Published in final edited form as:

Acta Physiol (Oxf). 2011 April ; 201(4): 457–466. doi:10.1111/j.1748-1716.2010.02226.x.

Effect of LKB1 deficiency on mitochondrial content, fiber type, and muscle performance in the mouse diaphragm

Jacob D. Brown¹, Chad R. Hancock², Anthony D. Mongillo¹, J. Benjamin Barton¹, Ryan A. DiGiovanni¹, Allen C. Parcell³, William W. Winder¹, and David M. Thomson¹

¹ Department of Physiology and Developmental Biology, Brigham Young University, Provo, UT 84602, USA

² Department of Nutrition, Dietetics and Food Science, Brigham Young University, Provo, UT 84602, USA

³ Department of Exercise Sciences, Brigham Young University, Provo, UT 84602, USA

Abstract

Aim—The Liver Kinase B1 (LKB1)/AMP-Activated Protein Kinase (AMPK) signaling pathway is a major regulator of skeletal muscle metabolic processes. During exercise, LKB1-mediated phosphorylation of AMPK leads to its activation, promoting mitochondrial biogenesis and glucose transport, among other effects. The roles of LKB1 and AMPK have not been fully characterized in the diaphragm.

Methods—Two methods of AMPK activation were used to characterize LKB1/AMPK signaling in diaphragms from muscle-specific LKB1 knockout (KO) and littermate control mice: (1) acute injection of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and (2) 5-min direct electrical stimulation of the diaphragm. Diaphragms were excised 60 minutes post-AICAR injection and immediately after electrical stimulation.

Results—AMPK phosphorylation increased with AICAR and electrical stimulation in control but not KO mice. Acetyl CoA carboxylase phosphorylation increased with AICAR in control but not KO mice, but increased in both genotypes with electrical stimulation. While the majority of mitochondrial protein levels were lower in KO diaphragms, uncoupling protein 3, complex I, and cytochrome oxidase IV protein levels were not different between genotypes. KO diaphragms have a lower percentage of IIX fibers and an elevated percentage of IIb fibers when compared to control diaphragms. While *in vitro* peak force generation was similar between genotypes, KO diaphragms fatigued more quickly and had an impaired ability to recover.

Conclusion—LKB1 regulates AMPK phosphorylation, mitochondrial protein expression, fiber type distribution, as well as recovery of the diaphragm from fatigue.

Keywords

AMPK; fatigue; skeletal muscle

Address for Correspondence: David M. Thomson, PhD; Department of Physiology and Developmental Biology, 589 WIDB, Brigham Young University, Provo, UT 84602; Phone: 801-422-8709; Fax 801-422-0700; david_thomson@byu.edu.

Conflict of Interest: The author report no conflict of interest relating to this study.

Introduction

The past 15 years of research has demonstrated the vital role that liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signaling plays in skeletal muscle metabolism (Koh et al., 2008, Winder and Thomson, 2007). LKB1 is a Ser/Thr kinase tumor suppressor (Sakamoto et al., 2004) that phosphorylates AMPK (Sakamoto et al., 2005, Thomson et al., 2007b, Koh et al., 2006) in skeletal muscle at Thr172 of the alpha subunit (Hawley et al., 1996). LKB1 also phosphorylates 12 additional AMPK-related kinases (Lizcano et al., 2004), further supporting its central role as a regulator of metabolic processes. AMPK is composed of heterotrimeric combinations of a catalytic α subunit and regulatory β and γ subunit isoforms (Woods et al., 1996). AMPK activity is regulated by AMP binding to the γ subunit (Cheung et al., 2000) which activates AMPK by preventing dephosphorylation (Davies et al., 1995). Thus, LKB1/AMPK signaling plays a key role in skeletal muscle metabolism at times when levels of AMP are elevated; such as contraction, hypoxia, and/or nutrient deprivation.

AMPK is present in multiple tissues and regulates a wide variety of pathways. Upon activation, AMPK promotes ATP-generating processes by stimulating fatty acid oxidation (Thomson et al., 2007a, Winder and Hardie, 1996) and glucose transport (Fisher et al., 2002, Kurth-Kraczek et al., 1999). AMPK also inhibits ATP-consuming pathways that promote fatty acid (Foretz et al., 1998, Gaidhu et al., 2006), cholesterol (Henin et al., 1995), and protein synthesis (Bolster et al., 2002, Gwinn et al., 2008, Reiter et al., 2005, Williamson et al., 2006). AMPK is activated in skeletal muscle during exercise (Winder and Hardie, 1996), electrical stimulation (Hutber et al., 1997), and treatment with the AMP mimetic 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (Corton et al., 1995, Merrill et al., 1997). Acetyl CoA Carboxylase (ACC), a potent inhibitor of fatty acid oxidation, is phosphorylated and inactivated by AMPK in skeletal muscle (Winder and Hardie, 1996) and ACC phosphorylation is widely regarded as a good indicator of AMPK activity *in vivo* (Branvold et al., 2008, Rubink and Winder, 2005, Winder et al., 1997, Thomson et al., 2007a). Previous studies have also shown the importance of LKB1/AMPK signaling in mitochondrial biogenesis and protein expression (Bergeron et al., 2001, Putman et al., 2003, Thomson et al., 2007b, Winder et al., 2000); specifically, activation of AMPK increases mitochondria-specific and PGC-1 protein levels while ablation of LKB1 causes them to decrease.

