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AMPK-independent pathways regulate skeletal muscle fatty acid oxidation

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The activation of AMP-activated protein kinase (AMPK) and phosphorylation/inhibition of acetyl-CoA carboxylase 2 (ACC2) is believed to be the principal pathway regulating fatty acid oxidation. However, during exercise AMPK activity and ACC Ser-221 phosphorylation does not always correlate with rates of fatty acid oxidation. To address this issue we have investigated the requirement for skeletal muscle AMPK in controlling aminoimidazole-4-carboxymide-1-β-D-ribofuranoside (AICAR) and contraction-stimulated fatty acid oxidation utilizing transgenic mice expressing a muscle-specific kinase dead (KD) AMPK α2. In wild-type (WT) mice, AICAR and contraction increased AMPK α2 and α1 activities, the phosphorylation of ACC2 and rates of fatty acid oxidation while tending to reduce malonyl-CoA levels. Despite no activation of AMPK in KD mice, ACC2 phosphorylation was maintained, malonyl-CoA levels were reduced and rates of fatty acid oxidation were comparable between genotypes. During treadmill exercise both KD and WT mice had similar values of respiratory exchange ratio. These studies suggested the presence of an alternative ACC2 kinase(s). Using a phosphoproteomics-based approach we identified 18 Ser/Thr protein kinases whose phosphorylation was increased by greater than 25% in contracted KD relative to WT muscle. Utilizing bioinformatics we predicted that extracellular regulated protein-serine kinase (ERK1/2), inhibitor of nuclear factor (NF)-κB protein-serine kinase β (IKKβ) and protein kinase D (PKD) may phosphorylate ACC2 at Ser-221 but during *in vitro* phosphorylation assays only AMPK phosphorylated ACC2. These data demonstrate that AMPK is not essential for the regulation of fatty acid oxidation by AICAR or muscle contraction.

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Skeletal muscle is a dynamic tissue that preferentially utilizes fatty acids as a fuel source during postprandial conditions. Defects in skeletal muscle fatty acid oxidation contribute to the pathogenesis of insulin resistance and obesity (Savage *et al.* 2007), therefore an understanding of the signalling pathways mediating fatty acid oxidation may yield therapeutic targets for the treatment of insulin resistance and associated disorders. The AMP-activated protein kinase (AMPK) is thought to regulate fatty acid oxidation in response to energy demand, nutrients and hormones by directly phosphorylating the muscle-specific isoform of acetyl-CoA carboxylase-2 (ACC2) on Ser-221 (corresponding to Ser-79 in ACC1) (for review see Steinberg *et al.* 2006*a*). ACC2 resides on the mitochondrial membrane catalysing the carboxylation of acetyl CoA to malonyl CoA, an allosteric inhibitor of the mitochondrial long-chain fatty-acyl CoA shuttle, carnitine palmitoyltransferase-1 (CPT-1) (Abu-Elheiga *et al.* 2000). Phosphorylation of ACC reduces enzyme activity (Carling *et al.* 1987) causing a reduction in malonyl CoA levels, a release of the malonyl CoA-mediated inhibition of CPT-1, and an increase in fatty acid β-oxidation by mitochondria (McGarry *et al.*

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1978). The importance of ACC2 in the regulation of skeletal muscle fatty acid metabolism is evident as mice lacking ACC2 have substantially enhanced rates of fatty acid oxidation, reductions in malonyl-CoA and remain lean despite hyperphagia (Abu-Elheiga *et al.* 2001). In both rodents and humans the activation of AMPK by aminoimidazole-4-carboxymide-1-β-D-ribofuranoside (AICAR) (Merrill *et al.* 1997; Koistinen *et al.* 2003; Steinberg *et al.* 2004), or muscle contractions (Vavvas *et al.* 1997; Winder *et al.* 1997; Chen *et al.* 2000; Fujii *et al.* 2000; Wojtaszewski *et al.* 2000) results in increased phosphorylation of ACC and reductions in malonyl-CoA. With obesity reduced skeletal muscle AMPK activity and ACC phosphorylation accompanied by increased levels of malonyl-CoA may contribute to the accumulation of intramuscular lipids (Bandyopadhyay *et al.* 2006; Steinberg *et al.* 2006*b*). However, it should be noted that reductions in AMPK signalling are not always observed in moderately obese humans (Steinberg *et al.* 2004) or in animals fed a high-fat diet (Martin *et al.* 2006; Watt *et al.* 2006*a*).

Despite the significant correlative evidence for a role of AMPK in regulating ACC2 phosphorylation/activity and fatty acid oxidation, direct genetic evidence supporting the role of AMPK in contraction-stimulated fatty acid oxidation has not been demonstrated. Indeed, several recent studies in both rodents and humans suggest that contraction-driven fatty acid oxidation is quite resilient to changes in AMPK signalling. One example is observed in LKB1-deficient mice who, despite no activation of AMPK α2 during muscle contraction, are able to maintain contraction-stimulated phosphorylation of ACC (Koh *et al.* 2006; Thomson *et al.* 2007*b*) and the ability to suppress malonyl-CoA (Thomson *et al.* 2007*a*) although it should be noted that this has not been observed in all studies (Sakamoto *et al.* 2005). Similarly, in mice with a deletion of the α2 (Jorgensen *et al.* 2003) or γ3 (Barnes *et al.* 2004) subunits, contraction-stimulated ACC phosphorylation is normal despite considerable reductions in AMPK phosphorylation in white muscle. It has also been reported that low intensity contraction can increase fatty acid oxidation independent of increased AMPK activity (Raney *et al.* 2005) and that AICAR and contraction may have additive effects on fatty acid oxidation (Smith *et al.* 2005). In humans, there are also several examples in which there is a dichotomy between AMPK activity, ACC phosphorylation and rates of fatty acid oxidation such as exercise training (McConell *et al.* 2005), high-intensity exercise (Chen *et al.* 2000) or exercise in women (Roepstorff *et al.* 2006). Similarly, intracellular concentrations of malonyl-CoA do not correlate with rates of fatty acid oxidation (Odland *et al.* 1996, 1998; Roepstorff *et al.* 2005). The aim of this study was to determine the requirement of AMPK in contraction-stimulated fatty acid oxidation using mice over-expressing a muscle-specific kinase dead (KD) form of AMPK α2, and to identify possible alternative signalling pathways regulating skeletal muscle fatty acid oxidation during muscle contraction.

