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Jacob D. Brown
Brigham Young University - Provo

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Jacob D. Brown

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Master of Science

David M. Thomson, chair
William W. Winder
Chad R. Hancock
Allen C. Parcell

Department of Physiology and Developmental Biology

Brigham Young University

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Date	David M. Thomson, Chair
Date	William W. Winder
Date	Chad R. Hancock
Date	Allen C. Parcell
Date	Dixon J. Woodbury, Graduate Coordinator
Date	Rodney J. Brown, Dean of Life Sciences

ABSTRACT

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Signaling in the Diaphragm

Jacob D. Brown

Department of Physiology and Developmental Biology

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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. Two methods of AMPK activation were used to characterize LKB1/AMPK signaling in diaphragms from muscle-specific LKB1 knockout (KO) and littermate control (C) mice: (1) acute injection of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and (2) 5-min direct electrical stimulation (ES) of the diaphragm. Diaphragms were excised 60 minutes post-AICAR injection and immediately after ES. AMPK phosphorylation increased with AICAR and ES in C but not KO mice. Acetyl CoA carboxylase (ACC) phosphorylation increased with AICAR in C but not KO mice, but increased in both genotypes with ES. While the majority of mitochondrial enzyme levels were lower in KO diaphragms, uncoupling protein 3 (UCP-3) levels were not different between genotypes. A IIx to IIb fiber type switch was observed in KO diaphragms. While *in vitro* peak force generation was similar between genotypes, KO diaphragms fatigued more quickly and had an impaired ability to recover. In conclusion, LKB1 regulates AMPK phosphorylation, mitochondrial enzyme expression, fiber type distribution, as well as recovery of the diaphragm from fatigue.

Key words: skeletal muscle, fiber type, fatigue, mitochondria

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PROSPECTUS

INTRODUCTION

LKB1/AMP-activated protein kinase (AMPK) signaling research is receiving more and more attention each year due to the wide variety of processes in which this pathway is involved (i.e. muscle metabolic pathways [1], cell cycle control [2, 3], food intake [4], etc.). Originally, the metabolic functions of AMPK were observed in the liver where it phosphorylated 3-hydroxy-3-methylglutaryl-CoA(HMG-CoA) reductase (cited in [5]). In 1989 further study showed that AMPK was present in skeletal muscle [5], but its function in that tissue was not understood. In 1996 it was shown that AMPK is activated during exercise in skeletal muscle[6]. Due to this discovery, it was hypothesized that LKB1/AMPK signaling may be the link between exercise and the benefits Type 2 diabetics receive from exercise; as well as being a potential target for Type 2 diabetic pharmaceuticals [7]. Since then, hundreds of papers have been published exploring the many roles that LKB1/AMPK signaling plays in skeletal muscle, including adaptation to exercise, insulin sensitivity, and muscle growth/atrophy. The following outlines some of this research as it relates to skeletal muscle.

LKB1 is a constitutively active serine-threonine kinase tumor suppressor [8]. LKB1 mutations are associated with Peutz-Jeghers disease, an autosomal-dominant disorder characterized by melanocytic macules of the lips, multiple gastrointestinal hamartomatous polyps and an increased risk for various neoplasms, including gastrointestinal cancer [9]. Recently, LKB1 has been shown to be an important regulator of skeletal muscle metabolic processes [10, 11]. This observation is explained by the role LKB1 plays as a major upstream activating-kinase of AMP-activated protein kinase (AMPK), a master switch activator of energy

producing systems in skeletal muscle [12]. One major relevance of the continued research of the LKB1-AMPK pathway is that when activated by muscle contraction a cascade of events allows the entrance of glucose into the cell, independent of insulin [13, 14]—an important advancement in the development of treatments for Type 2 Diabetes. Further evidence shows that LKB1 is up regulated during endurance exercise and may play a crucial role in muscle adaptation to exercise training [11, 15].

AMPK is composed of heterotrimeric combinations of the catalytic α subunit and regulatory β and γ subunit isoforms. When activated by contraction or nutritional stress, AMPK promotes ATP-generating processes by activating enzymes that stimulate fatty acid oxidation [6, 16], and promoting glucose transport by increasing insulin sensitivity [17] and GLUT4 translocation to the cell membrane [18]. At the same time, it inhibits ATP consuming pathways like lipid synthesis [19, 20], cholesterol synthesis [21], and protein synthesis [22-25]. AMPK also stimulates mitochondrial enzyme expression [26] and mitochondrial biogenesis [27] which explains how LKB1 promotes muscle adaptation to exercise.

Currently, we know of three upstream kinases for AMPK: LKB1 [28],TAK1 [29], CAMKK [30-32]. The predominant AMPK kinase (AMPKK) in skeletal muscle [12, 33] is LKB1 and will be the main focus for this study. AMPK activity is highly regulated by a balance between the phosphorylation of AMPK by LKB1 and dephosphorylation carried out most likely by protein phosphatase 2C (PP2C) [34]. LKB1 activates AMPK by phosphorylating the alpha subunit on the Thr172 residue [28]. PP2C deactivates AMPK by dephosphorylating Thr 172. An important step to maintaining AMPK in an active configuration is the binding of AMP to the 4-cystathionine-beta-synthase (CBS) domains located on the gamma subunit [35]. Originally it was thought that when AMP is bound to the CBS domains, the allosteric effects make AMPK a

better substrate for LKB1. However, more evidence supports the hypothesis that AMP binding prevents dephosphorylation of AMPK at Thr 172 by PP2C [36]. These observations demonstrate the way in which increased contractions during exercise, manifested by increased levels of AMP, can affect AMPK pathways.

AMPK is activated in limb muscle by electrical stimulation[6, 37], 5-aminoimidazole-4-carboxamide riboside (AICAR) injection [38], and treadmill running [39]. AICAR injection is a useful method to specifically activate AMPK without also activating other proteins that respond to either an exercise regimen or electrical stimulation of the muscle. AICAR is phosphorylated upon entering the cell and turned into ZMP, an AMP mimetic [38]. By giving the animal a proper dose of AICAR, the AMPK pathway is activated without perturbing AMP, ADP, or ATP levels [21, 40].