LKB1/AMPK signaling also functions in fiber type determination. For example, Rockl *et al.* observed a shift from IIb to IIa/x fibers in triceps of wild type (WT) mice after a six-week voluntary wheel running study; however, this transition was impaired in AMPK α 2-KO mice (Rockl et al., 2007). In another study, a 14-day AICAR treatment produced a decrease in type IIb fibers in rat extensor digitorum longus (EDL) muscles (Suwa et al., 2003). Therefore, LKB1/AMPK signaling seems to promote rearrangements that favor more oxidative fiber types.

The study of LKB1/AMPK signaling in skeletal muscle has been facilitated by the generation of a skeletal/cardiac muscle-specific LKB1 knock-out (KO) mouse (Koh et al., 2006, Thomson et al., 2007b, Sakamoto et al., 2005). These mice exhibit diminished AMPK activity, reduced phosphorylation of ACC, decreased voluntary wheel running, and lower levels of mitochondrial proteins (Thomson et al., 2007b). Peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1) protein levels are also attenuated which could explain the observed decrease in oxidative capacity of these animals (Koh et al., 2006, Thomson et al., 2007b) since PGC-1 is a major regulator of mitochondrial biogenesis. Although the diaphragm is well adapted to the metabolic demand of chronic contractions, it has been well established that increasing diaphragmatic stress promotes small, yet significant increases in

mitochondrial protein activity (Powers and Criswell, 1996). Given the essential, chronic, intermittent, contractile activity of the diaphragm, an important role for AMPK seems likely; especially during periods of increased stress (e.g. exercise, hyperventilation, chronic obstructive pulmonary disorder). In addition, it is possible that the decreased ability of the KO mice to run voluntarily could be due to the perturbations in diaphragm function caused by the LKB1 deficiency (Thomson et al., 2007b).

The majority of skeletal muscle LKB1/AMPK signaling research is focused on muscles of the limbs (e.g. soleus, quadriceps, triceps, etc.) while the muscles of respiration have not been studied in much depth. In this study, skeletal/cardiac muscle-specific LKB1-KO mice were used to determine the effects of LKB1 deficiency on: (1) AMPK phosphorylation after AICAR injection (sacrificed 1 hr post-injection) and a 5-min direct electrical stimulation of the diaphragm, (2) mitochondrial protein and PGC-1 protein levels of the diaphragm, (3) muscle fiber type distribution in the diaphragm, and (4) *in vitro* contractile properties of the diaphragm.

Materials and Methods

Animal care and development of muscle-specific knock-out mouse

Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University. All animals were housed in a temperature controlled (21–22°C) environment with a 12h:12h light-dark cycle and fed standard chow and water *ad libitum*. The skeletal/muscle-specific LKB1-knockout mouse (KO) model was generated through a Cre-Lox system in which Cre recombinase expression was activated by the muscle creatine kinase promoter (Thomson et al., 2007b). Control (C) mice contain the LoxP-flanked LKB1 gene, but exhibit the same phenotype and level of LKB1 and AMPK activation as wild-type mice. A western blot for LKB1 was performed on all muscles used for experimentation in order to verify the ablation of LKB1 from the KO mice. Male mice were used at an average of 20 weeks of age. Body weights were 26.2 g and 26.9 g for C and KO mice, respectively.

AICAR treatment

C and KO male mice were injected subcutaneously with either AICAR dissolved in saline (1 mg g⁻¹ BW) or an equivalent volume of saline without AICAR 1 hr before excision of muscles. Mice were anesthetized (intraperitoneal injection) with pentobarbital sodium (0.08 mg g⁻¹ BW) 30 minutes before excision of muscles to assure complete sedation and muscle equilibration. Diaphragms were excised immediately following the 1 hr AICAR/saline treatment. Muscles were then promptly freeze-clamped with metal-block tongs at liquid nitrogen temperature (–195 °C) and stored at –95 °C until homogenization and analysis.

Electrical stimulation (ES) of diaphragm

C and KO mice were anesthetized with pentobarbital sodium (0.08 mg g⁻¹ BW) 15 minutes before *in situ* electrical stimulation. Diaphragms from C (n=7) and KO (n=7) mice were electrically stimulated directly [train rate = 1 TPS, train duration = 500 ms, stim rate = 100 Hz, 2 volts] with an S48 Grass Stimulator (Astro-Med, Inc., West Warwick, RI, USA) for 5 minutes using a two-pronged probe designed for direct full-diaphragm stimulation. This protocol was developed in our lab and proved to activate AMPK. The diaphragm was accessed through an incision exposing the peritoneal cavity and inferior side of the diaphragm without tearing the diaphragm or opening the thoracic cavity. The peritoneal cavity of the sham-operated C mice was opened for five minutes without electrical stimulation to the diaphragm. Diaphragms were excised immediately after the treatment.

period and promptly freeze-clamped with metal-block tongs at liquid nitrogen temperature and stored at -95°C until homogenization and analysis.

