr, PCR levels and the estimated H\(^+\) concentration, which was calculated from a formula based on the muscle lactate content for dry muscle (Mannion et al. 1993). The observed equilibrium constant (\(K_{\text{obs}}\)) of 1.66 \(\times\) 10\(^9\) was used for creatine kinase (Lawson & Veech, 1979). An estimation of AMP\(_{\text{free}}\) was calculated from the measured ATP and estimated ADP\(_{\text{free}}\), using a \(K_{\text{obs}}\) of 1.05 for adenylate kinase (Lawson & Veech, 1979). Estimated ADP\(_{\text{free}}\) and AMP\(_{\text{free}}\) were expressed as \(\mu\)mol per kilogram of dry muscle mass (\(\mu\)mol kg\(^{-1}\) dry muscle).

**Immunoblotting.** Frozen skeletal muscle was homogenised in ice cold lysis buffer on ice [10 \(\mu\)l mg\(^{-1}\) tissue; 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 10\% v/v glycerol, 1\% v/v Triton X-100, 50 mM NaF, 5 mM Na\(_3\)P\(_2\)O\(_7\), 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 1 \(\mu\)l ml\(^{-1}\) trypsin inhibitor and 5 \(\mu\)l ml\(^{-1}\) protease inhibitor cocktail (P8340; Sigma, St Louis, MO, USA), incubated on ice for 20 min and centrifuged at 16 000 \(g\) for 20 min at 4\(^\circ\)C. The protein concentration of the supernatant was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) with BSA as the standard. All primary antibodies were diluted to a final concentration of 1:1000. Phosphospecific antibodies for AMPK \(\alpha\)Thr\(^{172}\) and ACC\(\beta\) Ser\(^{221}\) were purchased from Upstate Biotechnology (Lake Placid, NY, USA; catalogue no. 07–626 and 05–673, respectively). Polyclonal rabbit antibody specific for total AMPK \(\alpha\) protein was purchased from Cell Signalling Technology (Beverley, MA, USA; catalogue no. 2532). ACC\(\beta\) was detected using IRDye\(^{TM}\) 800-labelled streptavidin (Rockland, Gilbertsville, PA, USA; catalogue no. S000-45).

Skeletal muscle lysates (80 \(\mu\)g) were heated in SDS sample buffer and subjected to SDS-PAGE. Binding of purified proteins was detected by immunoblotting following an overnight incubation with primary antibody. Membranes were incubated in Odyssey anti-rabbit IRDye\(^{TM}\) 800- or anti-mouse IRDye\(^{TM}\) 700- labelled secondary antibody (Rockland, Gilbertsville, PA, USA), washed in PBS Tween 20 and were scanned for infra-red fluorescence using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). When both total protein and protein phosphorylation were measured, membranes were probed first for total protein, stripped of antibodies (2\% SDS in 25 mM glycine, pH 2.0) and re-probed with the anti-phospho antibody. Phosphorylation was expressed relative to the total protein of the specific protein of interest.

**AMPK activity.** Skeletal muscle lysates (50 \(\mu\)g) were combined with 15 \(\mu\)l of protein A sepharose beads (Pierce), bound to either AMPK \(\alpha_1\) (raised to the non-conserved region of the AMPK \(\alpha_1\) isoform, amino acid sequence 373–390 of rat AMPK \(\alpha_1\)) or AMPK \(\alpha_2\) (amino acid sequence 598.18

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**Table 2. Physiological responses during 120 min of steady-state exercise at \(\sim 65\% V_{\text{O2peak}}\)**

| Parameter          | Untrained | Trained |
|--------------------|-----------|---------|
| % of \(V_{\text{O2peak}}\) (ml.kg\(^{-1}\) min\(^{-1}\)) | 66 ± 4.5  | 65 ± 1.6 |
| Workload (watts)    | 100 ± 21* | 190 ± 15 |
| RER                | 0.91 ± 0.56 | 0.91 ± 0.38 |
| RPE                | 12.4 ± 1.2  | 11.0 ± 1.5 |
| Heart rate (beats min\(^{-1}\)) | 147 ± 9 | 143 ± 7 |