The discovery of the functions of LKB1/AMPK signaling in skeletal muscle has been facilitated by the generation of a muscle-specific LKB1 knock-out (KO) mouse. Some interesting observations have been made with respect to LKB1-deficiency and its effect on exercise. Thomson *et al.* [33] showed that LKB1-deficiency in cardiac and skeletal muscle causes a decrease in voluntary running and a reduction in muscle mitochondrial marker enzyme expression in mice. In this study, WT and KO mice showed an average running distance of 124 km and 36 km, respectively—a 3.4 fold difference between groups. Furthermore, KO mice forced to run on a treadmill could not keep a pace of 12 m/min, a pace that WT mice easily maintained. Other observations from this study showed that in the KO phospho-acetyl-CoA carboxylase (pACC), a downstream marker of pAMPK activity, and pAMPK were significantly lower in gastrocnemius (gastroc), white quad (WQ), and red quad (RQ) while hexokinase 2 (HK2) did not change in WQ or RQ. Citrate synthase activity was also significantly lower in

WQ, RQ, and Heart (Hrt) of the KO mice. With respect to other mitochondrial marker proteins, Cytochrome C (Cyto C) and peroxisome-proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1) were significantly lower in KO RQ muscle. In addition, Cyto C, PGC-1a, and glucose transporter 4 (GLUT4) tended to be lower in KO mice but not significantly. Overall, the literature demonstrates that LKB1/AMPK signaling strongly influences mitochondrial protein expression as well as fatty acid oxidation.

The literature shows that LKB1/AMPK signaling has been researched extensively in skeletal muscle. However, the studies have been limited mainly to limb muscles, while respiratory muscles have not been given much attention. Knowing that LKB1-deficiency causes the effects stated previously in limb and cardiac muscle, it must be supposed that the respiratory muscles are also being affected. Due to the constant contractions of the respiratory muscles, a consistent supply of energy molecules to these tissues is vital for the organism's survival. While running or lifting weights, ATP and other energy stores, like p-creatine (PCr), are consumed during contractions. If the energy supply is exhausted, the muscle fatigues, contractions become difficult and the animal stops exercise. Just like the limb muscles, the respiratory muscles are contracting at an increased rate during exercise. If they become exhausted, then breathing becomes more difficult. Normally, the diaphragm, like other skeletal muscle, increases its endurance capacity with regular exercise [41-44]. However, it is possible that LKB1-deficiency in the diaphragm does not allow this to occur—further exacerbating the problem. Since the respiratory muscles—especially the diaphragm—are vital to an organism's exercise capacity, further research of the bioenergetic ability and exercise tolerance of the diaphragm will allow us to further understand the role that LKB1/AMPK signaling plays in the overall process of an organism's adaptation to exercise.

The importance of this study is found in the application to treatments of patients who develop respiratory diseases due to mitochondrial dysfunction. Patients with chronic obstructive pulmonary disease (COPD) exhibit fiber type shifts and increases in mitochondrial oxidative enzymes [45] as well as increases in the concentration of mitochondria [46]. A link between these mitochondrial adaptations and these respiratory diseases could be found in studying LKB1/AMPK signaling. Since the LKB1/AMPK pathway has not been examined in depth in the diaphragm, this study will examine the importance of LKB1/AMPK signaling in the diaphragm. Specifically, we will address the effects of LKB1 on AMPK activation in AICAR treated and electrically stimulated diaphragms (explained hereafter). We will also compare the protein levels of mitochondrial enzymes, transcription factors, and co-activators in control (C) and KO diaphragms. MHC isoforms in C and KO diaphragms will also be determined. The data collected in this study should lay some groundwork for future studies ofLKB1/ AMPK signaling in the diaphragm and indirectly to further study of respiratory diseases caused by mitochondrial dysfunctions.

SPECIFIC AIMS AND PURPOSES

Specific Aim #1: to determine AMPK activity in the diaphragm basally and after treatment with AICAR and electrically stimulated contractions.

Hypothesis #1a

AMPK activity (indicated by AMPK phosphorylation) will increase in the diaphragm in response to AICAR injection and electrical stimulation while AMPK activity will be low at basal levels and remain low during both stimulations. AMPK activity increases in skeletal muscle after AICAR treatment and electrical stimulation. We propose that AMPK phosphorylation will increase in the diaphragm after AICAR treatment and electrical stimulation.

Hypothesis #1b

ACC, a well-established downstream target of AMPK, will have a higher level of phosphorylation after AICAR treatment and electrical stimulation. Previous research shows higher levels of ACC phosphorylation in red quad after electrical stimulation [33, 47]. Increased phospho-ACC (pACC) is also observed in AICAR treated [16] skeletal muscle. ACC phosphorylation has been repeatedly shown in the literature to be a good indicator of AMPK activity [16, 48-50].

Specific Aim #2: to compare mitochondrial enzyme, transcription factor, and co-factor levels, as well as citrate synthase activity in the diaphragms of control and KO mice.

Hypothesis #2a

KO diaphragms will have lower levels of hexokinase 2, mitochondrial enzymes (Cyto C, Complex I, Complex II, Core 2 of Complexes II and III, Cytochrome Oxidase I and IV, ATP

Synthase, LCAD, and UCP-3), co-factors (PGC-1) and related transcription factors (CREB). AMPK promotes mitochondrial enzyme expression through activation of PGC-1 and possibly CREB. Due to lower AMPK activity caused by LKB1 deficiency, mitochondrial protein, transcription factors, and co-activator levels should be negatively affected resulting in lower protein levels.

Hypothesis #2b

Citrate synthase activity will be lower in KO diaphragms. Citrate synthase activity is an indicator of mitochondrial activity. Since AMPK is known to affect mitochondrial biogenesis and mitochondrial enzyme expression, it should follow that in LKB1 deficient diaphragms AMPK activity will be low resulting in a decrease in mitochondrial citrate synthase activity. In comparison, LKB1-intact rat diaphragms showed an increase in citrate synthase activity with endurance exercise [44]. Lower citrate synthase activity was observed in heart muscle as well as red and white quadriceps of LKB1-KO mice [33]. We predict the same will occur in KO diaphragms.

Hypothesis #2c

CREB phosphorylation will be lower in KO diaphragms and raised in C diaphragms treated with AICAR or electrically stimulated. It was previously shown that CREB phosphorylation was increased after AICAR treatment in rat epitrochlearis muscle and HEK293 cells[51]. We predict that CREB phosphorylation in KO will be attenuated due to the absence of LKB1 and normal levels of AMPK activity. We also predict that an increase in AMPK activity caused by AICAR or ES will promote an increase in CREB phosphorylation.

Specific Aim #3: to determine fiber type composition of the diaphragm in C and KO mice.

Hypothesis #3a

C mice will have a higher level of type IIa muscle fibers than KO mice. The LKB1/AMPK pathway increases the oxidative capacity of muscle fibers by increasing mitochondrial biogenesis. It is quite possible that at the same time this signaling also effects MHC isoform expression; thus, promoting a shift in fiber type distribution. We predict that C mice will produce more 2a fibers than KO mice.

Hypothesis #3b

KO mice will have a higher frequency of IIb fibers when compared with C mice. Röckl *et al.* showed a training-induced increase in Type IIa/x fibers that was dependent on the presence of AMPK [52]. Suwa *et al.* also demonstrated a shift from IIb to IIx fibers in extensor digitorum longus (EDL) muscle after chronic AICAR administration [53]. We predict that by ablating LKB1 there will be a reverse shift to 2b fibers.

