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LKB1 Regulation of High-Fat Diet-Induced
Adaptation in Mouse Skeletal Muscle

Ting Chen

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

LKB1 Regulation of High-Fat Diet-Induced Adaptation in Mouse Skeletal Muscle

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Doctor of Philosophy

Ad libitum high-fat diet (HFD)-induced obesity leads to insulin resistance in skeletal muscle, altered gene expression, and altered growth signaling, all of which contributes to pathological changes in metabolism. Liver kinase B1 (LKB1) is an important metabolism regulator. The purpose of this dissertation was to understand how knocking out LKB1 influences HFD induced adaptations in mouse skeletal muscle. To do so, control and skeletal muscle LKB1 knock-out (LKB1-KO) mice were put on either standard diet (STD) or HFD for 1 week or 14 weeks, or put on the HFD for 14 weeks and then switched to STD for 1 week (switched diet).

The major differences in adaptation in the LKB1-KO mice include: 1) lower fasting blood glucose levels but impaired glucose tolerance compared to WT mice (although conflicting results are generated if the data is not normalized to fasting blood glucose levels), 2) altered expression of 16 HFD-induced genes, and 3) decreased muscle weight. The lower fasting blood glucose in LKB1-KO mice was likely due to elevated serum insulin levels, and the impaired glucose tolerance was associated with decreased phosphorylation of TBC1D1, an important regulator of insulin stimulated glucose uptake. 16 potential important target genes (metabolism, mitochondrial, cytoskeleton, cell cycle, cell-cell interactions, enzyme, ion channel) were identified in the context of HFD feeding and LKB1-KO. These genes were quantified by RT-PCR and grouped according to changes in their patterns of expression among the different groups. Among several other interesting changes in gene expression, the muscle growth-related protein, MyoD, was not affected by short-term HFD, but increased after long-term HFD, and did not decrease after switched diet, showing that its expression may be an important long-term adaptation to HFD. LKB1-KO promoted anabolic signaling through increasing t-eIF2 α and eIF4E expression, and promoted protein degradation through increasing protein ubiquitination. Because the degradation is the main effect and lead to muscle weight decrease. The effect of HFD and/or LKB1-KO on the LKB1-AMPK system was also determined. The results showed that knocking-out LKB1 decreased AMPK activity, decreased nuclear distribution for AMPK α 2 and increased AMPK α 1 expression. Long-term HFD increased t-AMPK expression in LKB1-KO mice, decreased the cytoplasm p-AMPK and nuclear p/t-AMPK ratio in CON mice.

Together the findings of this dissertation demonstrated HFD induced glucose/insulin tolerance, while LKB1-KO had a controversial effect on glucose/insulin sensitivity. Both HFD and LKB1-KO affect AMPK expression and cellular location, while LKB1-KO also affects AMPK activity. LKB1-KO promoted protein degradation through ubiquitination in skeletal muscle.

Keywords: high-fat diet, LKB1, skeletal muscle, AMPK, insulin/glucose tolerance test, GLUT4

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CHAPTER 1: Review of Literature

Liver Kinase B1: A Master Kinase Regulating Cell Metabolism

LKB1: A Master Kinase

Liver kinase B1 (LKB1), also known as Serine/threonine kinase 11 (STK11), is conserved throughout evolution from worms to mammals [1]. It has a long (50 kDa) and a short (48 kDa) isoform, but the short one appears to be restricted to testis [2]. Alone, LKB1 only has poor intrinsic kinase activity, but binding to the pseudo kinase STE-20 Related Adaptor (STRAD) dramatically improves its kinase activity and mouse protein25 (MO25) is another crucial binding partner that stabilizes the interaction between STRAD and LKB1 [3]. LKB1 binding with STRAD and MO25 results in extensive re-localization of LKB1 to the cytoplasm [3, 4].

