Skeletal muscle and heart LKB1 deficiency causes decreased voluntary running and reduced muscle mitochondrial marker enzyme expression in mice


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Thomson DM, Porter BB, Tall JH, Kim H-J, Barrow JR, Winder WW. Skeletal muscle and heart LKB1 deficiency causes decreased voluntary running and reduced muscle mitochondrial marker enzyme expression in mice. Am J Physiol Endocrinol Metab 292: E196–E202, 2007. First published August 22, 2006; doi:10.1152/ajpendo.00366.2006.—LKB1 has been identified as a component of the major upstream kinase of AMP-activated protein kinase (AMPK) in skeletal muscle. To investigate the roles of LKB1 in skeletal muscle, we used muscle-specific LKB1 knockout (MLKB1KO) mice that exhibit low expression of LKB1 in heart and skeletal muscle, but not in other tissues. The importance of LKB1 in muscle physiology was demonstrated by the observation that electrical stimulation of the muscle in situ increased AMPK phosphorylation and activity in the wild-type (WT) but not in the muscle-specific LKB1KO mice. Likewise, phosphorylation of acetyl-CoA carboxylase (ACC) was markedly attenuated in the KO mice. The LKB1KO mice had difficulty running on the treadmill and exhibited marked reduction in distance run in voluntary running wheels over a 3-wk period (5.9 ± 0.9 km/day for WT vs. 1.7 ± 0.7 km/day for MLKB1KO mice). The MLKB1KO mice anesthetized at rest exhibited significantly decreased phospho-AMPK and phospho-ACC compared with WT mice. KO mice exhibited lower levels of mitochondrial protein expression in the red and white regions of the quadriceps. These observations, along with previous observations from other laboratories, clearly demonstrate that LKB1 is the major upstream kinase in skeletal muscle and that it is essential for maintaining mitochondrial marker proteins in skeletal muscle. These data provide evidence for a critical role of LKB1 in muscle physiology, one of which is maintaining basal levels of mitochondrial oxidative enzymes. Capacity for voluntary running is compromised with muscle and heart LKB1 deficiency.

AMP-activated protein kinase (AMPK) is a major regulator of skeletal muscle energy metabolism (3, 5, 27, 29). It is activated in response to exercise and muscle contraction and other conditions of metabolic stress when AMP concentration increases (12, 19, 26, 30). When active, AMPK works to restore cellular energy balance by promoting ATP-generating processes such as fatty acid oxidation and glucose uptake, while inhibiting anabolic processes, such as protein synthesis, that consume ATP (3–5, 14, 27–29). In addition to its role in maintaining energy homeostasis during exercise, AMPK is also thought to play an important role in many adaptations to chronic exercise such as elevations in protein levels of GLUT4, hexokinase II, and mitochondrial proteins (2, 7, 9, 25, 31, 34). AMPK is a heterotrimer composed of a catalytic α-subunit and regulatory β- and γ-subunits. Binding of AMP to the γ-subunit of AMPK promotes phosphorylation at Thr172 on its α-subunit, which is requisite for its activity. Several upstream kinases have been shown to phosphorylate AMPK at Thr172, including LKB1 (6, 23, 33), Ca2+/calmodulin-dependent protein kinase (10, 32), and transforming growth factor β-activating kinase (15).

LKB1 was originally identified as a tumor-suppressor protein that is mutated in patients with Peutz-Jeghers syndrome (1). It is constitutively active when associated with the regulatory proteins Ste20-related adapter protein (STRAD) and mouse protein 25 (MO25) and phosphorylates AMPK in addition to several other related proteins (1, 6, 13, 20). Although multiple proteins are capable of phosphorylating AMPK, recent findings demonstrate that LKB1 is the major AMPK kinase in skeletal muscle (20, 21). Specifically, muscle-specific knockout of LKB1 prevents the normal phosphorylation and activation of AMPKα2 in response to in situ muscle contraction and to pharmacological treatment with 5-aminoimidazole-4-carboxamide riboside, a well-established AMPK activator (21). Furthermore, the lack of LKB1 in skeletal muscle results in elevated ADP-to-ATP and AMP-to-ATP ratios after muscle contractions, demonstrating the importance of the protein in maintaining cellular energy balance with contraction (21).

Given the important role of LKB1/AMPK signaling in the cellular response to exercise, the primary purpose of this study was to determine how LKB1 deficiency in cardiac and skeletal muscle would affect the voluntary wheel-running activity of mice. Our data demonstrate the functional importance of muscle LKB1 in regulating physical activity volume, and we also show that decreased mitochondrial protein content may contribute to the suppressed running volume in the LKB1-deficient mice.