Homogenization

Diaphragms were homogenized in a glass-on-glass homogenizer in 19-volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4; 250 mM mannitol; 50 mM NaF; 5 mM Sodium Pyrophosphate; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 50 mM B-glycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor). Aliquots of raw homogenate were freeze-thawed three times and saved for citrate synthase activity assays. The remaining raw homogenates were subjected to 2 cycles of freeze-thaw and then centrifuged at $1500 \times g$ for 10 minutes. Supernatants were then analyzed for protein content using the DC Protein Assay (Biorad Laboratories, Hercules, CA, USA). Supernatants were stored in microcentrifuge tubes at -95°C until analysis.

Western blot and immunodetection

Resting diaphragms were used to determine mitochondrial protein and PGC-1 protein levels. Homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β -mercaptoethanol, and 0.01% bromphenol blue) and then loaded on 5% [phosphorylated ACC (pACC), total ACC (tACC)], 7.5% [phosphorylated AMPK (pAMPK), total AMPK (tAMPK), LKB1, PGC-1, Cytochrome Oxidase-4 (COX4)], 10% [Uncoupling Protein-3 (UCP-3), Long Chain Acyl CoA Dehydrogenase (LCAD)], 15% [Cytochrome C], and 4–15% [Complex I, Complex II, Core 2, Cytochrome Oxidase-1 (COX1), ATP Synthase α subunit] Tris HCl gels (Bio-Rad Criterion System, Hercules, CA.) with 2.5 μg [Complex I, Complex II, Core 2, COX1, ATP Synthase α subunit], 5 μg [Cyto C]; 10 μg [PGC-1, LCAD, UCP-3, COX4], and 20 μg (LKB1, pAMPK, tAMPK, pACC, tACC,) of protein per well. Electrophoresis was applied for 45–55 min at 200 volts. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes at 100 volts for 1 hr. Membranes were stained with Ponceau S in order to verify equal protein loading. Membranes were then washed with Tris-buffered saline plus 0.1% Tween-20 (TBST), blocked with 5% non-fat dry milk in TBST for 1 hr, and probed overnight at 4°C with primary antibody diluted in 1% bovine serum albumin (BSA) dissolved in TBST, pH 7.6.

Primary antibody manufacturers and dilutions were as follows: pAMPK (#2531, 1:5000), tAMPK (#2532, 1:5000), pACC (#3661, 1:5000) from Cell Signaling Technology (Beverly, MA); Cyto C (#13156, 1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA); OxPhos Cocktail consisting of Complex I, Complex II, Core 2, COX1, and ATP Synthase α (#458099, 1:10000), COX4 (#21348A, 1:2000) from Invitrogen (Eugene, MA); UCP-3 (#PA1-055, 1:4000) from Affinity Bio Reagents (Golden, CO); LKB1 (#07-694, 1:5000) from Upstate (Charlottesville, VA); PGC-1 (#516557, 1:4000) from Calbiochem (La Jolla, CA); and LCAD (Gift from Dr. Daniel Kelly). All antibodies were diluted in 1% BSA in TBST with the exception of tACC. tACC was identified with streptavidin-horseradish peroxidase (HRP) conjugate (GE Healthcare, Buckinghamshire, UK, #RPN1231V, 1:20000) diluted in TBST.

Membranes were washed 4×5 min with TBST, after which they were probed with the appropriate HRP-conjugated, secondary antibody for 1 hr at room temperature. Membranes were then washed again 4×5 min with TBST. Membranes were covered with ECL Plus Western Blotting Detecting Solution (GE Healthcare Bio-Sciences, Piscataway, NJ) for 1.5 min. Blots were developed and then analyzed using AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA)

Citrate synthase activity assay

Citrate synthase was determined on freeze-thawed (3x) raw homogenates from resting diaphragms using the method described by Srere (Srere, 1969).

Myosin heavy chain (MHC) composition

MHC composition was assessed using a modified protocol by Talmadge and Roy (Talmadge and Roy, 1993). Separation of MHC proteins was achieved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel and an 8% separating gel using a Hoefer SE 600 gel electrophoresis system. Gels were loaded with 1.5 μ g of protein per well and run at 275 V for 22 hours at 4°C. After electrophoresis the gels were fixed with 50% ethanol, 10% glacial acetic acid, and 40% deionized water for at least 30 minutes. The gels were then linked in 10% glutaraldehyde for 30 minutes and rinsed 3×10 min and 2×20 min in distilled water and then silver stained using the protocol described by Giulian *et al.* (Giulian *et al.*, 1983). Protein bands were identified as MHC I, IIa, IIx, or IIb depending on their migration characteristics compared with a molecular weight standard and MHC standard. Analysis was performed with Quantity One 4.6.3 Imaging software (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of in vitro contractile properties of the diaphragm

Mice were anesthetized with sodium pentobarbital (0.08 mg g⁻¹ BW). The inferior portion of the diaphragm was exposed and the central tendon of the diaphragm was clamped with hemostats. A ~2mm strip was cut parallel with the muscle fibers with the central tendon at one end and costal connective tissue at the other. The central tendon was attached to a servomotor (300B Dual-Mode Lever System; Aurora Scientific, Aurora, Ontario, Canada) using 4-0 silk suture at the central tendon and the ribs were clamp-secured to a stationary platform in a jacketed tissue bath filled with Ringer's solution (137 mM NaCl, 24 mM NaCO₃, 11 mM D-glucose, 5 mM KCl, 2 mM CaCl₂, 1 mM NaH₂PO₄•H₂O, 1 mM MgSO₄, pH 7.4; aerated with 95% O₂/5% CO₂ at 37°C). Diaphragms were allowed to equilibrate to the bath for 10 minutes during which optimal length was determined from a resting level of 0.75g of force. Force frequency relationship was determined using 10 pulses at 10, 20, 40, 60, 80, 100, 150, 200, 250, and 300 Hz (S88X Grass Stimulator; Astro-Med, Inc., West Warwick, RI, USA), after which diaphragms were stimulated for 10 minutes at 200 Hz every two seconds with a train duration of 100 ms to determine the rate of fatigue. Acute diaphragm recovery was then determined by 5 additional stimulations after 1-min and 5-min. Diaphragm strips were then measured for length and weighed.