Research has shown LKB1 is involved in cell cell cycle arrest [5, 6], apoptosis [7], autophagia [8], energy metabolism [9] as well as in T cell maturation [10], liver glucose homaeostasis[11], tumor growth [12] and epithelial apicobasal polarity[13]. LKB1-knockout in skeletal muscle can increase insulin sentivity1. The heterotrimeric complex (LKB1- MO25-STRAD) was essential for the proficient activation of AMPK in vitro and in vivo [14, 15]. Active LKB1 exerts these cellular effects by activating AMP-activated protein kinase (AMPK) and its AMPK-related kinase (ARK) family members through phosphorylation at a conserved N-terminal residue [16] (Figure 1.1).

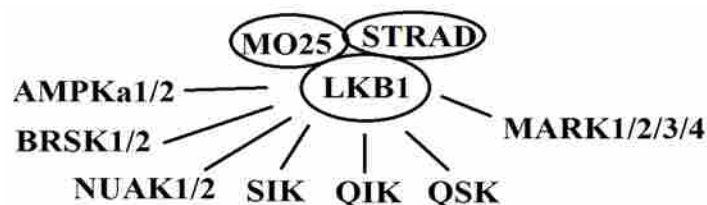


Figure 1.1: The LKB1 Complex (LKB1-STRAD-MO25) Works as the Master Upstream Kinase Controlling AMPK α 1/ α 2 and 11 Downstream ARKs (AMPK-related Kinases).

AMPK and AMPK-Related Kinase Family

AMPK is a well-characterized energy-sensing protein in the cell. It's a heterotrimeric protein formed by α , β , and γ subunits. The subunits also each have different isoforms and they are all expressed in skeletal muscle [17, 18]. AMPK can be phosphorylated and activated by AMPK kinase (AMPKKs), the most important of which are liver kinase B1 (LKB1) and calcium calmodulin-dependent protein kinase kinase (CaMKK). AMPK activation stimulates catabolic processes to generate ATP and inhibits ATP-consuming anabolic processes that are not required for the immediate survival of the cell [19].

Activation of AMPK in skeletal muscle, by phenformin or AICAR, requires LKB1 [19-21]. AMPK also requires LKB1 to phosphorylate HDAC4/5 and the amount of LKB1 can influence the phosphorylation state of HDAC4/5 [22]. Knocking out LKB1 in skeletal muscle decreases phosphorylated AMPK, AMPK- $\alpha 2$ activity (not $\alpha 1$), but increases AMPK- $\alpha 2$, $\beta 2$, and $\gamma 3$ protein expression [23].

The AMPK family also includes 12 other proteins of similar function called ARKs. The ARKs include nuclear AMPK-related kinase 1 (NUAK1/ARK5), NUAK2/SNARK, brain-specific kinase 1 (BRSK1), BRSK2, salt-inducible kinase 1 (SIK1/SNF1LK), SIK2/QIK, SIK3/QSK, microtubule affinity- regulating kinase 1 (MARK1), MARK2, MARK3, MARK4 and maternal embryonic leucine zipper kinase (MELK) (Figure 1.1).

MARK is also known as Par-1. Most ARKs are expressed in skeletal muscle [24-29], except for BRSK, which is only expressed in brain, testis and pancreas [30], and MELK, which is expressed only in undifferentiated, proliferating muscle precursor cells [31]. MARK2 protein expression is hard to detect in skeletal muscle [32], while MARK3 mRNA expression is stable, like a housekeeping gene [33]. ARKs play a role in cell polarity and motility [30], regulation of

metabolic processes, cell cycle control, insulin signaling, fertility, immune system homeostasis, learning & memory, tumor progression, and steroidogenic gene regulation [30]. ARKs also can regulate inflammation. For example, SIKs regulate induction of IL-10, an anti-inflammatory cytokine [34]. Increasing nuclear SNARK expression can change transcription level of many inflammatory genes, like FOS, FOSB, SLC39A14, MAPK14 and MEGF8 [35]. Overexpression of SIK1 and SIK3/QSK, but not SIK2/QIK, significantly inhibited NF- κ B activity in response to lipopolysaccharide stimulation and affected the expression of proinflammatory cytokines [36]. The expression of MARK proteins is associated with an increased inflammation [37]. SIKs and MARKs also can regulate some inflammation-related genes, like Srebp1, IRS1 [9]. Knockout study implicates Par-1b/MARK2/EMK in regulating immune homeostasis [38].