PROPOSED MATERIALS AND METHODS

Animal Care and Development of LKB1 Muscle-Specific Knock-Out Mouse.

Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University. All animals will be housed in a temperature controlled (21-22 °C) environment with a 12h:12h light-dark cycle and fed standard chow and water *ad libitum.* Mice will be obtained from current breeders from the Winder Lab. Muscle-specific LKB1 knockout (KO) mice and Control (C) littermate mice will be used for AICAR treatment and electrical stimulation experiments. A Cre-Lox system was employed in developing this muscle-specific LKB1 knock-out mouse (KO) model where a conditional LKB1 mouse (provided by R. DePinho and N. Bardeesy, Dana-Farber Cancer Institute, Boston, MA) with LoxP sites flanking the LKB1 allele (Control; C) was mated with a muscle creatine kinase-expressing (MCK-Cre) transgenic mouse (provided by C. R. Kahn, Joslin Diabetes Center, Boston, MA) in which Cre recombinase is constitutively activated under the creatine kinase promoter. When bred together, the Cre recombinase will excise the LKB1 gene specifically in skeletal and cardiac muscle tissue. C mice exhibit the same phenotype and level of AMPK activation as WT.

Acute AICAR Treatment

Two methods known to activate AMPK will be used in this study to demonstrate how AMPK responds to each in the diaphragm. In order to determine whether AMPK in the diaphragm is responsive to AICAR, C and KO mice will be injected subcutaneously with either AICAR dissolved in saline (1.0mg/g body weight) or with an equivalent volume of physiological saline solution without AICAR 1 hr before excision of muscles. Mice will be anesthetized

(intraperitoneally) with pentobarbital sodium (0.023mL/g) 30 minutes before excision of muscles to assure complete sedation and equilibration of the muscle tissues. Diaphragms will be excised immediately following the 1 hr AICAR/saline treatment. Muscles will then be promptly freeze-clamped with metal-block tongs at liquid nitrogen temperature and stored at -95°C until homogenization and analysis.

Electrical Stimulation (ES)

C and KO mice will be anesthetized with pentobarbital sodium (0.023 mL/g) 15 minutes before treatments. C (n=7) and KO (n=7) mice will be electrically stimulated [Stimulation Settings: Train Rate = 1 TPS, Train Duration = 500 ms, Stim Rate = 100 PPS, 2 volts] for 5 minutes using a two-pronged probe designed for full-diaphragm stimulation. The diaphragm will be accessed through an incision exposing the peritoneal cavity and inferior side of the diaphragm without tearing the diaphragm or opening the thoracic cavity. Two other groups of C (n=5) and KO (n=5) mice will be used as a sham, control group in which the peritoneal cavity will be opened for five minutes without receiving electrical stimulation. Diaphragms will be excised immediately after the 5 minute period of stimulation or no stimulation. Muscles will then be promptly freeze-clamped with metal-block tongs at liquid nitrogen temperature (-195°C) and stored at -95°C until homogenization and analysis.

Homogenization

Muscles will be glass-ground homogenized 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 ug/ml soybean trypsin inhibitor). Homogenates will then be freeze-thawed twice. A 500 µl aliquot of raw

homogenate will be taken at this time and stored in 0.6 ml microcentrifuge tubes to be used to determine citrate synthase activity. Homogenates will be centrifuged at 1500 x g for 10 minutes. The supernatant will then be placed in microcentrifuge tubes and stored at -95 ºC until needed for assays. A protein assay will be run using a modified Lowry assay (DC Protein Assay, Bio-Rad, Hercules, CA) to determine the correct amounts of homogenate needed to make samples for loading western blots.

Western blot and Immunodetection

Homogenates (supernatant)will be 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% (pACC, tACC), 7.5% (pAMPK, tAMPK, LKB1, PGC-1, HK2, COX4), 10% (pCREB, tCREB, MO25, UCP-3, GLUT4, LCAD, CPT-1), 15% (Cyto C), and 4-15% (Complex I, Complex II, Core 2, COX1, ATP Synthase α) Tris HCl gels (Bio-Rad Criterion System, Hercules, CA.)with 2.5 µg (Complex I, Complex II, Core 2, COX1, ATP Synthase α), 5 µg (CytoC); 10 µg (PGC-1, LCAD, UCP-3, COX-4), 20 µg (LKB1, pAMPK, tAMPK, pACC, tACC, GLUT4, pCREB, tCREB, HK2], 30 µg (MO25) of protein per well. Electrophoresis will be applied for 45-55 min at 200 volts. The proteins will then be transferred to polyvinylidene difluoride (PVDF) membranes at 100 volts for 1 hr. Membranes will be stained with Ponceau S in order to verify equal protein loading. Membranes will then be 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 4ºC with primary antibody diluted in 1% bovine serum albumin (BSA) dissolved in TBST, pH 7.6.

Primary antibody identification numbers and dilutions will be as follows: pAMPK (#2531, 1:5000), tAMPK (#2532, 1:5000), pACC (#3661, 1:5000), pCREB (#9191S, 1:2000),

tCREB (#9104, 1:1000) from Cell Signaling Technology (Beverly, MA); Cyto C (#13156, 1:2000), HK2 (#6521, 1:10000) 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), MO25 (Custom, 1:5000) 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), LCAD (Gift from Daniel Kelly). tACC was identified with streptavidin-horseradish peroxidase (HRP) conjugate (GE Healthcare, Buckinhamshire, UK, #RPN1231V, 1:20000) diluted in TBST.

Membranes will be washed 4x for 5 min with TBST, after which they will be probed with the appropriate HRP-conjugated, secondary antibody for 1 hr at room temperature. Membranes will then be washed again 4 x 5 min with TBST. Membranes will be covered with ECL Plus Western Blotting Detecting Solution (GE Healthcare Bio-Sciences, Piscataway, NJ) for 1.5 min and developed. AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA) will be used to measure relative protein levels.

Citrate Synthase Activity Assay

Citrate synthase will be determined using the method described by Srere *et al. [54]* on diluted raw homogenates after having been freeze-thawed three times.

Electrophoretic Separation of MHC Isoforms

Gel preparations will use a modified protocol from Talmadge and Roy[55]. Separation of the myosin heavy chain ATPases (MHC) will be achieved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4% stacking gel (30% glycerol, 4% acrylamide with a 50:1 acrylamide: N,N'-methlene-bis-acrylamide ratio, 70 mM Tris (pH 6.8), 4

mM EDTA, and 0.4% sodium dodecyl sulfate (SDS)) and an 8% separating gel (30% glycerol, 8% acrylamide with a 50:1acrylamide: N,N'-methylene-bis-acrylamide ratio, 0.2 M Tris (pH 8.8), 0.1 M Glycine, and 0.4% SDS) using a Hoefer SE 600 gel electrophoresis system. Once prepared the solutions will be degassed for 15 minutes, after which 0.2 ml or 0.3 ml of 10% ammonium persulfate and 0.02 ml or 0.015 ml of ,N,N',N'tetramethylethylenediameine (TEMED) will be added to the 4% and 8% solutions, respectively.