Methods

Ethical approval

All procedures were carried out and supervised in accordance with the St Vincent's Health Animal Ethics Committee as outlined by the Bureau of Animal Welfare (Victoria, Australia). For *ex vivo* and *in situ* contraction experiments mice were anaesthetized with sodium pentobarbital (6 mg (100 g body wt)$^{-1}$) delivered I.P. before muscles were removed as described below. Mice were then killed by cervical dislocation. For treadmill running experiments mice were killed by cervical dislocation before the removal of muscles for analysis. For metabolic treadmill running experiments mice were placed back in their cage at the completion of the exercise bout. Ninety-five mice were used for these experiments.

Animals

Transgenic mice over-expressing a kinase dead (KD) form of the AMPK α2 protein (AMPK KD) under the muscle creatine kinase promoter have been previously described (Mu *et al.* 2001). AMPK (KD) and littermate controls (WT) were used for experiments at 8–12 weeks of age. Mice were fed a standard rodent chow *ad libitum* and maintained on a 12 h light/12 h dark cycle with lights on at 07.00 h.

Ex vivo determination of fatty acid oxidation and AMPK signalling in isolated EDL and SOL muscle

Extensor digitorum longus (EDL) and soleus (SOL) muscles were carefully dissected tendon to tendon for muscle incubations as described (Steinberg *et al.* 2002). Fatty acid metabolism experiments were conducted using procedures previously described (Steinberg & Dyck, 2000; Steinberg *et al.* 2002). For all experiments, isolated EDL and SOL muscles were placed in warmed (30°C) Krebs-Henseleit buffer pH 7.4 containing 2 mM pyruvate, 4% fatty acid-free bovine serum albumin (Bovogen, VIC, Australia) and 1 mM palmitic acid (Sigma, St Louis, MO, USA). For resting experiments with or without AICAR (Sigma) EDL and SOL muscle incubation volume was 2 ml while for contraction experiments, the proximal and distal tendons of isolated EDL and SOL muscles were tied with silk suture and mounted to a force transducer in a 15 ml incubation reservoir (Radnoti, Monrovia, CA, USA). After an initial incubation of 20 min, the incubation buffer was replaced with the same

buffer described above supplemented with 0.5 μCi ml^{-1} of [1-^{14}C]palmitate (Amersham BioSciences, Little Chalfont, UK). For resting experiments fatty acid metabolism was determined over 90 min in the presence or absence of 2 mM AICAR. In contraction experiments fatty acid metabolism was measured in fused tetani of EDL (50 Hz, 350 ms pulse duration, 6 tetani min^{-1}) and SOL (30 Hz, 600 ms pulse duration, 18 tetani min^{-1}) over 25 min. This contraction protocol was selected as it has previously been demonstrated to maximally stimulate fatty acid metabolism in isolated muscles (Dyck & Bonen, 1998). Force was calculated from the integral of force × time for one contraction at a given time point using Powerlab software (ADInstruments, Colorado Springs, CO, USA). In separate experiments to obtain muscles for AMPK signalling the identical protocol was employed but in the absence of radiolabelled [1-^{14}C]palmitate. At the completion of the contraction protocol muscles were removed and snap-frozen in liquid nitrogen and stored at −80°C.

In situ muscle contractions

AMPK signalling was also determined in tibialis anterior (TA) muscles contracted *in situ* as previously described (Schertzer & Lynch, 2006). Briefly, the TA muscle was exposed by a single incision and the tendon tied securely to the lever arm of a dual-mode servomotor (Aurora Scientific, Richmond Hill, Ontario, Canada) and the foot was immobilized. The TA muscle was stimulated over 20 min by 10 V 0.2 ms square-wave pulses for 300 ms via two electrodes adjacent to the femoral nerve. A sham operation was performed on the contralateral leg which served as the resting control muscle. Following the contraction period both muscles were freeze clamped in liquid nitrogen and stored at −80°C.

Treadmill running

Prior to the exercise experiment, all mice were acclimatized to treadmill running at days −3 and −2 by 10 min at rest in the treadmill apparatus (Columbus Instruments International Exer4, USA) followed by running at a 10% slope for 5 min at 10 m min^{-1} and 1 min at 15 m min^{-1} as previously described (Jorgensen *et al.* 2005). On the experimental day, mice were randomly divided into a non-exercised basal group and a treadmill-exercised group which ran at a 10% slope for 10 min at 10 m min^{-1} followed by 15 m min^{-1} for 50 min. Muscles were quickly dissected and freeze clamped.

For determination of metabolic rate/fuel selection during treadmill exercise, mice were exercised at the same intensity as described above (10% slope for 10 min at 10 m min^{-1} followed by 15 m min^{-1} for 50 min). Expired gases were collected after steady state was reached between 15 and 20 min or 45 and 50 min using a Columbus Instruments Laboratory Animal Monitoring System (CLAMS) and sealed treadmill according to the manufacturer's recommendations.

Immunoblotting and AMPK activity

Protein lysates were prepared as described (Chen *et al.* 2000). Primary antibodies to detect AMPK α1, α2, total AMPK α, phosphorylation of AMPK at Thr-172 and phosphorylation of ACC2 on Ser-221 were used as previously described (Chen *et al.* 2000).

Liquid chromatography/mass spectrometry (LC/MS) analysis of malonyl CoA

Malonyl CoA was extracted from ∼20 mg of frozen TA muscle which was ground and powdered in liquid nitrogen using a mortar and pestle in 200 μl of 10% trichloroacetic acid. Each sample was supplemented with 100 pmol of propionyl CoA (Sigma) and prepared for analysis by solid phase extraction using 60 mg Oasis HLB cartridges (Waters). Standards containing 100 pmol proponyl CoA and 5–50 pmol malonyl CoA (Sigma) were prepared in an identical fashion. Malonyl CoA was eluted from the cartridges with 96% MeOH–4% NH_4 and frozen in liquid nitrogen before being concentrated by freeze drying. Dried samples were stored at −80°C until they were resuspended in 20 mM ammonium formate shortly before LC/MS analysis. Malonyl CoA and propionyl CoA were injected onto a 1 mm × 150 mm Acclaim Pepmap C18 column (LC Packings) equilibrated with 20 mM ammonium formate at 50 μl min^{-1} and resolved on a 0–30% MeOH gradient over 8 min. Mass spectrometry was performed with a Turboionspray source at 5000 V and 500°C attached to QSTAR Pulsar-i mass spectrometer (Applied Biosystems). Malonyl CoA and proponyl CoA were detected using an MRM strategy: malonyl CoA q1 = 854.2 q3 = 347.1, proponyl CoA q1 = 824.1 q2 = 317.1 as previously described (Gao *et al.* 2007). Mass spectrometry data was manually integrated from the standard curve (malonyl CoA_{peak}/proponyl CoA_{peak} + 0.0024)/0.004, $R = 0.9986$).