With the exception of MELK, all ARKs are activated by LKB1 at a site equivalent to threonine 172 in AMPK [16], although many of them are also regulated by other kinases. LKB1-KO decreases both SNARK protein expression and its activity [39].

High-Fat Diet (HFD)-Induced Obesity

Health Effects of HFD

It has been known for a long time that high-fat diet (HFD)-induced obesity is detrimental for health. It can either cause or increase the risk of obesity, chronic inflammation, [40] hypertriglyceridemia, insulin resistance, non-alcoholic fatty liver disease, [41] diabetes, Alzheimer's disease, stroke, schizophrenia, some types of cancer, [42] kidney disease [43], atherosclerosis, and osteopenia [44].

Ad libitum HFD feeding in rodents clearly leads to weight gain and increased fat mass [45-49], although one study did not find this [50]. Although the mass of the other organs, like the

heart and liver, can either increase [51, 52] or not change [53] after HFD-induced weight gain, the function for these organs (heart, brain, liver, kidney) usually becomes abnormal [54]. For instance, HFD-obesity can change the pancreas' function. 16 weeks of HFD feeding (60% fat) increased the fat content in the pancreas, which is a feature of non-alcoholic fatty pancreatic disease. Even with increased islet size and alpha and beta-cell number, the mice still became insulin resistant [55]. This may be related to increased autophagosome formation in the beta cells under HFD treatment [56]. Paternal [57] and maternal [58] HFD also can lead to β -cell dysfunction in the offspring.

HFD-Induced Obesity and Insulin Resistance

Insulin resistance is one of the major detrimental effects associated with HFD induced obesity [48, 55, 59, 60]. Insulin resistance is a condition in which the cells of the body, including skeletal muscle, do not respond to insulin normally. In insulin resistant animals or humans, fasting glucose levels are often elevated, and even if they are not, higher fasting insulin concentrations are required to maintain normoglycemia. Insulin resistance results at least in part from impaired intracellular insulin signaling.

When insulin binds with the insulin receptor, it activates the receptor and allows the signal to transfer down to glycogen synthesis and glucose transporter relocation. (Figure 1.2) Akt is the “hub” of the insulin pathway. It can be activated/phosphorylated through insulin-receptor-IRS-PI3K-PDK1 or insulin-receptor-IRS-PI3K-mTORC2. The activated Akt phosphorylates AS160 or TBC1D1, which allows glucose transporter 4 (GLUT4) vesicle translocation. When the GLUT4 moves to the cell membrane, it allows the transport of glucose into the cell by facilitated diffusion. The activated Akt also phosphorylates glycogen synthase kinase (GSK3) and

decreases its activity, and then leads to dephosphorylation of glycogen synthase (GS) to promote glycogen synthesis.