Samples will be loaded at a concentration of 1.5 ug protein/well. The gels will be placed in the electrophoresis chamber with electrode buffer (0.025 M Tris, 0.192 M Glycine and 0.1% SDS) and 2-mercaptoethanol which is added only to the upper electrode chamber at a final concentration of 10 mM. A constant voltage of 275 V will be applied for 22 hours at a temperature of 4 C.

After electrophoresis is completed, the gels will be placed in a fixing solution (50% ethanol, 10% glacial acetic acid, 40% deionized water) for at least 30 minutes. The gels will then be placed in a cross-linking solution (10 % gluteraldehyde and 90% deionized water) for another 30 minutes. A rinse procedure consisting of 3-10 minute and 2-20 minute rinses with distilled water will be performed.

Silver staining will follow the methods described in Giulian *et al[56]*. The gels were incubated in a silver stain solution (6 ml of 1.14 M AgNO3; 31.5 ml of 90 mM NaOH; 2.1 ml of 14.8 M NH4OH and 110 ml cold deionized water) for 5 minutes. Following staining, the gels will be rinsed in deionized water (3x2 min). Gel developing will use citric acid/formaldehyde solution (2.5 ml of 47.6 mM citric acid; 250 ul formaldehyde (37% formaldehyde/10% methanol) in 400 ml of deionized water). Once developed, the developing reaction will be stopped with a stopping solution (40% ethanol; 50% deionized water; 10% glacial acetic acid).

Protein bands will be identified as MHC I, IIa, IIx, or IIb depending on their migration characteristics compared with a molecular weight standard and MHC standard. Analysis will performed with densitometric computer software.

Statistics

Student *t* test or a two-way ANOVA will be used to determine statistical significance ($P \leq 0.05$) where appropriate, using Microsoft Excel or NCSS statistical analysis software (NCSS, Kaysville, UT).

Additional Costs

There are no plans for additional costs at this point.

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THESIS

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 (17, 45). LKB1 is a Ser/Thr kinase tumor suppressor (34) that phosphorylates AMPK (16, 35, 40) in skeletal muscle at Thr172 of the alpha subunit (13). LKB1 also phosphorylates 12 additional AMPK-related kinases (20), 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 (47). AMPK activity is regulated by AMP binding to the γ subunit (5) which activates AMPK by preventing dephosphorylation (7). 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 (39, 43) and glucose transport (8, 18). AMPK also inhibits ATP-consuming pathways that promote fatty acid (9, 10), cholesterol (14), and protein synthesis (3, 12, 31, 42). AMPK is activated in skeletal muscle during exercise (43), electrical stimulation (15), and treatment with the AMP mimetic 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (6, 23). Acetyl CoA Carboxylase (ACC), a potent inhibitor of fatty acid oxidation, is phosphorylated and inactivated by AMPK in skeletal muscle (43) and ACC phosphorylation is widely regarded as a good indicator of AMPK activity *in vivo* (4, 33, 39, 46). Previous studies have also shown the importance of LKB1/AMPK signaling in mitochondrial biogenesis and enzyme expression (2, 30, 40, 44).

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 AMPKα2-KO mice (32). In another study, a 14-day AICAR treatment produced a decrease in type Iib fibers in rat extensor digitorum longus (EDL) muscles (37). 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 (16, 35, 40). These mice exhibit diminished AMPK activity, reduced phosphorylation of ACC, decreased voluntary wheel running, and lower levels of mitochondrial enzymes (40). Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1) protein levels are also attenuated which could explain the observed decrease in oxidative capacity of these animals (16, 40) 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 enzyme activity (27). 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.

The majority of skeletal muscle LKB1/AMPK signaling research is focused on locomotor muscles (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 1hr post-injection) and a 5-min direct electrical stimulation of the diaphragm, (2) mitochondrial enzyme, PGC-1, and hexokinase II (HK2) 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 LKB1 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-knock-out mouse (KO) model was generated through a Cre-Lox system in which Cre recombinase expression was activated by the muscle creatine kinase promoter (40). Conditional (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.

AICAR Treatment

C and KO male mice were injected subcutaneously with either AICAR dissolved in saline (1mg 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 treatments. 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

Muscles 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 ug/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 x 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

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, Hexokinase 2 (HK2), 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 [CytoC]; 10 μg [PGC-1, LCAD, UCP-3, COX4], and 20 μg (LKB1, pAMPK, tAMPK, pACC, tACC, HK2] 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); CytoC (#13156, 1:2000), HK2 (#6521, 1:10000) 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, Buckinhamshire, UK, #RPN1231V, 1:20000) diluted in TBST.

Membranes were washed 4 x 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 x 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 using the method described by Srere (36).

Myosin Heavy Chain (MHC) Composition

MHC composition was assessed using a modified protocol by Talmadge and Roy (38). 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 ug 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% gluteraldehyde for 30 minutes and rinsed 3 x 10 min and 2 x 20 min in distilled water and then silver stained using the protocol described by Giulian *et al.* (11). 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 $NaCO_3$, 11 mM D-glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $NaH_2PO_4 \cdot H_2O$, 1 mM $MgSO_4$, pH 7.4; aerated with 95% O_2/5% CO_2 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 msec to determine the rate of fatigue. Acute diaphragm recovery was then determined by 5 additional stimulations after 1-min and 5-min. Muscles were then measured 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 ± standard error of the mean (S.E.M.). Statistical significance was set at $P \leq 0.05$ or $P \leq 0.001$.

RESULTS

Effects of LKB1 Deficiency on AMPK Phosphorylation in AICAR-Treated (AIC) and Electrically Stimulated (ES) Mouse Diaphragm

LKB1 protein content was absent in KO diaphragms as shown by western blotting (Fig. 1). AMPK phosphorylation (pAMPK) increased with AICAR and ES in C mice, but was virtually non-existent 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).

Effects of LKB1 Deficiency on PGC-1, Mitochondrial Enzyme Expression, and HK2 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 expression was decreased for: Complex 2 (67.5% of C), Core 2 of Complexes 2 and 3 (74.9% of C), CytoC (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). HK2 ($p = 0.07$; 83.8% of C) levels tended to be lower in KO diaphragms (data not shown).

Myosin Heavy Chain Composition

In both C and KO diaphragms, Type II fibers are predominant in mouse diaphragm, which is consistent with previous findings (26). MHC expression in C diaphragm was as follows (Fig. 4): Type I – 6.93% ± 2.28, Type IIa – 20.42 % ± 3.07, Type IIx – 44.54% ± 1.47, Type IIb – 28.12%

± 1.68. KO diaphragm analysis showed MHC expression as follows: Type I – 2.87% ± 1.30, Type IIa – 18.47 % ± 1.87, Type IIx – 40.61% ±1.02, Type IIb – 38.05% ± 1.71.There was no significant difference in I or IIa expression between C and KO diaphragms. However, there is a fiber type shift from IIx to IIb in KO diaphragms.