Kinexus antibody array

Tibialis anterior muscles from WT and AMPK KD mice were contracted *in situ* for 20 min as described above and prepared and analysed according to the methods of Kinexus Bioinformatic Incorporation (Vancouver, BC, Canada). The antibody-array utilized 350 pan antibodies detecting 190 protein kinases and 140 other proteins.

In vitro phosphorylation assays

Phosphorylation of recombinant baculovirus-expressed human ACC2 with a deletion of the first 20 amino acids (incorporating the mitochondrial membrane insertion region) was performed in kinase assay buffer (50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 5% glycerol, 0.05% Triton X-100) containing 1 mM DTT, 100 μM [γ^{32}-P]ATP (10 000 c.p.m. pmol^{-1}) (Amersham Biosciences, Little Chalfont, UK) and 50 μM AMP. Phosphorylation was initiated by addition of recombinant AMPK $\alpha\beta\gamma$ produced by baculovirus-driven expression and phosphorylated by CaMKKβ as previously described (Iseli *et al.* 2008) or by addition of recombinant IKKβ, ERK and PKD (Invitrogen, San Diego, CA, USA). The activity of all kinases was confirmed by examining the phosphorylation of myelin basic protein (AMPK, ERK and PKD), or in the case of IKKβ, autophosphorylation (data not shown). Samples were incubated at 30°C for 60 min with constant mixing. Phosphorylation was terminated by the addition of SDS sample buffer (67 mM Tris-HCl pH 6.8, 2% SDS, 2 mM EGTA, 0.07% bromophenol blue) followed by the heating of samples at 90°C for 5 min. Proteins were separated by SDS-PAGE and gels stained with Coomassie blue (Sigma) for 1 h before overnight de-staining (7% acetic acid, 12.5% ethanol) and autoradiography. Duplicate SDS-PAGE gels were used for ACC2 immunoblots.

Statistical analysis

All data are reported as mean ± standard error of the mean (S.E.M.). Results were analysed using Student's *t* test, paired *t* test or analysis of variance (ANOVA) procedures where appropriate using Graphpad Prism software. Tukey's *post hoc* test was used to test for significant differences revealed by ANOVA. Significance was accepted at $P \leq 0.05$. The quantity of palmitate oxidized was calculated using the specific activity of labelled palmitate in the incubation medium. Kinexus antibody microarray data were calculated as the difference in fold-change between resting and contracted muscles from WT relative to AMPK KD littermates.

Results

ACC phosphorylation is maintained with muscle contraction and AICAR in AMPK KD mice *ex vivo*

We investigated the importance of AMPK in the regulation of skeletal muscle fatty acid oxidation using transgenic muscle-specific AMPK α2 kinase dead (AMPK KD) mice (kindly provided by Professor Morris Birnbaum, University of Pennsylvania) and their wild-type (WT) littermates (Mu *et al.* 2001). In initial experiments glycolytic (extensor digitorum longus, EDL) and oxidative (soleus, SOL) muscles were removed and incubated *ex vivo* under basal/resting conditions, in the presence of AICAR (2 mM) or during electrically induced muscle contractions. Both AICAR and muscle contractions increased AMPK α2 activity in EDL and SOL muscles from wild-type mice (Fig. 1*A*). AMPK α1 activity was only increased with contraction in EDL muscle from wild-type animals (Fig. 1*B*). AMPK α2 activity was nearly eliminated and AMPK α1 was substantially suppressed at rest in AMPK KD SOL and EDL muscles, an effect that may have been due to the displacement of endogenous α1 from the $\beta\gamma$ heterotrimer as has previously been suggested (Mu *et al.* 2001). Importantly neither AMPK α1 or α2 activities were increased in AMPK KD mice by AICAR or muscle contraction in either muscle type (Fig. 1*A* and *B*). These data indicate that AMPK activity is markedly suppressed and is not increased in AMPK KD mice in response to AICAR or muscle contractions.

We then examined the phosphorylation of ACC2 and hormone-sensitive lipase (HSL). Hormone-sensitive lipase is a substrate of AMPK that regulates diacylglycerol hydrolysis, and is phosphorylated at Ser-565 with AICAR and muscle contractions (Roepstorff *et al.* 2004; Watt *et al.* 2005). Under basal conditions the phosphorylation of ACC was lower in both AMPK KD EDL and SOL muscles ($P < 0.05$) (Fig. 1*C*). Both AICAR and contraction increased the phosphorylation of ACC to a similar degree in muscle from WT and AMPK KD littermates although the absolute degree of phosphorylation was modestly reduced in AMPK KD mice (Fig. 1*C*). In contrast to the effects on ACC phosphorylation, AICAR stimulated HSL Ser-565 phosphorylation in WT but not AMPK KD muscle. However, muscle contraction increased HSL Ser-565 phosphorylation to a similar extent in both genotypes (Fig. 1*D*).