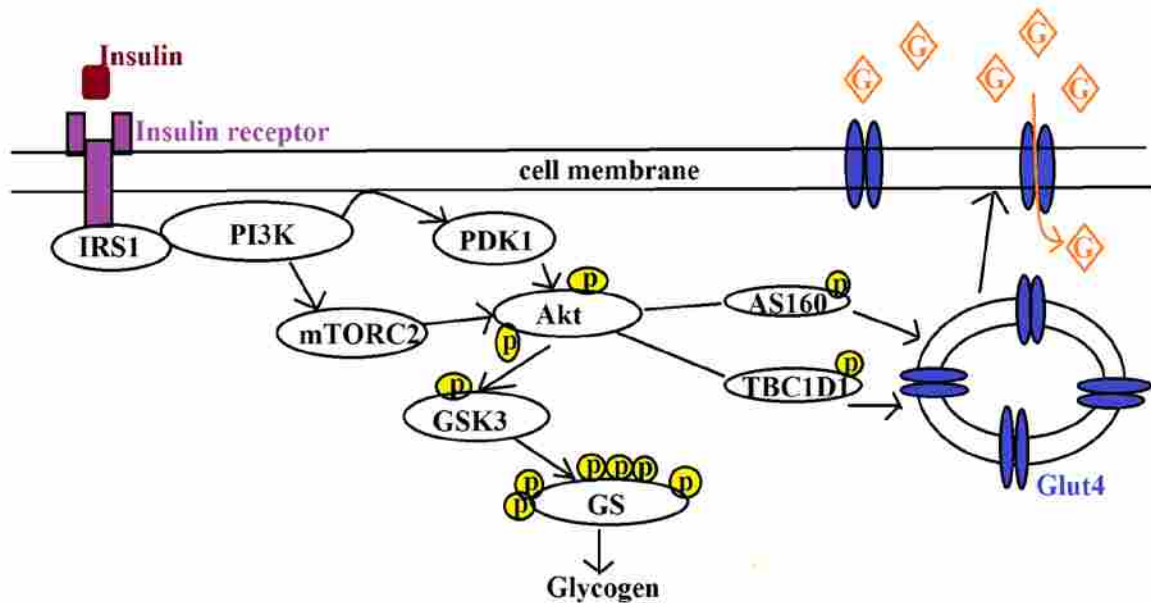


Figure 1.2: Insulin Signaling Pathways Regulating Glucose Transport and Glycogen Synthesis in Skeletal Muscle. IRS1=insulin receptor substrate 1; PI3K=phosphatidylinositol 3-kinase; mTORC2=mechanistic target of rapamycin complex 2; PDK1=phosphoinositide-dependent protein kinase-1; Akt= Protein kinase B/PKB; AS160= TBC1D4= TBC1 domain family member 4; TBC1D1= TBC1 domain family member 1; GLUT4= Glucose transporter type 4; G=glucose; GSK3= glycogen synthase kinase-3; GS=glycogen synthase.

HFD-induced obesity impairs the normal activation of the insulin signaling pathway by insulin. In cardiac muscle [61] and hippocampal neurons [62], HFD didn't affect the expression of insulin receptor (IR), while it decreased insulin stimulated auto-phosphorylation on the IR β subunit (IR β). In the skeletal muscle, HFD decreases insulin receptor substrate 1 (Irs1) protein level [63] and insulin-stimulated tyrosine phosphorylation of IR β [63] and Irs1 [63, 64], but increases phosphorylation of serine 307, 636/639 on Irs1 [64, 65]. Irs1 activity is inhibited by serine phosphorylation, but stimulated by tyrosine phosphorylation. Serine 307 and 302 phosphorylation on Irs1 also increased with HFD treatment in liver [66]. In hippocampal neurons, HFD increased total Irs1 and phosphorylation of serine 616 on Irs1 [67]. HFD feeding leads to increased serine 307 phosphorylation on Irs1 and decreased Irs1-associated PI3-kinase

(phosphatidylinositol 3-kinase) activity [64, 68, 69]. In epididymal adipocytes, total Irs1 and Irs2 decreased with high-fat feeding, but Irs3 increased [68]. However, in the pancreas islets, the expression of both IR and insulin receptor substrate 2 (Irs2) increased with HFD treatment [70]. Therefore, it appears that except for the pancreas, HFD decreases insulin response by decreasing or inactivating IR or Irs. For the pancreas, on the other hand, it's exactly the opposite.

HFD also impacts Akt activity, downstream of the IR and IRS1. In skeletal muscle, insulin-stimulated phosphorylation of Akt (p-Akt) decreased or was essentially unchanged after HFD [63, 69, 71, 72]. The reduced activity wasn't caused by decreased Akt expression [69]. In heart muscle [53] and prostate tissue [73], HFD increased basal p-Akt-serine 473. In aortic endothelial cells, short-term HFD increases meal-induced p-Akt, but long-term HFD decreases meal induced p-Akt [74]. In islets of pancreas, Akt1 expression didn't change with high-fat feeding [70]. In hypothalamic neurons, basal p-Akt increased with HFD treatment, but intracerebroventricular insulin induced p-Akt decreased [75]. When comparing how HFD influence insulin-induced p-Akt level between hypothalamic neuron, liver and skeletal muscle, the effect in hypothalamic neurons and skeletal muscle is similar. HFD impaired the ability of the cell to change the phosphorylation of p-Akt based on insulin concentration. [75] Overall, insulin induced Akt activity decreases with long-term HFD intervention.