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. 6). 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.

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 (32, 37). Since the development of muscle-specific LKB1-KO mice (16, 35, 40), 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 enzyme expression, fiber type determination, prolonged muscle contraction, and muscle recovery after training.

Consistent with previous findings in locomotor muscles, 5-min direct electrical stimulation of the diaphragm was sufficient to increase AMPK phosphorylation 2.5-fold in C diaphragms. Similarly, AICAR injection (1.0 mg/g) increased pAMPK almost 2-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. As observed previously in other skeletal muscle, ACC phosphorylation was unresponsive to AICAR but increased with ES in KO mice. This suggests that either ES leads to ACC activation in non-skeletal muscle cells (e.g. satellite cells, blood vessels, etc.) in the diaphragm or that there are LKB1-independent mechanisms (e.g. allosteric activation of AMPK, additional ACC kinases, etc.) that promote ACC phosphorylation during muscle contraction. These data demonstrated that responsiveness of AMPK and ACC phosphorylation in the diaphragm is similar to electrically-stimulated or AICAR-treated locomotor muscles.

To determine the effect of LKB1-deficiency on mitochondrial enzyme expression, we compared C and KO protein levels. Western blot analysis showed that the majority of

mitochondrial proteins analyzed were lower in KO mice. The lower level of PGC-1 in KO mice likely contributes to this observation due to the role of PGC-1 as a co-activator of the PPAR, NRF and CREB families of transcription factors (28, 29, 48). When activated, these transcription factor families increase mitochondrial enzyme expression and mitochondrial biogenesis.

Although some reports failed to find a role for AMPK in the regulation of fiber type (1, 30), 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 AMPKα2 (32). Likewise, chronic AICAR injection stimulated the same shift in rat EDL muscles (37). Consistent with these findings, we demonstrate here that LKB1-deficient diaphragms exhibit a fiber type shift from type IIx and possibly type I (P=0.12) to type IIb fibers, which is the opposite of what happens when LKB1/AMPK signaling is activated. Our data further supports the role that LKB1 plays in fiber type determination. Although the mechanism by which LKB1/AMPK regulates fiber type determination is not known, the study of epigenetic modifications promoted by LKB1 and AMPK could provide some answers. Recent evidence shows that AMPK is able to regulate histone deacetylases (21, 22) and that epigenetic modifications of the MHC promoters can promote fiber type shifts (25). We can deduce from this recent research that it is possible that LKB1 promotes fiber type shifts through its influence on epigenetic regulation.

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. Interestingly, both genotypes produced similar force frequency patterns. 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 enzymes—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.

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 enzyme expression, and fast to slow muscle fiber type shifts in the diaphragm (19, 24, 41); 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.

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 enzymes, 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 enzyme and PGC-1 levels, (3) LKB1 plays a role in fiber type determination, (4) LKB1 is required during endurance-type exercise to lower fatigue and promote rapid recovery, and (5) LKB1 is not essential for normal force production.

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FIGURES

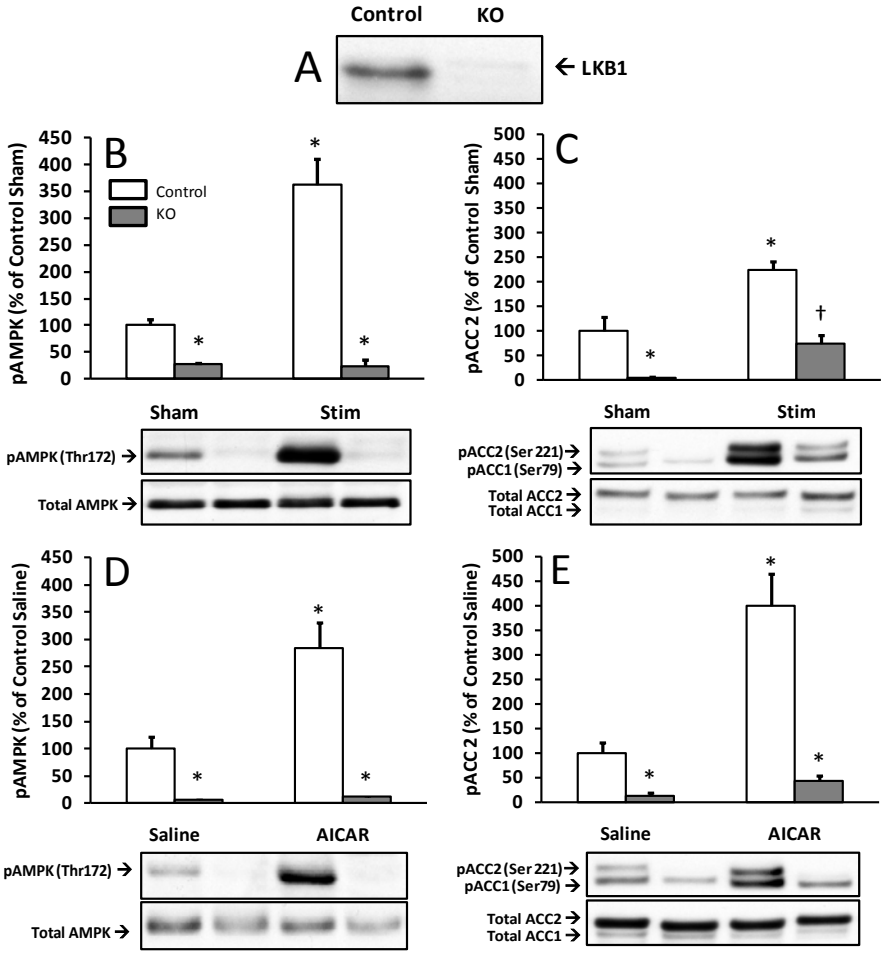

Figure 1— Effect of LKB1 deficiency upon AMPK and ACC phosphorylation in mouse diaphragm after 1hr post-AICAR injection or a 5-min direct electrical stimulation

Western blots show LKB1 protein levels (A) and phosphorylation states of AMP-activated protein kinase (AMPK; Thr172) and Acetyl CoA Carboxylase (ACC; Ser133) with electrical stimulation (B & C) or AICAR (D & E) treatments. Values are means ± SE (n = 5-8). *Significantly different from control sham/saline groups. †Significantly different from sham KO group ($P < 0.05$).