AICAR and muscle contraction increase fatty acid oxidation in AMPK KD mice *ex vivo*

Increases in ACC phosphorylation by AMPK are reported to regulate fatty acid oxidation by reducing malonyl-CoA content thereby relieving inhibition of CPT-1 and enhancing the flux of fatty acyl-CoA into the mitochondria (Merrill *et al.* 1997). We therefore measured fatty acid oxidation in resting and AICAR-treated SOL and EDL using the same procedure as used for the examination of AMPK signalling. Consistent with previous reports, SOL muscle had higher rates of fatty acid oxidation than EDL under resting conditions (Fig. 2*A*) (Dyck *et al.* 1997); however, rates of fatty acid oxidation were not different between WT and KD littermates in either muscle type at rest. AICAR stimulated fatty acid oxidation in both WT and KD EDL but not SOL muscle (Fig. 2*A*). We hypothesized that since high levels of fatty acids increase

oxidation through allosteric activation of AMPK (Watt *et al.* 2006*b*), that the effect of AICAR may be masked in SOL muscles due to higher rates of fatty acid uptake relative to EDL (Bonen *et al.* 1998). Therefore, we incubated SOL muscles with a lower concentration of fatty acid (0.25 mM palmitate) and despite lower absolute rates of fatty acid oxidation no increase in fatty acid oxidation was detected in SOL with AICAR (see Fig. 1 in online Supplemental material). During muscle contraction fatty acid oxidation was not different between wild-type and AMPK KD muscles (Fig. 2). There was no difference in force production between wild-type and AMPK KD EDL and SOL muscles (Supplemental Fig. 2*A* and *B*). In order to ensure that our assay system was sensitive enough to detect reductions in fatty acid oxidation during muscle contraction, isolated EDL and SOL muscles from WT mice were incubated in the presence of 50 μM etomoxir, an irreversible CPT-1 inhibitor. At rest, etomoxir had no significant effect on fatty acid oxidation; however, when etomoxir was included during muscle contraction fatty acid oxidation was reduced by ~50 and ~90% in EDL and SOL muscle, respectively (Fig. 2*B*) indicating the critical role of CPT-1 in the regulation of contraction-stimulated fatty acid oxidation.

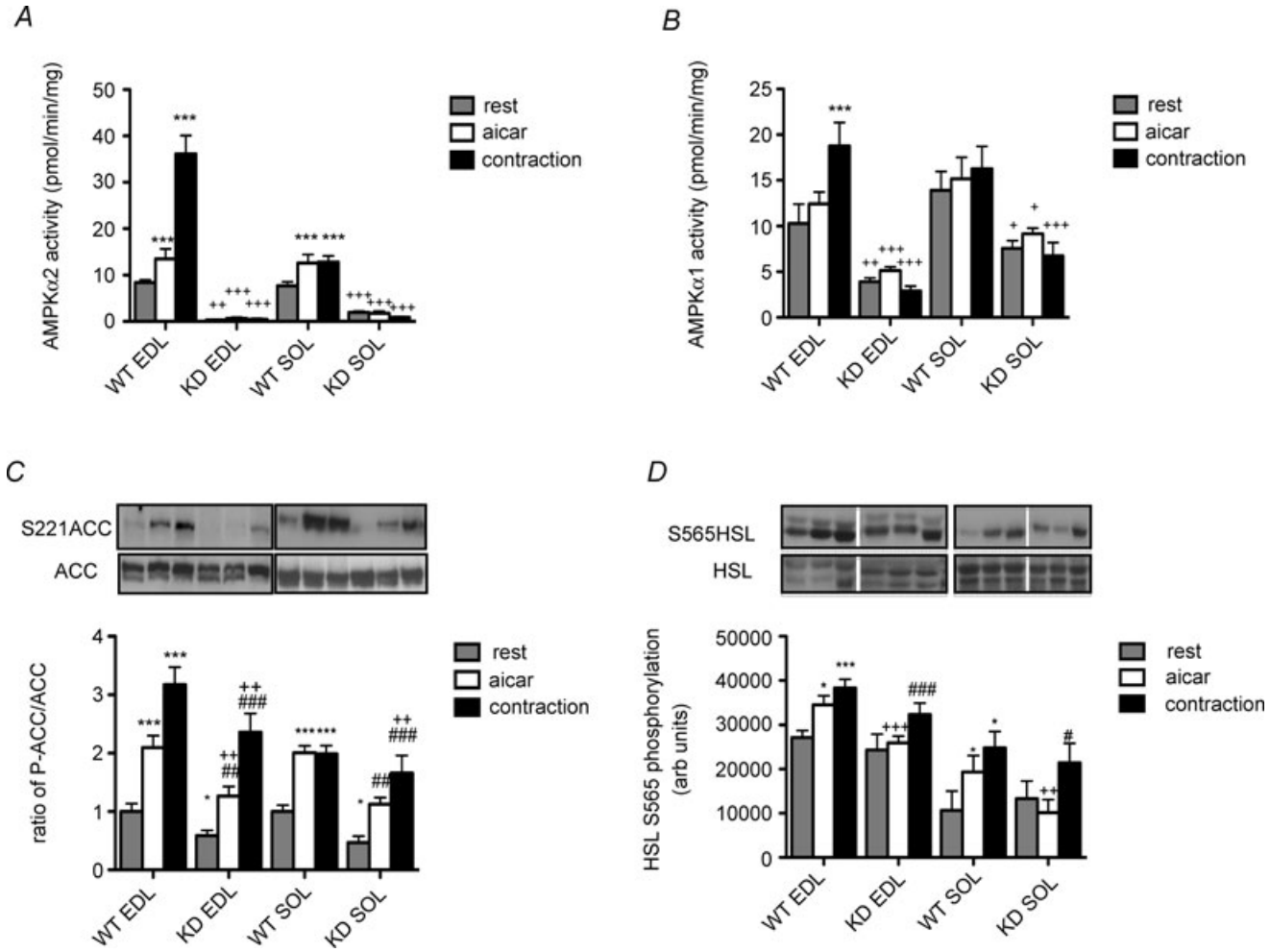

Figure 1. AMPK activity, and the phosphorylation of ACC2 Ser-221 (S221) and HSL Ser-565 (S565) in isolated EDL and SOL muscle

AMPK α2 activity (*A*) and AMPK α1 activity (*B*) in isolated EDL and SOL from WT and KD mice at rest, incubated with 2 mM AICAR, or contracted as described in Methods. *C*, representative Western blots and quantification of ACC S221 phosphorylation relative to total ACC. *D*, representative Western blots and quantification of HSL S565 phosphorylation relative to total HSL. All data are mean ± S.E.M., $n = 9$–12. $^{*}P < 0.05$ *versus* WT rest, $^{**}P < 0.01$ *versus* WT rest, $^{***}P < 0.001$ *versus* WT rest, $^{\#}P < 0.05$ *versus* KD rest, $^{\#\#}P < 0.01$ *versus* KD rest, $^{\#\#\#}P < 0.001$ *versus* KD rest, $^{+}P < 0.05$ *versus* corresponding WT group, $^{++}P < 0.01$ *versus* corresponding WT group, $^{+++}P < 0.001$ *versus* corresponding WT group.