As expected, given its effects on the insulin signaling pathway, HFD-induced obesity also impairs GLUT4 content and/or localization and glucose uptake. In epididymal (white) fat cells, insulin induced glucose transport rate decreases along with both glucose transporter 1 (GLUT1) and GLUT 4 expression after HFD, even though basal glucose transport rate increases [50]. In skeletal muscle, both basal and insulin induced GLUT4 content decreased after HFD in total homogenate, plasma membrane, T-tubules, and intracellular membranes [69, 71, 72], while

another study showed that the GLUT4 content and insulin-induced glucose transport activity didn't change with HFD treatment [76-78]. Basal glucose uptake also didn't change after HFD [76]. In the liver, the glucose transporter 2 (GLUT2) expression decreased with HFD treatment [79], while the glucose clearance didn't change [78]. Insulin stimulated glucose uptake is more insulin pathway dependent than the basal level, and it decreased in most insulin-stimulated organs after HFD. This is consistent with the results that the whole body glucose clearance rate decreased after HFD [77, 78]. That suggests that HFD induces insulin resistance. At the same time, the glucose-stimulated insulin secretion from islets increases to compensate the decreased glucose uptake in the whole body, and this did happen at all glucose levels [80].

HFD also affects glycogen synthesis, which decreased after HFD in muscle with GSI ratio (the ratio of glycogen synthase activity to the total activity at 0mM G-6-P) and the fractional velocity (at 0.1 mM G-6-P) of glycogen synthase activity also decreased, but the total glycogen synthase activity didn't change [77]. In both liver and muscle, the glycogen content didn't change under fed conditions after HFD [81-83]. Under fasting conditions, liver glycogen increased with HFD. Glycogen synthesis decreased in muscle, while glycogen synthase activity didn't change in liver after HFD [81, 84]. In muscle, the glycogen oxidation rate is reduced after HFD [85]. The HFD induced changes to glycogen utilization does not appear to be insulin pathway-dependent. After the body adapts to HFD, the body tends to decrease glycogen stores (not significant) and significantly decreases glycogen breakdown. This reflects more a shift in energy utilization and, not necessarily beneficial or detrimental.

Inflammation is a key biological response that is associated with HFD-induced obesity. Inflammation can be beneficial and detrimental. Inflammation is a response triggered by damaged living tissue or infection. It is a defense mechanism to localize and eliminate the

damage so that the body can initiate the healing process. However, chronic inflammation causes harm. Short-term HFD feeding is enough to cause immune cell infiltration into adipose tissue, but skeletal muscle needs long-term (more than 10 weeks) HFD to show that change [86-90]. The transcription of many inflammatory genes also changes very early in adipose tissue after HFD feeding, but requires a longer time to induce this change in skeletal muscle [86-88]. Chronic inflammation is thought to play an important role in many diseases including obesity, metabolic syndrome, cardiovascular disease, atherosclerosis, cancer, arthritis, autoimmune diseases, and most chronic noncommunicable diseases (CNCDs) [91, 92]. The diseases that are mentioned above include almost all of the top 10 leading causes of death in the United States as reported by the World Health Organization [86]. By causing chronic inflammation, long-term HFD decreases the body's ability to defend against chronic inflammation-related diseases.

Other Effects of HFD on Skeletal Muscle

Many other changes in skeletal muscle metabolism and function are associated with HFD-induced obesity. Loss of muscle strength, sensorimotor coordination and the peak force of twitch and tetanic contractions were seen after HFD [93-95]. Previous work also showed that muscle atrophy happened in soleus and gastrocnemius after HFD [93, 96]. The growth of regenerating myofibers was impaired, and myofiber maturation was delayed after HFD [97]. Myofiber area, satellite cells, and myonuclei of the muscle were decreased, while collagen deposition increased after HFD [93]. HFD also can alter the expression of some myokines: IL-6, and IL-15 expression are decreased [98], while apelin expression is increased [99].