Statistics

Two-group comparisons (C vs. KO) were made with Student *t* test or factorial ANOVA with repeated measures as appropriate, using Microsoft Excel (Microsoft Corp., Redmond, WA) or NCSS statistical analysis software (NCSS, Kaysville, UT), respectively. Data are presented as means $\pm$ standard error of the mean (S.E.M.). Statistical significance was set at $P \leq 0.05$.

Results

Effects of LKB1 deficiency on AMPK phosphorylation in AICAR-treated (AIC) and electrically stimulated (ES) mouse diaphragm

LKB1 protein was absent in KO diaphragms as shown by western blotting (Fig. 1). AMPK and ACC total protein levels did not differ between genotypes or treatment conditions. AMPK phosphorylation (pAMPK) increased with AICAR and ES in C mice, but was very low in diaphragms from all KO mice (Fig. 1). ACC phosphorylation (pACC) increased in C

mice after AICAR injection and ES while in KO it increased with ES but not AICAR (Fig. 1).

Effect of LKB1 deficiency on PGC-1, mitochondrial protein content, and citrate synthase activity in mouse diaphragm

PGC-1 levels were diminished in KO diaphragms (Fig. 2). Consistent with the role of PGC-1 as a controller of mitochondrial biogenesis, mitochondrial protein content was decreased for: Complex 2 (67.5% of C), Complex III core 2 (74.9% of C), Cyto C (83.4% of C), COX1 (74.7% of C), ATP synthase α subunit (56.2% of C), and LCAD (73.8% of C) (Fig. 2). On the other hand, Complex I (80.9% of C), COX4 (92.5% of C), and UCP-3 (117.1% of C) were not significantly different in KO diaphragms (data not shown). Citrate synthase activity was significantly lower in KO diaphragms (Fig. 3).

MHC composition

In both C and KO diaphragms, Type II fibers are predominant in mouse diaphragm, which is consistent with previous findings (Petrof et al., 1993). MHC expression in C diaphragm was as follows (Fig. 4): Type I – $6.93\% \pm 2.28$, Type IIa – $20.42\% \pm 3.07$, Type IIx – $44.54\% \pm 1.47$, Type IIb – $28.12\% \pm 1.68$. KO diaphragm analysis showed MHC expression as follows: – Type I $2.87\% \pm 1.30$, Type IIa $18.47\% \pm 1.87$, Type IIx – $40.61\% \pm 1.02$, Type IIb – $38.05\% \pm 1.71$. The percentage of MHC type IIx was significantly lower, and that of type IIb was significantly higher in KO vs. C muscles. The percentage of type I and IIa MHC tended to be lower in KO vs. C muscles, but this difference was not significant.

The effects of LKB1 deficiency on force production, fatigability, and recovery

Force frequency relationship between C and KO was not different (data not shown) demonstrating that both genotypes were able to generate similar forces. Peak tetanic force generation was not different between groups (1.07 ± 0.14 and 1.01 ± 0.10 g tension/mg muscle in C and KO muscle, respectively). During a ten minute contraction period KO diaphragms displayed earlier fatigue than C diaphragms (Fig. 5). Area below the curve analysis confirmed this observation ($P = 0.02$; data not shown). Measurements of diaphragm force generation after 1 and 5 minutes of recovery showed that C diaphragms increased force production to 63% (1 min) and 81% (5 min) and KO diaphragms demonstrated no significant recovery of force after cessation of stimulation (Fig. 6).

Discussion

LKB1/AMPK signaling has been established as a vital component in skeletal muscle metabolism, and recent evidence shows its importance in fiber type expression (Rockl et al., 2007, Suwa et al., 2003). In addition to metabolic disorders, LKB1/AMPK signaling could have clinical implications related to individuals with chronic obstructive pulmonary disease (COPD). Patients with COPD exhibit increases in mitochondrial density, mitochondrial protein content, and fast to slow muscle fiber type shifts in the diaphragm (Levine et al., 2001, Orozco-Levi et al., 1999, Wijnhoven et al., 2006); phenotypic changes similar to rodent models where LKB1/AMPK signaling is activated. Given that LKB1/AMPK signaling plays a major role in mitochondrial and fiber type adaptations in other skeletal muscle, it is possible that this pathway could be involved in the adaptation of the diaphragm to COPD. Since the development of muscle-specific LKB1-KO mice (Koh et al., 2006, Sakamoto et al., 2005, Thomson et al., 2007b), most research has focused on locomotor muscles, without consideration of respiratory muscles. By using a muscle-specific LKB1 KO mouse, we were able to determine that the presence of LKB1 in the diaphragm is important in AMPK and ACC phosphorylation, mitochondrial protein content, fiber type determination, prolonged muscle contraction, and muscle recovery after training.