Data are the mean ± SD, \(n = 11\) untrained and 7 exercise trained participants. \(V_{\text{O2}}\), oxygen consumption, RER, respiratory exchange ratio, RPE, rating of perceived exhaustion. *Significantly different to corresponding trained value (\(P < 0.05\)).
Table 3. Measured and calculated muscle metabolites at rest (0 min), and after 30 min and 120 min of steady-state exercise at ~ 65% of $V_{O2peak}$

| Metabolite | Untrained | Trained | Untrained | Trained | Untrained | Trained |
|------------|-----------|---------|-----------|---------|-----------|---------|
| Lactate    | 4.6 ± 1.2 | 3.8 ± 2.1 | 70.1 ± 15.5 | 78.9 ± 13.7 | 38.5 ± 7.0 | 39.1 ± 6.1 | 0.64 ± 0.07 | 0.66 ± 0.07 |
| (mmol kg$^{-1}$ dm$^{-1}$) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| PCr        | 131 ± 36  | 109 ± 20 | 338 ± 239 | 228 ± 66 | 338 ± 239 | 228 ± 66 | 338 ± 239 | 228 ± 66 |
| (mmol kg$^{-1}$ dm$^{-1}$) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| Cr         | 2.6 ± 0.8 | 2.3 ± 0.8 | 4.1 ± 2.0  | 3.8 ± 2.0 | 4.1 ± 2.0 | 3.8 ± 2.0 | 4.1 ± 2.0 | 3.8 ± 2.0 |
| (mmol kg$^{-1}$ dm$^{-1}$) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| PCr:(PCr + Cr) | 0.6 ± 0.3 | 2.4 ± 1.5 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 |
| ATP        | 24.3 ± 2.2 | 24.1 ± 2.5 | 24.3 ± 3.5 | 24.3 ± 3.5 | 24.3 ± 3.5 | 24.3 ± 3.5 | 24.3 ± 3.5 | 24.3 ± 3.5 |
| (mmol kg$^{-1}$ dm$^{-1}$) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| ADPfree    | 131 ± 36  | 109 ± 20 | 338 ± 239 | 228 ± 66 | 338 ± 239 | 228 ± 66 | 338 ± 239 | 228 ± 66 |
| (μmol kg$^{-1}$ dm$^{-1}$) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| AMPfree    | 0.6 ± 0.3 | 2.4 ± 1.5 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 | 3.2 ± 2.6 |
| (μmol kg$^{-1}$ dm$^{-1}$) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| AMPfree:ATP| 0.04 ± 0.02 | 0.08 ± 0.03 | 0.56 ± 0.17 | 0.56 ± 0.17 | 0.56 ± 0.17 | 0.56 ± 0.17 | 0.56 ± 0.17 | 0.56 ± 0.17 |
| (Untrained) | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |
| (Trained)  | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ | $\dagger$ |

Values are the mean ± SD, $n = 11$ untrained and 7 exercise trained participants. PCr, creatine phosphate; Cr, creatine; ADPfree, free adenosine diphosphate; AMPfree, free adenosine monophosphate, dm, dry muscle. *Significantly different to corresponding trained value. §Main effect for time. †Main effect for untrained compared to trained ($P < 0.05$).

sequences 351–366 and 490–516 of rat AMPKα2 polyclonal antibodies (a gift from Professor Bruce Kemp, St Vincent’s Institute of Medical Research, Fitzroy, VIC, Australia) and incubated for 2 h at 4 °C. Immunocomplexes were washed in lysis buffer containing 0.5 M NaCl and resuspended in 25 μl of 0.05 M Tris buffer (pH 7.5). To commence the assay, 25 μl of reaction mixture containing (final concentrations) 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM $[^{32}P]ATP$ (~200 cpm/pmol; PerkinElmer Life and Analytical Sciences, Boston, MA, USA), 30 μM AMARA peptide (Upstate Biotechnology) and 200 μM AMP was added to each sample at 30°C for 20 min with agitation. Then, 40 μl of each sample was transferred onto P81 chromatography paper and washed 3 × 10 min in 75 mM H₃PO₄, once in 100% ethanol, and air dried. P81 paper was then placed in organic scintillation fluid (Opti-Fluor O; PerkinElmer) and radioactivity was counted on a β counter (PerkinElmer). AMARA peptide has the same AMPK phosphorylation site as ACCβ; therefore, AMPK activities were calculated as units of γ-$[^{32}P]$-ATP incorporated into the AMARA peptide [ACCα (73-87)A77] min$^{-1}$ mg$^{-1}$ total protein subjected to immunoprecipitation.