HFD induces mitochondrial dysfunction in skeletal muscle, and some studies suggest that skeletal muscle mitochondria dysfunction plays a key role in HFD-induced insulin resistance [59]. However, not all the studies succeeded in showing that the HFD can change the

mitochondria function, and even when it changed, it is not always in the detrimental direction. For example, skeletal muscle mitochondrial respiration increased after HFD exposure [100, 101], which is generally considered a positive adaptation. On the other hand, mitochondrial density, mitochondrial DNA and subsarcolemmal mitochondrial antioxidant enzyme activities were decreased in mouse after HFD [102, 103]. However, some research showed the opposite results: mitochondrial enzyme activity, and mitochondrial DNA copy number increased after HFD in rat's skeletal muscle [82, 104-107]. Expression of several important mitochondrial genes was altered after HFD. Oxidative phosphorylation (ATP processing pathway) genes, like NDUFB3, NDUFB5, NDUFS1, NDUFV1, SLC25A12, and SDHB, were downregulated in skeletal muscle after HFD [108, 109]. Peroxisome proliferator-activated receptor γ coactivator-1 (PGC1) α and β , a transcriptional coactivator to induce mitochondrial biogenesis, were showed to be able to increase or decrease after HFD [102, 106, 109]. Some mitochondrial protein (CS, COX1, COX4, LCAD, UCP3, ALAs, NUO, SUO, Cyt c) increased after HF diet [106, 107]. In all, whether the HFD is beneficial or detrimental for the mitochondria is equivocal.

Interaction of LKB1 and HFD-Induced Obesity

Given the prominent role of LKB1 and its targets in regulating metabolism, it will be important to understand how the LKB1/AMPK system is affected by HFD feeding, and what role it plays in HFD-induced effects.

Effect of HFD Feeding on the LKB1/AMPK Pathway

There are some studies about how HF diet affects LKB1, but they showed opposite results. Some articles showed LKB1 protein didn't change after HF diet [110, 111], while others showed that LKB1 mRNA or protein decreased after HFD [112-115]. All articles show LKB1

phosphorylation after HFD changes, although some show an increase and some show a decrease [110, 111, 113]. People also report that there is a tendency for LKB1 to move into the nucleus instead the cytoplasm after HFD [116], and this may be because the phosphorylation state of LKB1 can influence its subcellular localization [117].

Long-term HFD is able to change AMPK expression or activity or both, but the results are not consistent. Liu et al. showed that long-term HFD feeding decreases expression (mRNA and protein) of AMPK- α , phosphorylated AMPK and AMPK activity in rat skeletal muscle [118]. However, other two studies show that long-term HFD has the tendency to increase phosphorylated AMPK in rat skeletal muscle [119, 120]. Therefore, the conclusion about how HFD affects AMPK is not clear.

LKB1 or AMPK -Knockout Influences HFD-Induced Adaptation

Adipocyte-specific LKB1 knockout mice are more resistant to HFD-induced obesity, have increased brown adipose tissue mass, improved glucose tolerance and insulin sensitivity after HFD feeding compared to wild-type (WT) mice [121]. LKB1 knockout in adult β cells also leads to improved HFD induced glucose tolerance compared to WT mice, with increased β cell mass and insulin secretion [122].

The effects of HFD in skeletal muscle specific LKB1-KO mice have not been reported. However, the effect of HFD in muscle specific-AMPK knockout mice has been studied. Three studies used muscle (heart and skeletal muscle) specific AMPK α 2 knockout (AMPK α 2-KO) mice, and put them on the HFD for more than 13 weeks. Two studies put the young mice (18 weeks old) on the HFD [123, 124], while the other study used the old mice (18 months old) [125]. After the HFD treatment, the young AMPK α 2-KO mice did better in both glucose and insulin tolerance test than the wildtype young mice, but this is not true for the old mice. When

the young AMPK α 2-KO mice were put on the HFD, they gained less body weight with less food intake. They also gained less white adipose and less muscle weight, but not less brown adipose. Fasting plasma insulin decreased in the old AMPK α 2-KO mice, not in the young KO mice. Even though AMPK α 2 is the main AMPK α isoform, there is a HF study that used young muscle specific AMPK α 1 knockout (AMPK α 1-KO) mice [124]. Interestingly, after HFD, the fasting serum insulin was lower in the AMPK α 1-KO mice than wildtype and AMPK α 2-KO mice. AMPK α 1-KO mice did even better than AMPK α 2-KO mice in the insulin tolerance test. One study also put AMPK γ 3 knockout mice on the HFD for 12 weeks. The HFD decreased glucose uptake, but this was unaffected by AMPK γ 3 knockout [126].