A 5-min direct electrical stimulation of the diaphragm was sufficient to increase AMPK phosphorylation 3.5-fold. Similarly, AICAR injection (1.0 mg/g) increased pAMPK almost 3-fold. Due to the lack of LKB1, basal pAMPK was very low in KO diaphragms and did not increase with AICAR or ES; indicating that LKB1 is the major upstream kinase of AMPK in the diaphragm under these conditions. pACC levels increased in C groups with both treatments. ACC phosphorylation was unresponsive to AICAR but increased with ES in KO mice. The lack of ACC response to AICAR in the diaphragm from KO mice is consistent with previous findings in isolated EDL muscles from muscle-specific LKB1-KO mice (Sakamoto et al., 2005), suggesting that responsiveness of AMPK and ACC phosphorylation in the diaphragm is similar to electrically-stimulated or AICAR-treated locomotor muscles. However, our findings conflict with other studies in which ACC phosphorylation increased mildly in isolated EDL muscles from both dominant-negative AMPK-expressing mice (AMPK-DN) and muscle-specific LKB1-KO mice after AICAR incubation (Koh et al., 2006, Dzamko et al., 2008). The responsiveness of ACC to AICAR in these studies, and to electrical stimulation in the present study, may be explained by an unknown ACC kinase (Dzamko et al., 2008). It is likely, however, that part or all of this effect is due to allosteric activation of AMPK since there is a low level of active AMPK in both muscle-specific LKB1 knockouts (Koh et al., 2006) and AMPK-DN (Dzamko et al., 2008) muscles that would be subject to allosteric activation by AMP (in the case of contraction) or ZMP (derived intracellularly from AICAR).

To determine the effect of LKB1-deficiency on mitochondrial protein content, we compared C and KO protein levels for a set of mitochondrial proteins as well as PGC-1, a transcriptional co-activator of mitochondrial gene expression. Western blot analysis showed that the majority of mitochondrial proteins analyzed were lower in KO mice which may be due to the lower levels of PGC-1 protein. PGC-1 is a co-activator of the PPAR, NRF and CREB families of transcription factors (Puigserver et al., 1999, Puigserver et al., 1998, Wu et al., 1999). When activated, these transcription factor families increase mitochondrial protein content and mitochondrial biogenesis. Activation of AMPK has been shown to increase PGC-1 levels in rat triceps, C2C12 cells, and L6E9 myotubes (Winder et al., 2006); possibly through the activation of CREB (Thomson et al., 2008). Not all mitochondrial proteins were decreased in the KO diaphragms, including somewhat surprisingly, UCP-3, the expression of which has been shown to be regulated by AMPK (Suwa et al., 2003). The precise reason for the lack of effect is not known. This does not seem to be due to the location of the genes since COX1 (which is mitochondrially encoded) and many proteins encoded by the nucleus were decreased in KO muscles, while COX4 (a nuclearly encoded protein) was not affected in KO muscles. Presumably, the differences have to do with varying degrees of influence that LKB1/AMPK linked transcription factors and cofactors have upon the promoters of the various proteins combined with variable rates of protein degradation. Regardless, our results support a previous study (Thomson et al., 2007b) that demonstrated that the ablation of LKB1 decreases PGC-1 protein content in skeletal muscle. Although the absence of LKB1 and lower levels of PGC-1 may account for the decreased levels of mitochondrial proteins, it may be that this observation also results from the elevated level of type IIb fibers in the KO diaphragms. It is well established that type IIb fibers have less mitochondria than type I, IIa, and IIx fibers and thus would have a decreased amount of proteins associated with mitochondria. On the other hand, altered mitochondrial function may likewise affect fiber type.

Although some reports failed to find a role for AMPK in the regulation of fiber type (Bamford et al., 2003, Putman et al., 2003), others have demonstrated that LKB1/AMPK signaling promotes shifts favoring oxidative fiber types. Voluntary wheel running caused a shift in fiber type in mouse triceps from type IIb to IIx/a, which was dependent, in part, on the presence of AMPK α 2 (Rockl et al., 2007). Likewise, chronic AICAR injection

stimulated the same shift in rat EDL muscles (Suwa et al., 2003). We demonstrate here that LKB1-deficient diaphragms have elevated percentages of type IIb MHC and decreased percentage of type IIx MHC. Type I and IIa MHC tended to be lower in KO muscles, but not significantly so with greater variability. Still, the magnitude of difference between C and KO MHC IIb percentage suggests that lower type I and IIa in the KO also contributed to the shift in MHC composition. Given the increased fatigability of KO diaphragms that we show here, and the previously reported finding that voluntary wheel running distance is lower in KO mice, it might be surmised that the apparent shift to IIb MHC is due to decreased overall activity of the KO mice. However, ambulatory activity in young female (Thomson et al., 2010) and male (unpublished results from our laboratory) KO mice is not different than that of C mice. Therefore, the alteration in fiber-type and mitochondrial protein content does not appear to be due to decreased physical activity, assuming that respiratory activity tracks well with ambulation in these mice. Taken together, these results demonstrate the importance of LKB1 in both promoting increases in oxidative fiber types as well as preserving oxidative fibers. Thus, while activation of LKB1/AMPK signaling favors increases in oxidative fiber types, the ablation of this pathway promotes a more glycolytic fiber type distribution demonstrating that LKB1 is important in the preservation of oxidative fiber types.