In situ muscle contraction increases ACC phosphorylation and reduces muscle malonyl-CoA

In previous experiments EDL and SOL muscle were contracted *ex vivo* therefore we wanted to examine whether the maintenance of ACC phosphorylation in the absence of AMPK activation was also observed during muscle contractions performed *in situ*. *In situ* muscle contraction experiments were conducted by exposing the sciatic nerve in both hindlimbs and stimulating one tibialis anterior (TA) muscle over a 20 min time period while the TA muscle of the contralateral limb served as a resting control. Over the contraction period fatigue of the TA muscles was comparable between genotypes (Supplemental Fig. 3). AMPK $\alpha 2$ activity was significantly lower in the TA muscles from AMPK KD mice at rest but was not elevated with muscle contractions as observed in WT littermates (Fig. 3*A*). Contraction did not increase AMPK $\alpha 1$ activity in either wild-type or AMPK KD muscles (Fig. 3*B*). However, during contraction, increases in ACC Ser-221 phosphorylation in AMPK KD TA were similar to increases observed in WT mice (Fig. 3*C*).

The phosphorylation of ACC2 at Ser-221 inhibits its activity (Davies *et al.* 1990) leading to reductions in malonyl-CoA which is a potent inhibitor of CPT-1 (McGarry *et al.* 1978). Surprisingly, muscle malonyl-CoA levels were lower in AMPK KD mice than in wild-type littermates under resting conditions ($P < 0.05$). With muscle contraction the malonyl-CoA content tended to be reduced by ~25% in both wild-type ($P = 0.07$) and KD mice ($P = 0.08$) (Fig. 3*D*). These findings support studies suggesting a critical role for malonyl-CoA in the regulation of fatty acid oxidation (Winder & Hardie, 1996; Ruderman *et al.* 1999) and are consistent with the maintenance of ACC2 Ser-221 phosphorylation in AMPK KD mice during muscle contraction.

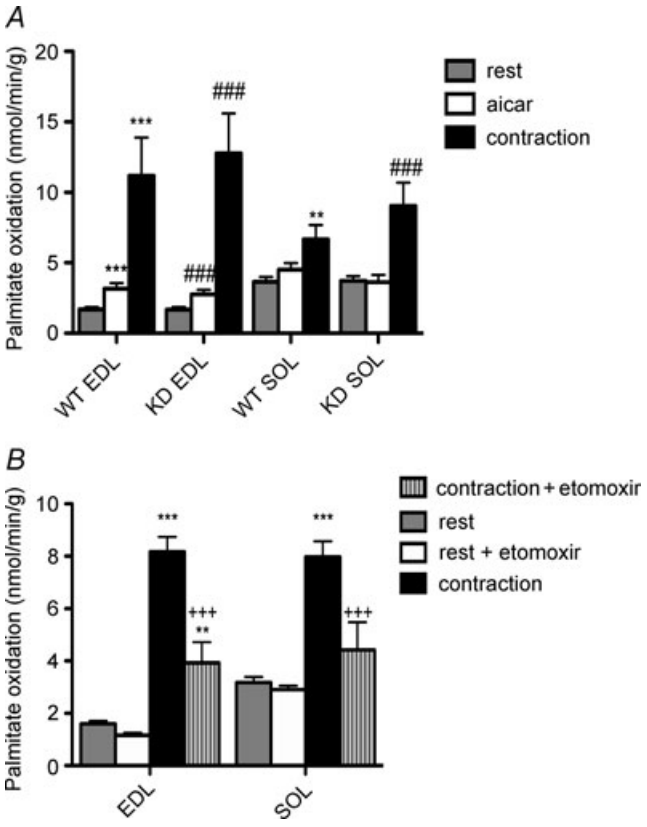

Figure 2. Fatty acid oxidation is maintained in AMPK KD muscle at rest and with muscle contraction but is impaired in WT mice with etomoxir

A, rates of palmitate oxidation in isolated EDL and SOL from WT and KD mice at rest, incubated with 2 mM AICAR, or contracted as described in Methods. All data are mean ± S.E.M., $n = 9$–12. *B*, isolated EDL and SOL muscles from WT mice were incubated at rest or contracted in the presence or absence of 50 μM etomoxir as described in Methods. Data are mean ± S.E.M., $n = 4$. $^{*}P < 0.05$ *versus* WT rest, $^{**}P < 0.01$ *versus* WT rest, $^{***}P < 0.001$ *versus* WT rest, $^{\#}P < 0.05$ *versus* KD rest, $^{\#\#}P < 0.01$ *versus* KD rest, $^{\#\#\#}P < 0.001$ *versus* KD rest, $^{+}P < 0.05$ *versus* corresponding WT group, $^{++}P < 0.01$ *versus* corresponding WT group, $^{+++}P < 0.001$ *versus* corresponding WT group.

Fatty acid oxidation is maintained during treadmill exercise in AMPK KD mice

We examined ACC phosphorylation and fatty acid oxidation in muscle from AMPK KD mice during treadmill running. Pilot experiments were conducted to ensure that WT and AMPK KD mice were capable of performing equal amounts of work since it has been reported that AMPK KD mice have reduced exercise tolerance/capacity (Mu *et al.* 2003). Both wild-type and KD littermates either rested in a cage beside the treadmill or ran for 50 min at 15 m min^{-1} and 10% gradient. AMPK $\alpha 2$ activity was increased in gastrocnemius muscle with treadmill running relative to resting wild-type controls, an effect that was not observed in gastrocnemius muscle from AMPK KD mice (Fig. 4*A*). AMPK $\alpha 1$ activity was not different between genotypes or with exercise (Fig. 4*B*). The increase in AMPK $\alpha 2$ in treadmill-exercised wild-type mice was relatively modest and did not lead to increases in ACC phosphorylation (Fig. 4*C*) probably due to the relatively low workload/intensity which was required due to the impaired exercise capacity of AMPK KD mice. Importantly despite a lack of activation of AMPK $\alpha 2$ activity in KD mice, ACC phosphorylation was increased with treadmill running (Fig. 4*C*). Fatty acid and glucose oxidation during treadmill running was assessed in wild-type and AMPK KD mice by collecting expired respiratory gases, which during submaximal endurance exercise is indicative of muscle metabolism. Oxygen uptake ($\dot{V}_{O_2}$) and the respiratory exchange ratio (RER) (Table 1) were not different between genotypes during treadmill exercise, indicating that the proportion of fat and

carbohydrate oxidized was comparable between wild-type and KD mice (Fig. 4*D*).