Skeletal muscle, as the organ that has the body's largest mass, uses a large amount of energy every day. It is also the largest insulin-stimulated organ. LKB1 is the master kinase of the energy-sensing protein (AMPK), and involved in many other biological processes. HFD provides concentrated energy to use or store. Therefore, it is important to see how knocking out LKB1 in skeletal muscle influences HFD induced effects. This has not been reported previously.

Aims

Ad libitum HFD feeding leads to obesity and impacts metabolism and cell function in many different ways. LKB1 and its targets of the AMPK family play an important role in the control of metabolism and is also involved in so many other biological functions. Skeletal muscle, as the organ that has the body's largest mass, uses a huge amount of energy every day. It is also the largest insulin-stimulated organ. Therefore, the overriding purpose of this dissertation was to determine how the lack of LKB1 in skeletal muscle influences HFD-induced adaptation.

The specific purpose in Chapter 2 was to determine how skeletal muscle specific LKB1 knockout (skmLKB1-KO) affects short- and/or long-term HFD-induced body weight, glucose

and insulin tolerance, and insulin signaling in skeletal muscle. To do this, skmLKB1-KO mice and littermate controls were placed on short (1 week) or long-term (14 weeks) high-fat or standard chow diets, with a third group switching back to standard chow after long-term high-fat feeding. The hypotheses were:

- 1). Long-term HFD-induced weight-gain should be attenuated by skmLKB1-KO. This would not occur in the short-term HFD-fed mice.
- 2). Long-term HFD-induced impairment in glucose and insulin tolerance would be attenuated in skmLKB1-KO mice.
- 3). HFD-induced impairment in insulin pathway signaling would be attenuated by LKB1-KO.
- 4). HFD feeding would not change LKB1 expression, but would induce LKB1 movement into the nucleus. LKB1 activity would also decrease with HFD treatment.
- 5). Knocking out LKB1 would decrease AMPK activity, but increase its expression and let less AMPK move into the nucleus.
- 6). HFD would decrease AMPK expression, with more AMPK moving into the nucleus. AMPK activity should also decrease with HFD treatment.

The specific purpose in Chapter 3 was to determine potential genes that play an important role in long-term HFD-induced effects and/or LKB1-mediated effects in skeletal muscle. After selecting the potential genes, and to determine whether their protein levels also showed the same change in expression. The hypotheses were:

- 1). HFD and LKB1-KO would lead to altered gene expression.
- 2). The protein levels for those potential genes would show the same change as mRNA.

The specific purpose in Chapter 4 was to find out if HFD and/or knocking-out LKB1 affect muscle growth, anabolic and catabolic signaling in skeletal muscle. The hypotheses were:

- 1). Both HFD and LKB1-KO would decrease muscle size.
- 2). Markers of protein synthase would decrease with high-fat feeding or LKB1-KO, while the protein degradation would show the opposite change.

Impact

This dissertation is the first to study how LKB1-KO in skeletal muscle can influence high-fat diet induced adaptation. A better understanding of the molecular signaling events that are affected by HFD-induced changes in physiology will allow for improved therapeutic treatments for obesity, diabetes, and other conditions affected by over-nutrition.