METHODS

Animal care and generation of LKB1KO mice. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Mice were bred and housed in a temperature-controlled (21–22°C) room with a 12:12-h light-dark cycle, with free access to standard chow and water. Muscle-specific knockout of LKB1 (LKB1KO) was achieved by cross-breeding LKB1 conditional mice (provided by R. DePinho and N. Bardeesy, Dana-Farber Cancer Institute, Boston, MA) in which the LKB1 allele is flanked by loxP sites with MCK-Cre transgenic mice (provided by C. R. Kahn, Joslin Diabetes Center, Boston, MA) in which Cre recombinase is expressed constitutively in skeletal muscle and heart under the creatine kinase promoter. In the resultant LKB1KO mice, the skeletal muscle-specific expression of Cre leads to loxP recombi-
nation and deletion of the LKB1 gene. Presence of the floxed LKB1 and the MCK-Cre genes was assessed by PCR ear-snip analysis. Primers for detection of Cre were 5’-CCATGAGTGCCCGAACCTGG-3’ and 5’-TGATGAGGTCTGGAAAGACC-3’. Primers for detecting the floxed LKB1 gene were 5’-TCTAACAATTGCGCTCATCGTCATCTCGCC-3’ and 5’-GAGATTGGTACCAGGAGTTGGGCT-3’. The wild-type (WT) LKB1 gene was detected with the primers 5’-GGGCTTACACTGGTGCCAGCGT-3’ and 5’-GAGATTGGTACCAGGAGTTGGGCT-3’.

Verification of muscle LKB1 knockout in each muscle was also performed by Western blotting and immunodetection of LKB1 protein (see RESULTS).

Muscle stimulation protocol. Mice were anesthetized with pentobarbital sodium (0.08 mg/g body wt) for at least 20 min before beginning the procedure. The right gastrocnemius was isolated and frozen using aluminum block tongs at liquid nitrogen temperature. The left tibial nerve was isolated and stimulated at a frequency of 1/s, 10-ms duration, 15 volts, for 5 min. The left gastrocnemius was then clamp-frozen. The muscles were kept at −95°C until analyzed for phospho-AMPK and phospho-acetyl-CoA carboxylase (ACC).

Muscle collection. Mice were anesthetized with pentobarbital sodium (0.08 mg/g body wt). Gastrocnemius, red quadriceps, white quadriceps, and heart muscles were harvested. Tissues were frozen between metal blocks cooled to the temperature of liquid nitrogen and stored at −10°C until processing. Muscles were weighed and then homogenized in 9 vol of homogenization buffer (50 mM Tris-HCl, 250 mM mannitol, 50 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml soybean trypsin inhibitor; pH 7.4). An aliquot of whole homogenate was separated for the citrate synthase activity assay and GLUT4 protein determination, and the rest was clarified by centrifugation at 1,200 g and 4°C.

Citrine synthase activity. Citrate synthase activity was determined on diluted frozen and thawed (3 times) whole muscle homogenates by the method described by Sere (24).

Western blotting and immunodetection. Homogenates were diluted in sample loading buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.1% bromophenol blue) and loaded (6 μl homogenate/lane except for GLUT4, which was 2 μl raw homogenate) on 5% [phospho-(p)-ACC, ACC, hexokinase II], 7.5% (p-AMPK, AMPKα1, AMPKα2, PGC-1, LKB1, GLUT4), or 15% (cytochrome c) Tris-HCl gels (Bio-Rad Criterion System, Hercules, CA.). Electrophoresis was applied for 55 min at 200 volts, after which the proteins were transferred to polyvinylidene difluoride membranes at 100 volts for 1 h. Membranes were stained with Ponceau S to ensure that the proteins were transferred to polyvinylidene difluoride membranes at 100 volts for 1 h. Membranes were stained with Ponceau S to ensure that the proteins were transferred to polyvinylidene difluoride membranes. Primers for detection of Cre were 5’-CCATGAGTGCCCGAACCTGG-3’ and 5’-TGATGAGGTCTGGAAAGACC-3’. Primers for detecting the floxed LKB1 gene were 5’-TCTAACAATTGCGCTCATCGTCATCTCGCC-3’ and 5’-GAGATTGGTACCAGGAGTTGGGCT-3’.

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Total AMPK α2-subunit protein measured by Western blot was not statistically different either in response to voluntary running or in response to LKB1 deficiency (Table 1). Total AMPK α1-subunit protein was significantly increased by 50% in the red quadriceps of WT mice in response to wheel running but was not influenced by lack of LKB1 (Table 1). Voluntary running did not influence the amount of phospho-AMPK detected in the muscles (Fig. 4); wheels were removed from cages at least 1 h before sampling of the muscle. Phosphorylation of the downstream target for LKB1-STRAD-MO25, ACC, was significantly lower in the MLKB1KO muscles than in the WT muscles (Fig. 5). Total ACC was not changed significantly in either white quadriceps or red quadriceps (Table 1).