Taken together these data demonstrate that muscle contractions performed *ex vivo*, *in situ* or during treadmill exercise activates an alternative kinase capable of phosphorylating ACC Ser-221 and stimulating fatty acid oxidation in the absence of increases in AMPK activity.

Screening for alternative ACC kinases in contracting muscles

To identify alternative ACC2 kinase(s) we performed an antibody microarray on muscle from resting and *in situ* contracted WT and AMPK KD mice. We hypothesized that an alternative ACC2 kinase capable of substituting for AMPK would be activated to a greater degree during muscle contraction in AMPK KD mice compared with WT littermates. We identified 18 Ser/Thr kinases whose phosphorylation was up-regulated by greater than 25% in contracted muscles from AMPK KD mice (Table 2). Of these candidates we then utilized *in silico* screening using both ScanSite (Obenauer *et al.* 2003) and Predikin (Brinkworth *et al.* 2003) prediction-based software, which utilize substrate-specificity databases and structural databases, respectively, to assess which of these protein kinases might phosphorylate ACC2 Ser-221. The results of this survey suggested three possible candidates: extracellular regulated protein-serine kinase (ERK) 1/2 (also known as p44/p42 MAP kinases), inhibitor of NF-κB protein-serine kinase α/β (IKKα/β) and protein kinase D (PKD-formerly known as PKCμ) all of which have previously been demonstrated to be activated in skeletal muscle during muscle contraction (Sherwood *et al.* 1999; Ryder *et al.* 2000; Turcotte *et al.* 2005; Luiken *et al.* 2008).

To test if these kinases could phosphorylate ACC2 we conducted *in vitro* experiments utilizing

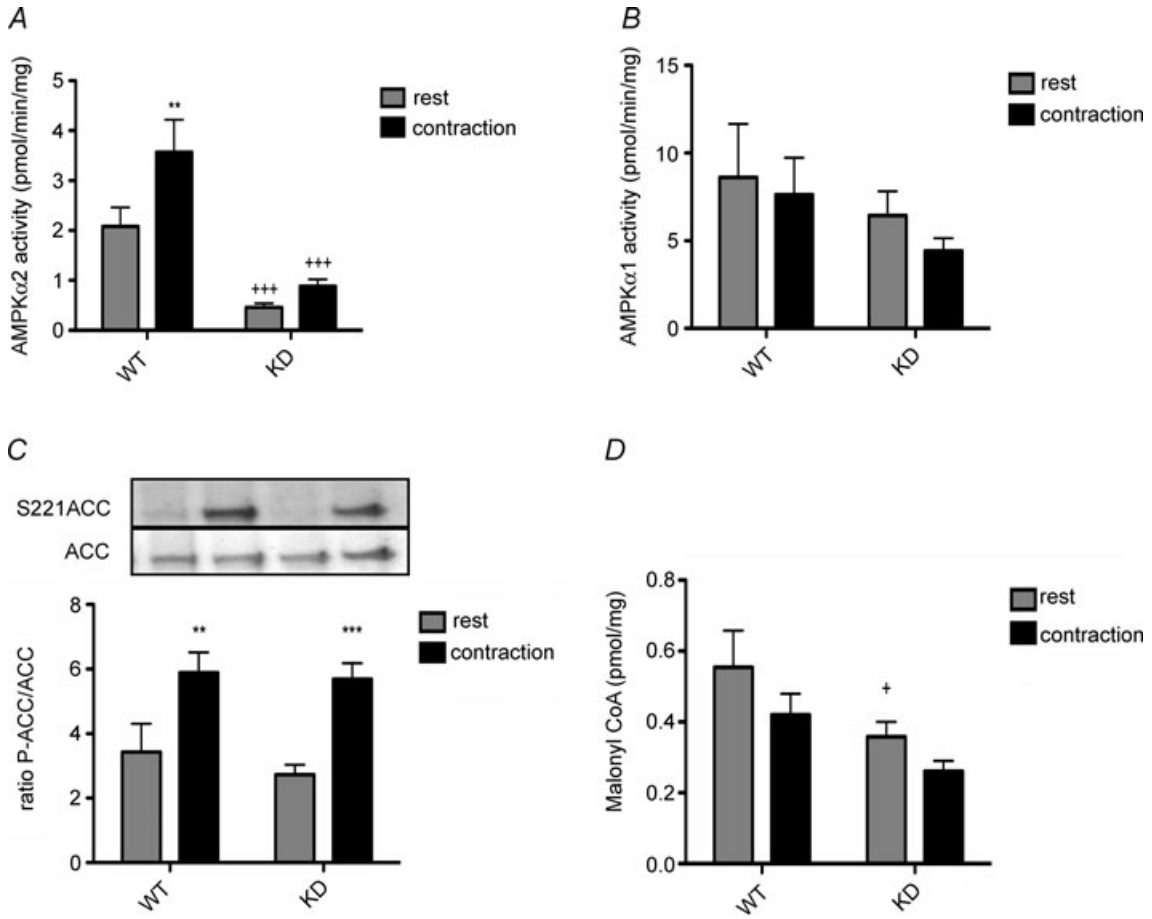

Figure 3. AMPK signalling and malonyl-CoA during *in situ* muscle contraction
AMPK α2 (*A*) and α1 (*B*) activity in TA muscle from WT and KD mice at rest, or contracted *in situ* as described in Methods. *C*, representative Western blots and quantification of ACC S221 phosphorylation relative to total ACC from TA muscle. *D*, malonyl CoA levels in TA muscle from WT and KD mice at rest, or contracted *in situ* as described in Methods. All data are mean ± S.E.M., $n = 8$. $^{*}P < 0.05$ *versus* rest, $^{**}P < 0.01$ *versus* rest, $^{+}P < 0.05$ *versus* corresponding WT group, $^{+++}P < 0.001$ *versus* corresponding WT group.