Knowing that LKB1-deficient muscle tissue exhibits lower amounts of mitochondria and possibly a decreased ability to promote mitochondrial biogenesis, we measured the force generation and fatigability of C and KO diaphragms. Because this study sought to determine if AMPK is regulated by LKB1 in the diaphragm we adapted a high-intensity stimulation protocol (Comtois et al., 1999) for the effects to be clearly visible. Both genotypes produced similar force frequency patterns which is consistent with another *in vitro* study measuring force production of the extensor digitorum longus muscle in KO mice (Sakamoto et al., 2005). For the initial 60s of the fatigue protocol, the decline in force is similar in KO and C diaphragms; after which KO diaphragms fatigued more than C diaphragms. The inability of the KO diaphragms to recover at all after five minutes was surprising when at least a partial recovery was expected. It is likely that lower levels of mitochondria and proteins—and thus an impaired ability to replenish energy stores—explain the rapid fatigue of KO diaphragms as well as the impaired ability to recovery after the fatigue protocol. The potential role of fatty acid (FA) oxidation might also be considered in the context of fatigability. Phosphorylation of ACC in KO muscles increased with electrical stimulation *in situ*, albeit not to the levels observed in the C muscles. This suggests, however, that FA oxidation can be stimulated by contraction somewhat in the KO muscles. Since FA was not included in the incubation media in our *in vitro* fatigue experiment, it is not known whether the upregulation of FA oxidation would affect the fatigue properties of KO muscle. Future experiments testing this possibility would be valuable.

In conclusion, this study demonstrates that AMPK is phosphorylated in the diaphragm in an LKB1-dependent manner. LKB1 deficiency causes lower protein levels of PGC-1, mitochondrial proteins, as well as a fiber type shift from type IIx to type IIb muscle fibers. We present evidence that the absence of LKB1 increases the rate of fatigue and a decreased ability of the diaphragm to recover after a 10-min training session. We conclude the following regarding this signaling pathway in the diaphragm: (1) LKB1 is necessary for proper AMPK phosphorylation, (2) LKB1 is a regulator of mitochondrial protein and PGC-1 levels, (3) LKB1 plays a role in fiber type determination, (4) LKB1 is required to lower fatigue and promote rapid recovery in the diaphragm, and (5) LKB1 is not essential for normal force production.

Acknowledgments

We thank Hoon Kim for animal husbandry and genotyping. This study was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-051928.

References

- Bamford JA, Lopaschuk GD, MacLean IM, Reinhart ML, Dixon WT, Putman CT. Effects of chronic AICAR administration on the metabolic and contractile phenotypes of rat slow-and fast-twitch skeletal muscles. *Can J Physiol Pharmacol* 2003;81:1072–82. [PubMed: 14719043]
- Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF, Shulman GI. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *American Journal of Physiology-Endocrinology and Metabolism* 2001;281:E1340–E1346. [PubMed: 11701451]
- Bolster DR, Crozier SJ, Kimball SR, Jefferson LS. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J Biol Chem* 2002;277:23977–80. [PubMed: 11997383]
- Branvold DJ, Allred DR, Beckstead DJ, Kim HJ, Fillmore N, Condon BM, Brown JD, Sudweeks SN, Thomson DM, Winder WW. Thyroid hormone effects on LKB1, MO25, phospho-AMPK, phospho-CREB, and PGC-1alpha in rat muscle. *J Appl Physiol* 2008;105:1218–27. [PubMed: 18669938]
- Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochem J* 2000;346(Pt 3):659–69. [PubMed: 10698692]
- Comtois AS, El-Dwairi Q, Laubach VE, Hussain SN. Lipopolysaccharide-induced diaphragmatic contractile dysfunction in mice lacking the inducible nitric oxide synthase. *Am J Respir Crit Care Med* 1999;159:1975–80. [PubMed: 10351947]
- Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem* 1995;229:558–65. [PubMed: 7744080]
- Davies SP, Helps NR, Cohen PT, Hardie DG. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett* 1995;377:421–5. [PubMed: 8549768]
- Dzamko N, Schertzer JD, Ryall JG, Steel R, Macaulay SL, Wee S, Chen ZP, Michell BJ, Oakhill JS, Watt MJ, Jorgensen SB, Lynch GS, Kemp BE, Steinberg GR. AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. *J Physiol* 2008;586:5819–31. [PubMed: 18845612]
- Fisher JS, Gao J, Han DH, Holloszy JO, Nolte LA. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *Am J Physiol Endocrinol Metab* 2002;282:E18–23. [PubMed: 11739078]
- Foretz M, Carling D, Guichard C, Ferre P, Foulfelle F. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. *J Biol Chem* 1998;273:14767–71. [PubMed: 9614076]
- Gaidhu MP, Fediuc S, Ceddia RB. 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside-induced AMP-activated protein kinase phosphorylation inhibits basal and insulin-stimulated glucose uptake, lipid synthesis, and fatty acid oxidation in isolated rat adipocytes. *J Biol Chem* 2006;281:25956–64. [PubMed: 16816404]
- Giulian GG, Moss RL, Greaser M. Improved methodology for analysis and quantitation of proteins on one-dimensional silver-stained slab gels. *Anal Biochem* 1983;129:277–87. [PubMed: 6189421]
- Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* 2008;30:214–26. [PubMed: 18439900]
- Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J Biol Chem* 1996;271:27879–87. [PubMed: 8910387]