A marked difference in voluntary running was observed between the WT and MLKB1KO mice (Fig. 6). Over the 21-day running period, WT mice ran an average of 124 km, whereas MLKB1KO mice ran only 36 km, a 3.4-fold difference. The difference in daily running distance between WT and MLKB1KO mice was statistically significant for all days except day 4. In a separate experiment (data not shown), an attempt was made to run mice on a rodent treadmill. The MLKB1KO mice had difficulty keeping pace at 12 m/min, a work rate easily maintained by the WT mice. In that study, phospho-AMPK was undetectable in muscles of the

### Table 1. AMPK, ACC, and GLUT4 in RQ and WQ of WT and muscle-specific LKB1 knockout mice

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Values are means ± SE; n = 7–9 mice/group. AMPK, AMP-activated protein kinase; RQ, red quadriceps; WQ, white quadriceps; ACC, acetyl-CoA carboxylase; WT, wild type; Sed, sedentary; KO, knockout. *Significantly different from WT Sed, P < 0.05.
MLKB1KO mice, even after 30 min of treadmill exercise (data not shown).

GLUT4 was not influenced by LKB1 deficiency. This protein was significantly increased in the WT red quadriceps but not in the white quadriceps (Table 1). Hexokinase II was increased in response to wheel running in both red and white regions of the quadriceps but was not significantly changed in response to LKB1 deficiency (Fig. 7).

Citrate synthase activity was determined to be lower in both sedentary and wheel-running MLKB1KO mice than in corres-

sponding WT mice (Fig. 8). This enzyme was significantly increased in response to wheel running in the red region of the quadriceps in both the WT and MLKB1KO mice. Cytochrome c showed a similar pattern, except that no statistically signifi-

Fig. 5. Phospho-ACC in white and red regions of the quadriceps of WT and MLKB1KO mice. Values are means ± SE (n = 7–9/group). *Significantly different from wild-type treatment groups, P < 0.05.

Fig. 6. Effect of LKB1 deficiency on running performance in voluntary exercise wheels. Values are means ± SE (n = 7–9/group). MLKB1 values are all significantly different from WT values with the exception of day 4, P < 0.05.

Fig. 7. Hexokinase II in white and red regions of the quadriceps of WT and MLKB1KO mice. Values are means ± SE (n = 7–9/group). *Significantly different from WT sedentary, P < 0.05.

Fig. 8. Citrate synthase activity in white and red regions of the quadriceps of WT and MLKB1KO mice. Values are means ± SE (n = 7–9/group). *Significantly different from WT control, P < 0.05. †Significantly different from sedentary MLKB1KO, P < 0.05.
were not statistically significant. Although trends were noted in the same direction for cytosynthase activity in the knockout mice compared with WT. Reduced in MLKB1 KO mice to 20% of that seen in WT sedentary, *Significantly different from WT sedentary, P < 0.05.

![Fig. 9. Cytochrome c in white and red regions of the quadriceps of WT and MLKB1KO mice. Values are means ± SE (n = 7–9/group). *Significantly different from WT sedentary, P < 0.05.](image)

![Fig. 10. PGC-1α in red quadriceps of WT and MLKB1KO mice. Values are means ± SE (n = 7–9/group). *Significantly different from WT sedentary, P < 0.05.](image)

cant increase was observed in red quadriceps in response to wheel running in the knockout mice (Fig. 9). PGC-1α protein levels were reduced in the sedentary knockout mice in red quadriceps (Fig. 10), but no significant differences were observed in white quadriceps (data not shown).

Data from the heart are shown in Table 2. Heart LKB1 was reduced in MLKB1 KO mice to ~20% of that seen in WT mice. Of particular note is the considerable decrease in citrate synthase activity in the knockout mice compared with WT. Although trends were noted in the same direction for cytochrome c, hexokinase, GLUT4, and PGC-1α, these changes were not statistically significant.