Table 1. $\dot{V}_{O_2}$ and respiratory exchange ratio (RER) during treadmill exercise at 15 m min^{-1} and 10% gradient in WT and AMPK KD mice measured at 15 and 45 min of exercise

	15 min		45 min	
	WT	AMPK KD	WT	AMPK KD
$\dot{V}_{O_2}$ (ml kg^{-1} h^{-1})	5490 ± 360	4760 ± 195	4905 ± 205	5080 ± 85
RER ($\dot{V}_{CO_2}$/ $\dot{V}_{O_2}$)	0.75 ± 0.01	0.77 ± 0.02	0.71 ± 0.0	0.71 ± 0.01

All data are mean ± S.E.M., $n = 6$.

recombinant baculovirus-expressed ACC2 as a substrate for active recombinant AMPK, ERK2, IKKβ and PKD. AMPK markedly stimulated [^{32}P]ATP-mediated phosphorylation of ACC2. PKD caused a minor stimulation of ACC2 phosphorylation (only 5–10% of that caused by AMPK) while neither IKKβ nor ERK2 increased ^{32}P incorporation into ACC2. Importantly, only AMPK was able to increase ACC2 Ser-221 phosphorylation indicating that ERK2, IKKβ or PKD are not ACC2 kinases (Supplemental Fig. 4).

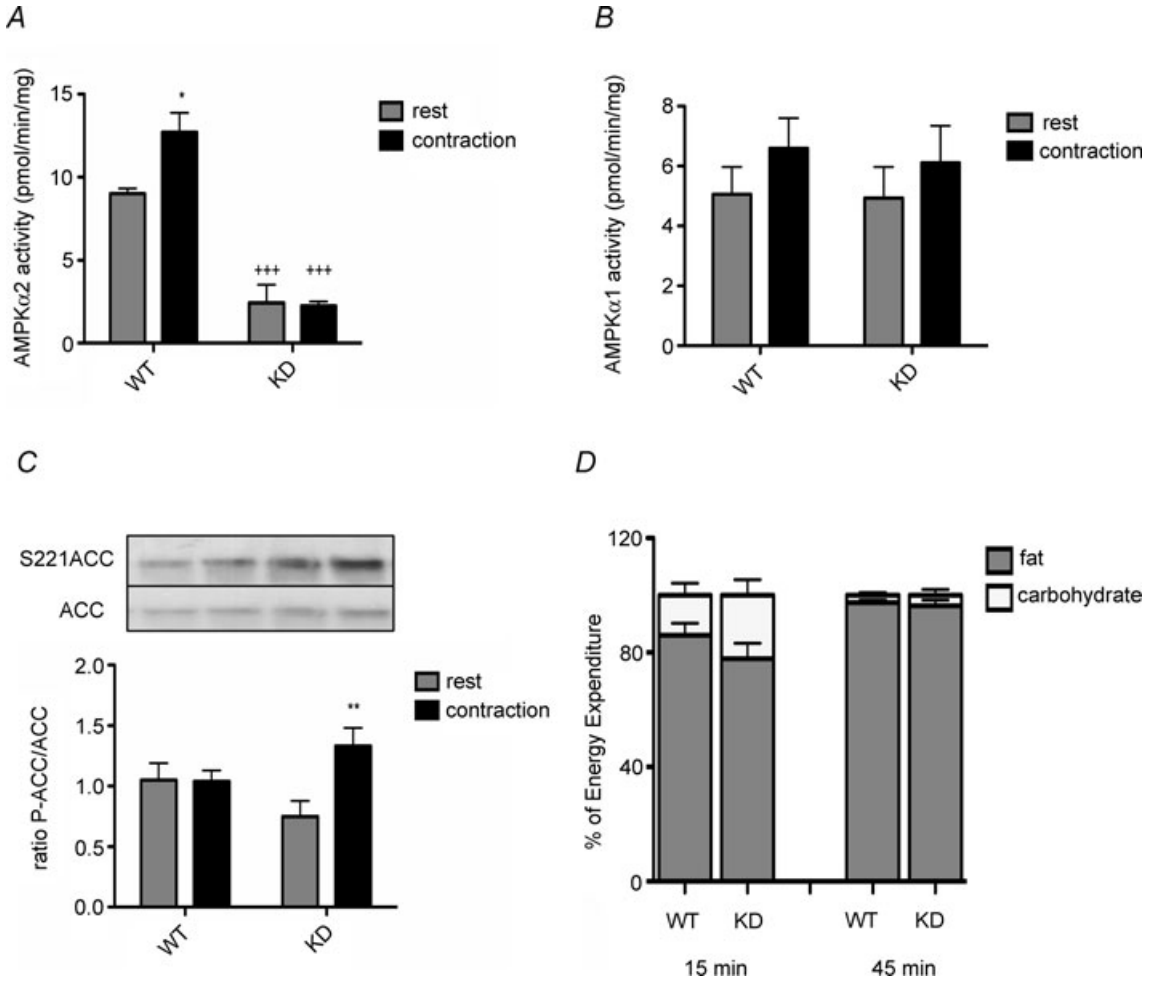

Figure 4. Fatty acid oxidation is not altered in AMPK KD mice during submaximal treadmill running
AMPK α2 (*A*) and α1 (*B*) activity in gastrocnemius muscle following treadmill running. *C*, representative Western blots and quantification of ACC S221 phosphorylation relative to total ACC in gastrocnemius muscle following treadmill running. *D*, estimated percentage fat or carbohydrate utilization determined at 15 or 45 min during treadmill exercise. All data are mean ± S.E.M., $n = 6$–8. $^{*}P < 0.05$ *versus* rest, $^{**}P < 0.01$ *versus* rest, $^{+++}P < 0.001$ *versus* corresponding WT group.

Table 2. Summary of Ser/Thr kinases up-regulated by greater than 25% in contracted AMPK KD skeletal muscle