- Henin N, Vincent MF, Gruber HE, Van den Berghe G. Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. *FASEB J* 1995;9:541–6. [PubMed: 7737463]
- Hutber CA, Hardie DG, Winder WW. Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am J Physiol* 1997;272:E262–6. [PubMed: 9124333]
- Koh HJ, Arnolds DE, Fujii N, Tran TT, Rogers MJ, Jessen N, Li Y, Liew CW, Ho RC, Hirshman MF, Kulkarni RN, Kahn CR, Goodyear LJ. Skeletal muscle-selective knockout of LKB1 increases insulin sensitivity, improves glucose homeostasis, and decreases TRB3. *Mol Cell Biol* 2006;26:8217–27. [PubMed: 16966378]
- Koh HJ, Brandauer J, Goodyear LJ. LKB1 and AMPK and the regulation of skeletal muscle metabolism. *Curr Opin Clin Nutr Metab Care* 2008;11:227–32. [PubMed: 18403917]
- Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 1999;48:1667–71. [PubMed: 10426389]
- Levine S, Nguyen T, Shrager J, Kaiser L, Camasamudram V, Rubinstein N. Diaphragm adaptations elicited by severe chronic obstructive pulmonary disease: lessons for sports science. *Exerc Sport Sci Rev* 2001;29:71–5. [PubMed: 11337826]
- Lizcano JM, Goransson O, Toth R, Deak M, Morrice NA, Boudeau J, Hawley SA, Udd L, Makela TP, Hardie DG, Alessi DR. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J* 2004;23:833–43. [PubMed: 14976552]
- Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* 1997;273:E1107–12. [PubMed: 9435525]
- Orozco-Levi M, Gea J, Lloreta JL, Felez M, Minguella J, Serrano S, Broquetas JM. Subcellular adaptation of the human diaphragm in chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:371–8. [PubMed: 10065684]
- Petrof BJ, Stedman HH, Shrager JB, Eby J, Sweeney HL, Kelly AM. Adaptations in myosin heavy chain expression and contractile function in dystrophic mouse diaphragm. *Am J Physiol* 1993;265:C834–41. [PubMed: 8214039]
- Powers SK, Criswell D. Adaptive strategies of respiratory muscles in response to endurance exercise. *Med Sci Sports Exerc* 1996;28:1115–22. [PubMed: 8882998]
- Puigserver P, Adelmant G, Wu Z, Fan M, Xu J, O'Malley B, Spiegelman BM. Activation of PPARgamma coactivator-1 through transcription factor docking. *Science* 1999;286:1368–71. [PubMed: 10558993]
- Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 1998;92:829–39. [PubMed: 9529258]
- Putman CT, Kiricsi M, Pearcey J, MacLean IM, Bamford JA, Murdoch GK, Dixon WT, Pette D. AMPK activation increases uncoupling protein-3 expression and mitochondrial enzyme activities in rat muscle without fibre type transitions. *J Physiol* 2003;551:169–78. [PubMed: 12813156]
- Reiter AK, Bolster DR, Crozier SJ, Kimball SR, Jefferson LS. Repression of protein synthesis and mTOR signaling in rat liver mediated by the AMPK activator aminoimidazole carboxamide ribonucleoside. *Am J Physiol Endocrinol Metab* 2005;288:E980–8. [PubMed: 15613684]
- Rockl KS, Hirshman MF, Brandauer J, Fujii N, Witters LA, Goodyear LJ. Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. *Diabetes* 2007;56:2062–9. [PubMed: 17513699]
- Rubink DS, Winder WW. Effect of phosphorylation by AMP-activated protein kinase on palmitoyl-CoA inhibition of skeletal muscle acetyl-CoA carboxylase. *J Appl Physiol* 2005;98:1221–7. [PubMed: 15579580]
- Sakamoto K, Goransson O, Hardie DG, Alessi DR. Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am J Physiol Endocrinol Metab* 2004;287:E310–7. [PubMed: 15068958]

- Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, Alessi DR. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J* 2005;24:1810–20. [PubMed: 15889149]
- Srere, PA. *Methods in Enzymology*. New York: Academic Press; 1969. Citrate Synthase; p. 3-6.
- Suwa M, Nakano H, Kumagai S. Effects of chronic AICAR treatment on fiber composition, enzyme activity, UCP3, and PGC-1 in rat muscles. *J Appl Physiol* 2003;95:960–8. [PubMed: 12777406]
- Talmadge RJ, Roy RR. Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J Appl Physiol* 1993;75:2337–40. [PubMed: 8307894]
- Thomson DM, Brown JD, Fillmore N, Condon BM, Kim HJ, Barrow JR, Winder WW. LKB1 and the regulation of malonyl-CoA and fatty acid oxidation in muscle. *American Journal of Physiology-Endocrinology and Metabolism* 2007a;293:E1572–E1579. [PubMed: 17925454]
- Thomson DM, Hancock CR, Evanson BG, Kenney SG, Malan BB, Mongillo AD, Brown JD, Hepworth S, Fillmore N, Parcell AC, Kooyman DL, Winder WW. Skeletal muscle dysfunction in muscle-specific LKB1 knockout mice. *J Appl Physiol* 2010;108:1775–85. [PubMed: 20360428]
- Thomson DM, Herway ST, Fillmore N, Kim H, Brown JD, Barrow JR, Winder WW. AMP-activated protein kinase phosphorylates transcription factors of the CREB family. *Journal of Applied Physiology* 2008;104:429–438. [PubMed: 18063805]
- Thomson M, Porter BB, Tall JH, Kim HJ, Barrow JR, Winder WW. Skeletal muscle and heart LKB1 deficiency causes decreased voluntary running and reduced muscle mitochondrial marker enzyme expression in mice. *American Journal of Physiology-Endocrinology and Metabolism* 2007b;292:E196–E202. [PubMed: 16926377]
- Wijnhoven JH, Janssen AJ, van Kuppevelt TH, Rodenburg RJ, Dekhuijzen PN. Metabolic capacity of the diaphragm in patients with COPD. *Respir Med* 2006;100:1064–71. [PubMed: 16257195]
- Williamson DL, Bolster DR, Kimball SR, Jefferson LS. Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. *Am J Physiol Endocrinol Metab* 2006;291:E80–9. [PubMed: 16760336]
- Winder WW, Hardie DG. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *American Journal of Physiology-Endocrinology and Metabolism* 1996;33:E299–E304.
- Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M, Holloszy JO. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *Journal of Applied Physiology* 2000;88:2219–2226. [PubMed: 10846039]
- Winder WW, Taylor EB, Thomson DM. Role of AMP-activated protein kinase in the molecular adaptation to endurance exercise. *Medicine and Science in Sports and Exercise* 2006;38:1945–1949. [PubMed: 17095928]
- Winder WW, Thomson DM. Cellular energy sensing and signaling by AMP-activated protein kinase. *Cell Biochem Biophys* 2007;47:332–47. [PubMed: 17652779]
- Winder WW, Wilson HA, Hardie DG, Rasmussen BB, Hutber CA, Call GB, Clayton RD, Conley LM, Yoon S, Zhou B. Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J Appl Physiol* 1997;82:219–25. [PubMed: 9029219]
- Woods A, Cheung PC, Smith FC, Davison MD, Scott J, Beri RK, Carling D. Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J Biol Chem* 1996;271:10282–90. [PubMed: 8626596]
- Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 1999;98:115–24. [PubMed: 10412986]

Fig. 1. Effect of LKB1 deficiency upon AMPK and ACC phosphorylation in mouse diaphragm after 1hr post-AICAR injection or a 5-min direct electrical stimulation

Western blots show LKB1 protein levels (A) and phosphorylation states of AMP-activated protein kinase (pAMPK; Thr172) and Acetyl CoA Carboxylase (pACC1 & pACC2; Ser 79 & Ser221, respectively), with electrical stimulation (B & C) or AICAR (D & E) treatments. Values are means $\pm$ SE ($n = 5-8$). *Significantly different from control sham/saline groups. †Significantly different from sham KO group ($P < 0.05$).

Fig. 2. Protein content of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1) and mitochondrial proteins in LKB1-deficient mouse diaphragms

Western blots showing protein levels of PGC-1, Complex II, Complex 3 Core 2 (Core 2), Cytochrome C, Cytochrome Oxidase I (COX1), ATP Synthase α -subunit (ATP Synthase), Long Chain Acyl Dehydrogenase (LCAD). Values are means $\pm$ SE (n = 13–15).

*Significantly different from control mice ($P < 0.05$).

Fig. 3. Citrate synthase (CS) activity in LKB1-deficient mouse diaphragm

Spectrophotometric determination of CS activity based on the conversion of oxaloacetate and acetyl CoA to citrate. Values are means $\pm$ SE (n = 5–6). *Significantly different from control mice ($P < 0.05$).

Fig. 4. Myosin heavy chain-isoform (MHC) percentage distribution in mouse diaphragms lacking LKB1

Electrophoresis of homogenized diaphragms followed by silver staining. Molecular weight and MHC-isoform standards were used to identify the various isoforms. Top: Representative image of the MHC bands. KO = muscle-specific LKB1-knockout mice; C = littermate control mice. Bottom: Mean MHC distribution $\pm$ SE (n=13–15/group), expressed as the percentage of the total MHC signal comprised by each MHC isoform. *Significantly different from control mice ($P < 0.05$).

Fig. 5. 10-minute *In vitro* stimulation of LKB1-deficient diaphragms

Rate of fatigue was determined by stimulating a diaphragm strip (~2mm) at 200 Hz every 2 seconds (100 m sec train duration) for ten minutes. Values are means $\pm$ SE ($n = 7-10$).

*Significantly different from control mice ($P < 0.05$).

Fig. 6. Force production of LKB1-deficient diaphragms at 0 min, 1 min, and 5 min after 10-minute *in vitro* stimulation

After the ten minute fatigue protocol, diaphragms were stimulated for 5 additional pulses at 1-min and 5-min to determine ability to recover force production. Values are means $\pm$ SE ($n = 7-10$). *Significantly different from corresponding genotype at 0 min ($P < 0.001$). † Significantly different from control at corresponding time point ($P < 0.05$).