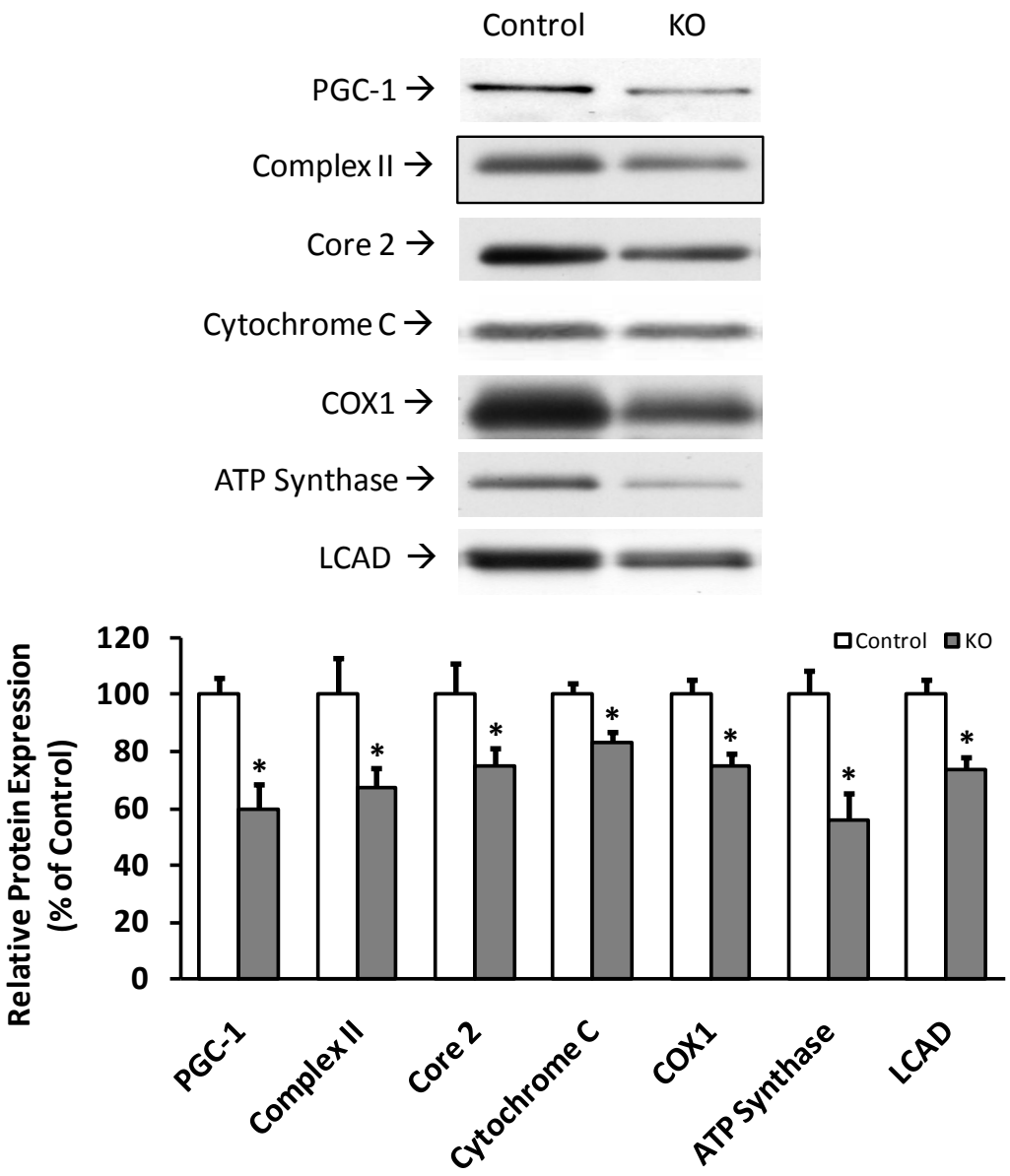

Figure 2—Protein expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1) and mitochondrial enzymes in LKB1-deficient mouse diaphragms

Western blots showing protein levels of PGC-1, Complex II, Core 2 of Complexes II and III of electron transport chain (Core 2), Cytochrome C, Cytochrome Oxidase I (COX1), ATP Synthase α-subunit (ATP Synthase), Long Chain Acyl Dehydrogenase (LCAD). Values are means ± SE (n = 13-15). *Significantly different from control mice ($P < 0.05$).

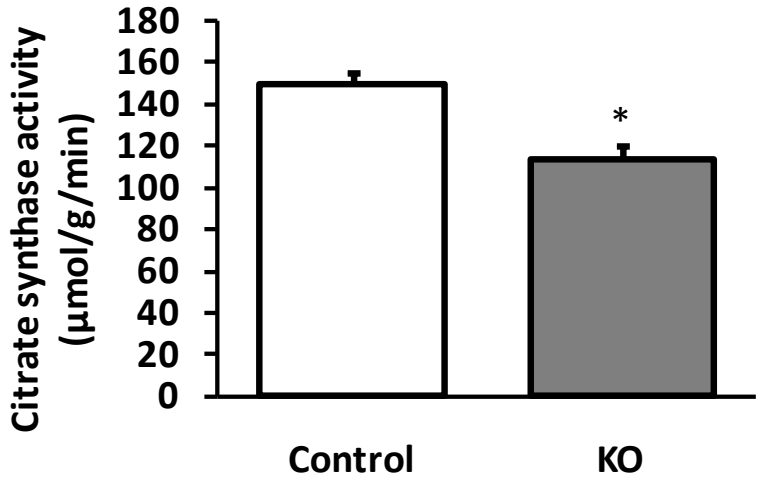

Figure 3—Citrate synthase (CS) activity in LKB1-deficient mouse diaphragm

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

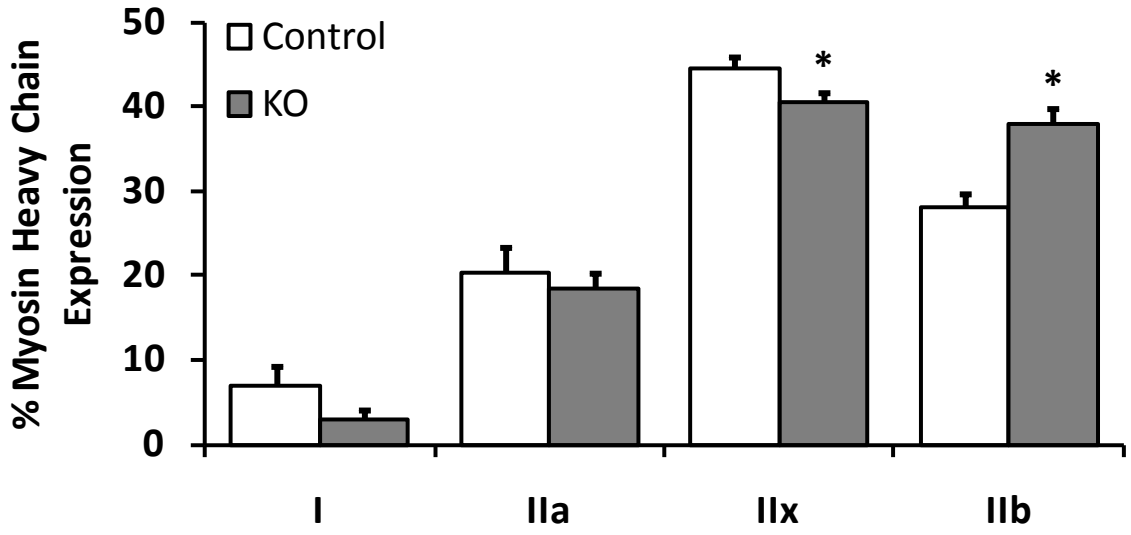

Figure 4—Myosin heavy chain-isoform (MHC) percentage distribution in mouse diaphragms lacking LKB1

Electrophoresis of homogenized diaphragms followed by silver staining. Molecular weight and MHC-isoform markers were used to determine the various isoforms. Values are means ± SE (n = 13-15). *Significantly different from control mice ($P < 0.05$).