**Statistical analysis.** Data are expressed as the mean ± SD. Untrained and exercise trained group values were compared using a two-factor (training × time) repeated measures ANOVA and, if there was a significant interaction, the ANOVA was followed by a post hoc comparison using a least significant difference test. $P < 0.05$ was considered statistically significant.

**Results**

**Subjects**

There was no significant difference in age, weight, height or body mass index between the untrained and exercise trained groups (Table 1). However, as expected, $V_{O2peak}$ was significantly higher in the endurance trained group ($P < 0.05$) (Table 1) and the trained group cycled on average at almost twice the workload of the untrained group ($P < 0.05$) (Table 2). Importantly, the relative exercise intensity was the same between groups, with no significant difference for $V_{O2}$ (as a percentage of $V_{O2peak}$), heart rate or rating of perceived exhaustion (Table 2).

**Muscle metabolites**

Measured muscle lactate and Cr and estimated ADPfree, AMPfree and the AMPfree:ATP ratio all increased progressively with exercise in both groups (Table 3). The exercise induced increase in muscle lactate, Cr and AMPfree was attenuated in the exercise trained compared to the untrained group ($P < 0.05$) (Table 3). Skeletal muscle AMPfree increased ($P < 0.05$) 17-fold in the untrained group and significantly ($P < 0.05$) less (5-fold) in the endurance trained group following 120 min of exercise and the muscle lactate concentration increased by 5.9-fold in
the untrained and 3.1-fold in the well trained individuals \((P < 0.05)\) (Table 3). PCR and the PCr:(PCr+Cr) ratio decreased progressively in both groups, and this decrease was greater in untrained subjects \((\sim 26\% \text{ decrease in untrained, } \sim 44\% \text{ decrease in endurance trained groups, } P < 0.05)\) (Table 3). There was no difference in measured ATP levels during exercise or between groups \((P > 0.05)\) (Table 3).

Resting muscle glycogen levels were 93\% higher \((P < 0.05)\) in the exercise trained group, and remained higher than the untrained values at 30 min \((130\%, P < 0.05)\) and 120 min of exercise \((440\%, P < 0.05)\) (Fig. 1). However, net glycogen utilization during exercise was similar between the two groups.

**Plasma glucose lactate, insulin, glycerol and NEFA**

Plasma glucose concentration remained at a similar level during exercise in untrained and endurance trained subjects \((P > 0.05)\) (Fig. 2A). Plasma lactate was similar at rest in the two groups; however, it was elevated by \(\sim 110\%\) in the untrained group during exercise, which was significantly higher than the trained group \((P < 0.05)\) (Fig. 2B). Plasma insulin decreased progressively throughout exercise in both groups; however, fasting plasma insulin was 40\% higher in the untrained compared to the trained group at rest, and remained \(\sim 25\%\) higher than the trained values throughout 120 min of exercise \((P < 0.05)\) (Fig. 2C). Both plasma glycerol and NEFA increased progressively throughout exercise in both groups; however, NEFA levels were attenuated in the exercise trained group at 90 and 120 min \((P < 0.05)\) (Fig. 3).