Accession	Name	Short name	Phosphorylation site	Fold change
P27361	Extracellular regulated protein-serine kinase 1/2 (p44/p42 MAP kinases)	ERK1/2	T202 + Y204/ T185 + Y187	3.46
P46734	MAP kinase protein-serine kinase 3/6 (MKK3/6)	MEK3/6 (MAP2K3/MAP2K6)	S189/S207	3.06
O15111	Inhibitor of NF-κB protein-serine kinase α (CHUK)/β	IKKα/β	S180/S181	2.22
P23443	p70 ribosomal protein-serine S6 kinase alpha /p85 ribosomal protein-serine S6 kinase 2	S6Ka (p70 S6Ka)/ S6K2 (p85 S6K2)	T229/T252	2.03
P49137	Mitogen-activated protein kinase-activated protein kinase 2	MAPKAPK2	T334	2.02
P13861	cAMP-dependent protein-serine kinase regulatory type 2 subunit α	PKA R2α	S98	1.71
P49841	Glycogen synthase-serine kinase 3 α/β	GSK3α/β	Y279/Y216	1.71
Q15139	Protein-serine kinase C μ (protein kinase D)	PKCμ (PKD)	S910	1.66
P17252	Protein-serine kinase C α	PKCα	S657	1.66
P24723	Protein-serine kinase C θ	PKCθ	S674	1.64
Q15418	Ribosomal S6 protein-serine kinase 1/2	RSK1/2	S380/S386	1.60
P31749	Protein-serine kinase B α	PKBα (Akt1)	S473	1.47
Q02750	MAPK/ERK protein-serine kinase 1 (MKK1)	MEK1 (MAP2K1)	T291	1.43
O75582	Mitogen and stress-activated protein-serine kinase 1	Msk1	S376	1.35
P41743	Protein-serine kinase C λ/ι	PKCl λ/ι	T555	1.31
P36507	MAPK/ERK protein-serine kinase 2 (MKK2)	MEK2 (MAP2K2)	T394	1.30
Q02156	Protein-serine kinase C ε	PKCε	S729	1.29
Q15139	Protein-serine kinase C μ (protein kinase D)	PKCμ (PKD)	S738 + S742	1.26
P05129	Protein-serine kinase C γ	PKCγ	T514	1.25

Discussion

Despite the well-recognized clinical importance of exercise in the prevention and treatment of insulin resistance and type 2 diabetes, the molecular mechanisms mediating the positive effects of exercise have remained elusive (Diabetes Prevention Program Research Group, 2002). The discovery that AMPK is activated by exercise in both rodents (Winder & Hardie, 1996; Vavvas *et al.* 1997) and humans (Chen *et al.* 2000; Fujii *et al.* 2000; Wojtaszewski *et al.* 2000) provided a possible molecular mechanism explaining the beneficial effects of exercise on metabolism. In the current study we demonstrate that the activation of AMPK by both AICAR and muscle contraction is not required for stimulating fatty acid oxidation in skeletal muscle, suggesting that alternative pathways exist that are capable of compensating for AMPK in the control of fatty acid oxidation.

During exercise, increased ATP demands by contracting sarcomeres and Ca^{2+} pumps in the sarcoplasmic reticulum can increase ATP turnover by in excess of 100-fold (Spriet & Dyck, 1996). This dramatic increase in ATP demand is tightly matched by the rapidly increasing oxidative and non-oxidative metabolism of substrates derived from intra- and extracellular lipid and carbohydrate stores (Spriet & Dyck, 1996). Studies in AMPK α2 (Jorgensen *et al.* 2005) and LKB1 null mice (Sakamoto *et al.* 2005) have highlighted the importance of AMPK signalling in the maintenance of energy charge as both models have an elevated muscle AMP : ATP ratio during treadmill running and *ex vivo* muscle contractions, respectively. During endurance exercise fatty acid oxidation is the predominant substrate for working skeletal muscle. Therefore, our findings that muscle fatty acid oxidation was not impaired during muscle contraction both *ex vivo* or during endurance treadmill exercise suggests that this is not the primary defect contributing to a reduced ability to buffer ATP in the absence of AMPK. Indeed we also found that carbohydrate oxidation was also maintained during treadmill exercise supporting previous findings demonstrating that glucose uptake is not impaired in skeletal muscle of mice with AMPK deficiency (Jorgensen *et al.* 2003; Barnes *et al.* 2004; Fujii *et al.* 2005; Thomson *et al.* 2007*b*). Taken together these data support the concept that reduced exercise capacity in AMPK-deficient mice (Jorgensen *et al.* 2003; Thomson *et al.* 2007*b*) may be due to secondary effects on cardiac metabolism which reduces cardiac output or perfusion (Mu *et al.* 2003).

ACC was one of the first substrates of AMPK identified and is commonly used as a surrogate marker of *in vivo* AMPK activity (Carling *et al.* 1987). Activation of AMPK by muscle contraction is associated with increased phosphorylation and deactivation of ACC2 (Winder, 1996; Vavvas *et al.* 1997). Surprisingly, the phosphorylation of ACC on Ser-221 was significantly increased in KD muscles with both AICAR and contraction despite no detectable increase in AMPK activity. Although it cannot be ruled out

completely, we consider that residual AMPK activity in KD mice is not responsible for the phosphorylation of ACC during AICAR treatment or contraction since no increase in AMPK α1 or α2 activity could be detected under these conditions. In particular, AMPK α2 activity, the isoform predominantly activated by contraction and AICAR, was severely blunted in kinase dead mice. In addition, in the same muscles in which ACC Ser-221 phosphorylation was examined, the phosphorylation of HSL was impaired in AMPK KD muscle following AICAR treatment, but not contraction. This suggests that the maintenance of ACC phosphorylation following both AICAR and contraction was not due to residual AMPK activity as it would be expected that HSL would have been phosphorylated following AICAR treatment in AMPK KD mice if this was the case.

In addition to control by ACC2, malonyl-CoA levels are also regulated by malonyl-CoA decarboxylase (MCD) (Dyck *et al.* 1998). The depletion of MCD in muscle increases malonyl-CoA and reduces fatty acid oxidation (Bouzakri *et al.* 2008; Koves *et al.* 2008). MCD activity is increased in response to AICAR and contraction in skeletal muscle (Saha *et al.* 2000; Park *et al.* 2002) but this regulation appears to be indirect as AMPK does not phosphorylate MCD (Habinowski *et al.* 2001). Our data demonstrating reduced levels of malonyl-CoA in AMPK KD mice, both at rest and following muscle contraction, could be due to MCD activation but by a mechanism independent of AMPK. Clearly, further studies examining the regulation of malonyl-CoA and MCD with AICAR and muscle contraction are warranted.

In conclusion these studies demonstrate that AMPK is dispensable for the regulation of skeletal muscle fatty acid oxidation in response to muscle contraction and treadmill exercise. Importantly, we demonstrate that AICAR can also regulate fatty acid oxidation independent of AMPK activation highlighting the need for more specific activators of AMPK. Future studies investigating the entire muscle kinome or genetic evidence examining the role of ACC2 phosphorylation will be important to identify novel pathways regulating muscle fatty acid oxidation.

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Supplemental material

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