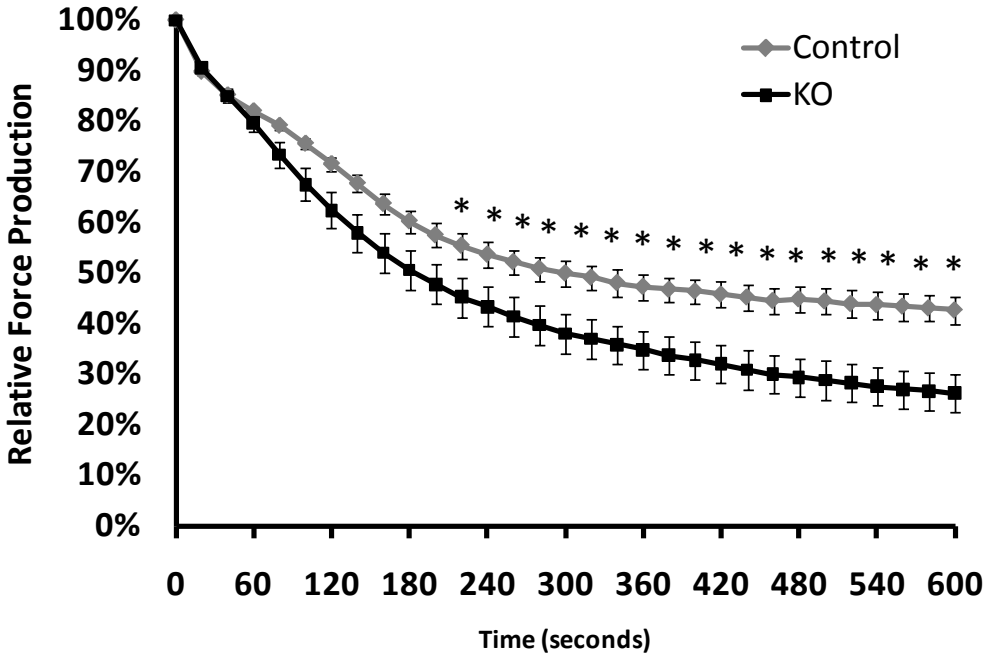

Figure 5—10-minute in vitro stimulation of LKB1-deficient diaphragms

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

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

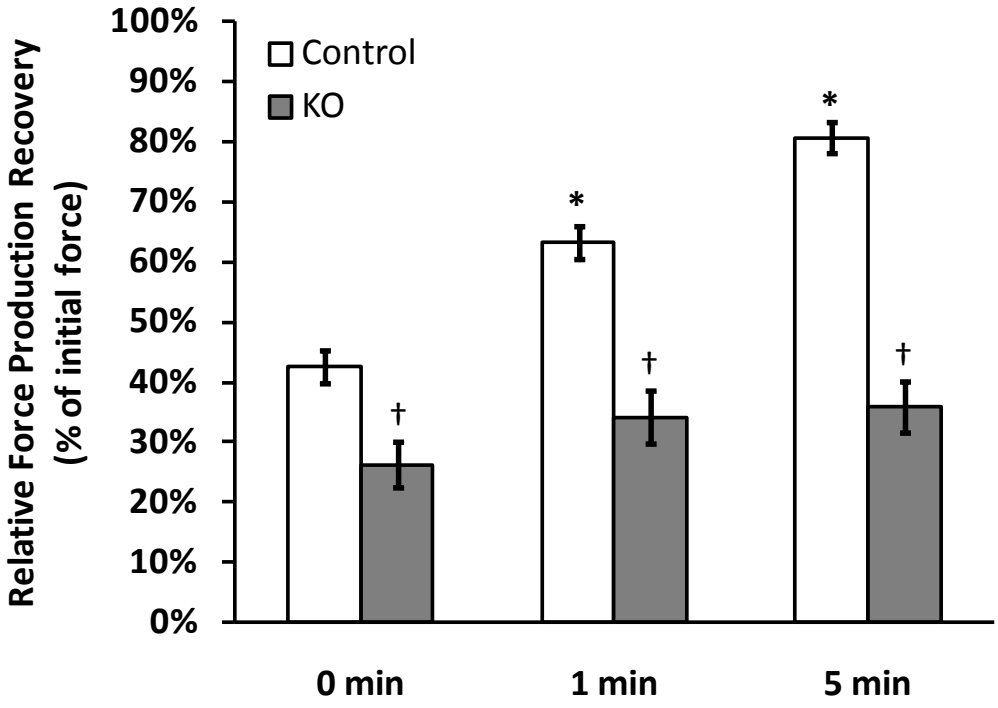

Figure 6—Force production at 0 min, 1 min, and 5 min after 10-minute in vitro stimulation

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 ± SE (n = 7-10). *Significantly different from corresponding genotype at 0 min ($P < 0.001$). † Significantly different from control at corresponding time point ($P < 0.05$).

CURRICULUM VITAE

Jacob D. Brown

Curriculum Vitae
August 2010

Home Address: 1963 N. Canyon Rd. #207 Provo, UT, 84604
Home Phone: (480) 586-0542
Work Address: 594 WIDB
Brigham Young University
Department of Physiology and Developmental Biology
Provo, UT 84602
Work Phone: (801) 422-3322
Date of Birth: October 6, 1982
Birthplace: Mesa, Arizona
Electronic Mail: jacob.brown247@gmail.com
United States Citizen

Education

BS, Physiology and Developmental Biology *April 2008*
Brigham Young University, Provo, UT
Minor: Business Management
Related course work: Anatomy, Advanced Physiology with Lab, Reproductive Physiology, Histology, Pathophysiology, Endocrinology, Biochemistry, Human Dissection, Developmental Biology, and Advanced Cell Biology.