**AMPK signalling**

Basal AMPK \(\alpha1\) activity was 90\% higher \((P < 0.05)\) in the exercise trained group, whereas there was no difference in basal AMPK \(\alpha2\) activity between the two groups (Fig. 4). AMPK \(\alpha1\) activity increased by 220\% and AMPK \(\alpha2\) activity increased by 370\% during exercise in the untrained
group at 120 min ($P < 0.05$). However, neither AMPK $\alpha_1$, nor AMPK $\alpha_2$ activity increased during exercise in the endurance trained group ($P > 0.05$) (Fig. 4). AMPK $\alpha$Thr$^{172}$ (115%, $P < 0.05$) phosphorylation and ACC$\beta$ Ser$^{222}$ phosphorylation (100%, $P < 0.05$) increased with exercise in the untrained group after 120 min of exercise (Fig. 5). However, there was no significant increase in AMPK $\alpha$Thr$^{172}$ phosphorylation or ACC$\beta$ Ser$^{222}$ phosphorylation during exercise in the trained group ($P > 0.05$) (Fig. 5).

**Discussion**

The results of the present study show that AMPK activity is not increased during prolonged steady-state, moderate intensity exercise in endurance trained individuals. Indeed, AMPK $\alpha_1$ and $\alpha_2$ activity was significantly elevated following 120 min of exercise in the untrained group (220% and 370%, respectively), whereas no increase in AMPK activity was observed during exercise in the trained participants (Fig. 4). Given that there is a substantial amount of glucose and fat oxidised during 120 min of exercise at 65% $\dot{V}_O_2$peak in endurance trained individuals (Romijn et al. 1993; van Loon et al. 2001), these results indicate that AMPK activation is not important for exercise metabolism under these circumstances.

Almost every study investigating AMPK states in their Introduction that AMPK regulates glucose uptake and fat oxidation during exercise, despite much evidence to the contrary. Indeed, our current findings are consistent with our previous results indicating that, after 10 days of exercise training, there was no increase from rest in skeletal muscle AMPK activity, AMPK $\alpha$Thr$^{172}$ phosphorylation or ACC$\beta$ phosphorylation during 120 min of exercise at 65% of pre-training $\dot{V}_O_2$peak despite substantial glucose disposal and higher fat oxidation during exercise after (compared to before) exercise training (McConnell et al. 2005). Other studies similarly report in humans that skeletal muscle AMPK activation is barely increased during exercise at 65% $\dot{V}_O_2$peak after 12 weeks of exercise training (Mortensen et al. 2013). Although not universally the case (Mu et al. 2001; Sakamoto et al. 2005; Lee-Young et al. 2009), many rodent studies have found that there are normal increases in glucose uptake and fat oxidation during contraction or during exercise in AMPK kinase dead or AMPK knockout mice (Jorgensen et al. 2004;
Unlike the present study, it was reported previously that skeletal muscle AMPK activity increases similarly in untrained and well trained people cycling for 20 min at 80% $\dot{V}O_2\text{peak}$ (Nielsen et al. 2003). In addition, Clark et al. (2004) found AMPK activity increased similarly during repeated bouts of exercise at 85% $\dot{V}O_2\text{peak}$ after exercise training despite evidence of lower energy deficit after the exercise training. In the present study, exercise was performed at $\sim 65\%$ of $V_O_2\text{peak}$ (untrained 66%; trained 65%) (Table 2); some may argue that this workload is quite easy for trained individuals and so no increase in AMPK activation would be expected. However, because there are 2–3-fold increases from rest in whole-body glucose uptake and fat oxidation during moderate intensity exercise at $\sim 65\%$ $V_O_2\text{peak}$ in endurance trained individuals (Romijn et al. 1993; van Loon et al. 2001), factors that are important to mediating exercise metabolism should be enhanced at these workloads. Therefore, activation of skeletal muscle AMPK is probably not necessary for increases in glucose uptake and fat oxidation during moderate intensity exercise in humans. These findings are also supported by other human studies that found dissociations between AMPK activation and glucose uptake and fat oxidation during prolonged exercise at 40–45% $V_O_2\text{max}$ (Chen et al. 2000; Wojtaszewski et al. 2002).