![Table 2. Effects of LKB1 deficiency on heart protein content](image)

### DISCUSSION

The finding that LKB1 complexed with STRAD and MO25 is one of the major upstream kinases for AMPK activation in mammalian tissues (6, 23, 33) represents a major advancement in the study of AMPK signaling. Although other AMPK kinases have been identified, strong evidence supports the idea that LKB1 is essential for AMPK phosphorylation in response to muscle contraction. Sakamoto et al. (21) were the first to report data on the muscle-specific LKB1 knockout mouse. Without LKB1, no activation occurs in the AMPK complexes in response to electrical stimulation of the muscle. Contraction-stimulated glucose uptake is prevented in these mice along with marked reduction in phosphorylation and inactivation of ACC (21). Disturbances in the energy charge induced by contraction are exacerbated in LKB1-deficient muscle. AMPK-to-ATP and IMP-to-ATP ratios were considerably higher in electrically stimulated LKB1-deficient muscle compared with WT muscle (21). Similar increases in these ratios were observed in ischemic LKB1-deficient hearts compared with hearts from WT mice (22). This implies that the capacity to make ATP in response to an energy challenge is inadequate in the LKB1-deficient muscle.

The current study was designed to determine if voluntary running performance is influenced by LKB1 deficiency. LKB1 deficiency was confirmed by the following two methods: genotyping and Western blotting. In addition, activation of muscle contraction by stimulation of the tibial nerve produced marked increases in these ratios were observed in ischemic LKB1-deficient hearts compared with hearts from WT mice (22). This implies that the capacity to make ATP in response to an energy challenge is inadequate in the LKB1-deficient muscle.

We emphasize that the MLKB1KO mice were not completely inactive. Without LKB1 in their muscles, these mice were running ~2.4 km/day at the end of the 3-wk voluntary running period. The MLKB1KO mice show no outward phenotypic difference compared with the WT mice, including body weight, muscle weight, and heart weight, yet they were running much less than the WT mice. The reason for this difference in performance is of considerable interest, since it may point to critical roles of LKB1 in skeletal muscle and heart.

We considered one possibility for the reduced running volume to be a reduction in oxidative capacity of the muscle. We and others have previously published data suggesting that...
AMPK activation results in an increase in muscle mitochondrial oxidative enzymes, hexokinase, and GLUT4 (2, 7, 9, 17, 18, 25, 31). If AMPK were essentially inactive in the muscle because of absence of the upstream kinase, LKB1, would critical enzymes of energy-producing pathways be compromised? We decided therefore to measure a few of these proteins. We were somewhat surprised to see that GLUT4 and hexokinase were not deficient in the MLKB1KO muscle. It is clear, however, that redundant signals may control levels of these proteins (8, 16) so that absence of one signal is compensated by another. Citrate synthase and cytochrome c are classical mitochondrial marker enzymes that have been measured to assess the degree of training of the muscle. Both of these mitochondrial marker proteins were diminished in the MLKB1KO muscle. In addition, the transcriptional coactivator involved in inducing increases in gene expression of mitochondrial proteins was also reduced in the MLKB1KO red quadriceps. If additional oxidative enzymes are also reduced, this may explain in part the reduced voluntary running performance and the exaggerated changes in energy charge that occur in response to contraction reported by Sakamoto et al. (21, 22).

In addition to the reduced oxidative capacity of the muscle, it is likely that acute signals for making substrate available for oxidation are also deficient. Although the MLKB1KO muscle shows an increase in glucose uptake in response to insulin, contraction-stimulated glucose uptake is deficient (21). With the reduction in ACC phosphorylation/inactivation, it is possible that malonyl-CoA would remain elevated and carnitine acyl-transferase I inhibited during exercise, thus decreasing the rate of fatty acid oxidation. There is the potential therefore for reduction of availability of blood glucose and reduction in availability of fatty acyl-CoA derivatives entering the mitochondrial matrix for oxidation in the MLKB1KO muscle. Low work rates could be supported but not high work rates. With the reduction in oxidative capacity in the heart, oxygen delivery to the working muscle may also be impaired. Although the current study was not designed to address these issues, they must be considered as distinct possibilities for explaining the reduction in performance.

The fact that citrate synthase activity increased in response to 3 wk of voluntary running in red quadriceps of both the WT and MLKB1KO mice is worthy of mention. Because this enzyme increased in the MLKB1KO muscle, signals in addition to AMPK activation are responsible for mitochondrial biogenesis in response to endurance exercise.

In summary, the MLKB1KO mouse exhibits no increase in AMPK phosphorylation and markedly reduced ACC phosphorylation in response to muscle contraction. These mice show markedly reduced running performance in cages equipped with voluntary running wheels over a 3-wk period. They also have difficulty running on the treadmill. Muscles from the MLKB1KO mice have lower levels of mitochondrial marker enzymes. These data provide evidence for a critical role of LKB1 in muscle physiology, one of which is maintaining basal levels of mitochondrial oxidative enzymes. Without LKB1, capacity for high work rates appears to be compromised.

ACKNOWLEDGMENTS

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GRANTS

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