MS, Physiology and Developmental Biology *August 2010*
Brigham Young University, Provo, UT
Mentor: Dr. David Thomson
Committee Members: *William Winder, Chad Hancock, Allen Parcell*
Thesis: LKB1/AMP-Activated Protein Kinase in the Diaphragm
Course work: Cellular Signal Transduction, Physiology, Renal/Gastrointestinal Physiology, Genes and Cancer, Cardiopulmonary Physiology, and Molecular Biology of the Cell

Ph.D., Medical Pharmacology and Physiology *Projected 2014*
University of Missouri, Columbia, MO
Mentor: Dr. Frank Booth

Work Experience

Research Assistant—Exercise Endocrinology/Metabolism *May 2006—Present*
Brigham Young University, Provo, UT
Dr. Dave Thomson/Dr. William Winder Labs
General Focus: Roles of AMP-activated protein kinase (AMPK) in skeletal muscle, targeting of AMPK for prevention and treatment of Type 2 diabetes and obesity, and the study of intracellular signaling pathways controlling skeletal muscle growth and metabolism
Laboratory Skills: Western Blot, protein quantification, tissue sectioning, immunohistochemistry, immunoprecipitation, enzyme activity, spectrophotometry, quantitative RT-PCR, transmission electron microscopy
Models Used: Rat, mouse, and cell culture

Teaching Assistant—Pathophysiology (Dr. Allan Judd) *April 2009—June 2009*
Brigham Young University, Provo, UT
Description: Provide out-of-class help to university students by reviewing the physiological cause of disease in one-on-one sessions and large group discussions/review sessions.

Physiology Lab Instructor *May 2008—July 2008*
Provo College, Provo, UT
Description: Created and directed ten learning exercises related to basic physiological processes (i.e. gradients, hematology, respiration, etc.) for 24 pre-nursing students.

Teaching Assistant/Tutor—Anatomy/Physiology *July 2007—April 2008*
Provo College, Provo, UT
Description: Assisted students in understanding fundamental physiological processes as well as identifying anatomical structures within all organ systems through review sessions and open lab settings.

Teaching Assistant—Pathophysiology (Dr. James Porter) *January 2007 – April 2007*
Brigham Young University, Provo, UT
Description: Provided out-of-class help to university students by reviewing the physiological cause of disease in one-on-one sessions and large group discussions/review sessions.

Teaching Assistant—Anatomy Lab (Dr. Robert Seegmiller *Sept. 2005—Dec. 2006*
Brigham Young University, Provo, UT
Description: Taught approximately 1000 anatomical structures to multiple groups of university students using cadavers, models, and diagrams in structured and open lab settings.

Publications

David Thomson, Chad R. Hancock, Bradley G. Evanson, Steven G. Kenney, Brandon B. Malan, Anthony D. Mongillo, Jacob D. Brown, Squire Hepworth, Natasha Fillmore, Allen C. Parcell, David L. Kooyman, and William W. Winder. **Skeletal Muscle Dysfunction in Muscle-Specific LKB1 Knockout Mice.** *J Appl Physiol.* 2010 Jun;108(6):1775-85.

Thomson DM, Brown JD, Fillmore N, Ellsworth SK, Jacobs DL, Winder WW, Fick CA, Gordon SE. **AMP-Activated protein kinase response to contractions and AICAR treatment in young adult and old skeletal muscle.** *J Physiol.* 2009 May 1;587(Pt 9):2077-86.

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-1{alpha} in Rat Muscle.** *J Appl Physiol*. 2008 Oct;105(4):1218-27.

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.** *J Appl Physiol*. 2008 Feb;104(2):429-38.

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.** *Am J Physiol Endocrinol Metab.* 2007 Dec;293(6):E1572-9.

In Review

Jacob Brown, Chad Hancock, Anthony Mongillo, Ryan DiGiovanni, Allen Parcell, William Winder, and David Thomson **LKB1/AMPK Signaling in the Diaphragm.** Submitted to Acta Physiologica.

Oral Presentations

American Diabetes Association Scientific Sessions, Chicago, IL *June 22-26, 2007*

Thomson DM, Brown JD, Kim HJ, Chesser DG, Fillmore N, Porter BB, Tall JH, Barrow JR, and Winder WW. **LKB1 is Required for AICAR-induced Elevations in Hexokinase II Content in Skeletal Muscle.** (Presenter: Dr. David Thomson)

Poster Presentations

Experimental Biology 2009, New Orleans, LA *April 18-22, 2009*

Brown JD, Mongillo AD, DiGiovanni RA, Lane MD, Parcell AC, Winder WW, and Thomson DM **LKB1/AMPK Signaling in the Diaphragm.**

FASEB Summer Research Conference, Copenhagen, Denmark *August 10-15, 2008*
AMPK: In Sickness and Health – From Molecule to Man

Thomson DM, Fillmore N, Ellsworth SK, Brown JD, Fick CA, Winder WW, Gordon SE. **Contraction-induced AMPK Activity is Elevated in Aged Skeletal Muscle.**

American Diabetes Association Scientific Sessions, Chicago, IL *June 22-26, 2007*

Branvold DJ, Allred DR, Beckstead DJ, Thomson DM, Kim HJ, Fillmore N, Condon BM, Brown JD, and Winder WW. **Regulation of LKB1-STRAD-MO25 Complex Expression and Activation of AMPK in Skeletal Muscle by Thyroid Hormone.**

FASEB Summer Research Conference, Snowmass, CO *August 12-17, 2006*
AMPK: Impact on Mammalian Metabolism and Disease
Thomson DM, Porter BB, Tall J, Kim H-J, Brown J, Thompson B, Anderson J, Allred D, Condon B, Fillmore N, Branvold D, Stone DE, Barrow J, Winder WW. **Muscle-specific LKB1 Knockout Results in Decreased Voluntary Running and Mitochondrial Enzyme Expression.**

Awards

Life Science Fellowship *August 2010-2014*
University of Missouri Life Science Fellowship Program

Graduate Research Assistant *April 2008-August 2010*
NIH Grant-William W. Winder

Research Presentation Award *April 2009*
Brigham Young University Graduate Student Society

Eagle Scout Award *November 1998*
Project: Collection and delivery of non-perishable food and toiletry items for The Salvation Army.

Volunteer Work

Youth Mentor—Health Professions Mentoring *September 2006—March 2007*
Brigham Young University, Provo, UT

Volunteer—Emergency Room and Wound Care *April 2004—August 2005*
Utah Valley Regional Medical Center, Provo, UT

LDS Mission—Northern New Jersey *October 2001—October 2003*
The Church of Jesus Christ of Latter-day Saints
***Mission Headquarters*:** Morristown, New Jersey

Certifications

Phlebotomy *November 9, 2005*
Utah School of Phlebotomy, Provo, UT

Languages

English (Read, write, and speak)
Spanish (Read, write, and speak)

Interests and Hobbies

Reading, History, Sports (Ultimate Frisbee, Football, Racquetball, etc.), Playing Guitar, Songwriting, and Cooking