AMPK activity is increased by upstream kinase phosphorylation and allosterically by increases in AMP (Hardie, 2004; Sakamoto et al. 2005). In addition, ADP increases AMPK activity by preventing dephosphorylation of AMPK (Xiao et al. 2011). Exercise increases skeletal muscle free ADP and AMP (McConell et al. 1999) and exercise training attenuates the normal increases in ADP and AMP during exercise compared to untrained muscle (Gollnick & Hermansen, 1973). Indeed, in the present study, we report an attenuated increase in calculated AMP and ADP during exercise in the well trained compared to the untrained participants (Table 3) and thus would
have expected a similar attenuated response in AMPK activity. In the present study, despite the five-fold increase in $\text{AMP}_{\text{free}}$ during exercise in the trained individuals, AMPK activity was not increased from rest. Future studies should investigate whether there is a reduction in AMPK sensitivity to AMP and ADP during moderate intensity exercise after exercise training. This could be carried out in highly trained endurance subjects by using exercise intensity to titrate the free AMP levels to compare the threshold for AMPK activation during exercise in endurance trained vs. untrained individuals.

It is possible that AMPK activation during exercise in the trained individuals was restrained by the higher muscle glycogen content since muscle glycogen content has also been implicated as a potential regulator of AMPK activation during exercise. Indeed, the $\beta$ subunit contains a glycogen binding domain that associates with glycogen in cell free assays (Hudson et al. 2003; Polekhina et al. 2003). High muscle glycogen has been shown to inhibit contraction induced AMPK activation without influencing glucose uptake in rats (Derave et al. 2000). However, there is evidence to suggest that AMPK does not associate with glycogen in vivo (Viollet et al. 2003; Parker et al. 2007). Indeed our laboratory has shown that muscle glycogen is not responsible for abolished AMPK activity during exercise in humans. Following short-term exercise training, there is no increase in AMPK activity during exercise commenced with normal or high glycogen levels (McConell et al. 2005). In the present study, muscle glycogen content was 93% higher in the trained subjects at rest, and remained higher than untrained values throughout the exercise trial (Fig. 1). However, given the findings of our short-term exercise training study (McConell et al. 2005), as well as other evidence of a lack of importance of AMPK for in vivo muscle glycogen content (Viollet et al. 2003), the higher muscle glycogen content in the endurance trained individuals was probably not responsible for the abolished increase in AMPK activity during exercise.

It is not clear why activation of AMPK $\alpha$Thr$^{172}$ phosphorylation and AMPK activity did not occur during exercise in the exercise trained individuals; however, there is some evidence to suggest that training is associated with changes in the regulation of upstream AMPKK(s). For example, endurance training in rats decreases basal LKB1 activity (Hurst et al. 2005) and decreases AMPK activity during exercise (Durante et al. 2002). It is also not known what effect exercise training has on PP2C activity, and it may be that increases in PP2C activity following training are preventing AMPK $\alpha$Thr$^{172}$ phosphorylation and increases in AMPK activity during exercise. PP2C has previously been shown to inhibit the activity of both AMPK $\alpha_1$ and $\alpha_2$ isoforms (Davies et al. 1995); however, further studies are required to determine whether changes in PP2C activity occur following exercise training.

In summary, the present study found that, unlike untrained individuals, endurance trained men have no increase in skeletal muscle AMPK activity, AMPK phosphorylation or ACC phosphorylation during 120 min of cycling exercise at ~65% $\dot{V}_{\text{O}_2\text{peak}}$. This finding is consistent with results following short-term exercise training where no increase in AMPK activation during moderate intensity exercise is also observed (McConell et al. 2005). Given that skeletal muscle AMPK is not activated but fat oxidation and glucose uptake are substantial during moderate intensity exercise after exercise training, these results indicate that AMPK does not regulate metabolism under these circumstances.

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**Additional information**

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Author contributions**

GKM, KCL and GDW designed the study. GKM, KCL conducted the exercise tests. GKM, KCL, GDW and KLP conducted the experiments. KCL and GDW conducted the analysis. GKM and KCL drafted the manuscript. All authors revised the manuscript critically.

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**Keywords**

AMPK activity, endurance, exercise, metabolism, trained, training, signalling

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Statistical Summary Document**
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