CHAPTER 2: The Effect of High-Fat Diet-induced Obesity and/or Skeletal Muscle Specific LKB1 Knockout on Insulin Induced Glucose Handling

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Abstract

Many studies have shown that a long-term high-fat diet (HFD) can induce glucose and insulin intolerance in mice. In skeletal muscle, Liver kinase B1 (LKB1) is a master kinase regulating the AMPK family of protein kinases, which are major regulators of metabolism. LKB1-knockout (LKB1-KO) in muscle has been shown to improve insulin sensitivity. This study's purpose was to determine how LKB1-KO from skeletal muscle can affect short-term and/or long-term HFD induced changes in body weight, glucose tolerance, insulin tolerance, and skeletal muscle insulin signaling, among other measures. In order to do so, control and skeletal muscle specific LKB1 knock-out (LKB1-KO) mice were put on either standard diet (STD) or HFD for 1 week or 14 weeks, or put on the HFD for 14 weeks and then switched to STD for 1 week (switched diet). We found that HFD increased body weight and fat mass, and this was related to increased caloric consumption and less daily activity. LKB1-KO decreased AMPK activity, decreased nuclear distribution for AMPK $\alpha 2$ and increased AMPK $\alpha 1$ expression. Long-term HFD increased t-AMPK expression in LKB1-KO mice, decreased the cytoplasm p-AMPK and nuclear p/t-AMPK ratio in CON mice. By decreasing TBC1D1 phosphorylation and GLUT4 expression, HFD causes insulin resistance in adult mice. LKB1-KO mice had lower fasting blood glucose levels that were likely due to increased fasting insulin concentration and an associated increase in AS160 phosphorylation to induce more GLUT4 vesicle exocytosis.

Glucose tolerance testing showed impaired glucose tolerance in the LKB1-KO mice which was related to decreased TBC1D1 phosphorylation, while insulin tolerance testing showed improved insulin tolerance in the LKB1-KO mice which was related to increased AS160 phosphorylation. In conclusion, HFD impaired insulin induced glucose handling, but LKB1 knockout had an opposite effect.

Introduction

Ad libitum high-fat diet (HFD) feeding leads to weight gain and increased fat mass in rodents. This accumulation of increased body fat is associated with the development of insulin resistance [48, 55, 59, 60], and eventually the development of diabetes with increased fasting blood glucose and insulin levels [46, 78, 99, 127-129].

Liver kinase B1 (LKB1), also known as Serine/threonine kinase 11 (STK11), is a major regulator of metabolism through its downstream targets of the AMP-activated protein kinase (AMPK) family. It is conserved throughout evolution from worms to mammals [1]. LKB1 is involved in cell cycle arrest [5, 6], apoptosis [7], autophagia [8], energy metabolism [9] as well as in T cell maturation [10], liver glucose homeostasis[11], tumor growth [12] and epithelial apicobasal polarity[13]. Full LKB1 activity requires association with its binding partners, the pseudo kinase STE-20 Related Adaptor (STRAD) and mouse protein 25 (MO25), which stabilizes the interaction between STRAD and LKB1 [3].

Active LKB1 exerts these cellular effects by activating AMP-activated protein kinase (AMPK) and its family members (ARKs) through phosphorylation at a conserved N-terminal residue [16].

AMP-activated protein kinase (AMPK) is a well-characterized cellular energy-sensing protein. It is a heterotrimeric protein formed by α , β , and γ subunits, each of which also have

different isoforms ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$) [17, 18]. AMP binds with the γ subunit of AMPK which leads to conformational change in AMPK to induce AMPK activation [130-132]. In the AMP-bound state, AMPK can be phosphorylated and activated by AMPK kinases (AMPKKs), the most important of which is liver kinase B1 (LKB1), although calcium calmodulin-dependent protein kinase kinase (CaMKK) plays an important role under some circumstances. AMPK activation stimulates catabolic processes to generate ATP and inhibits ATP-consuming anabolic processes that are not required for the immediate survival of the cell [19].

Activation of AMPK in skeletal muscle, by phenformin or the AMP agonist AICAR, requires LKB1 [19-21]. LKB1 is also required for AMPK-mediated phosphorylation HDAC4/5 and the amount of LKB1 can influence the phosphorylation state of HDAC4/5 [22]. Knocking out LKB1 in skeletal muscle decreases phosphorylated AMPK, AMPK- $\alpha 2$ activity (not $\alpha 1$), but increases AMPK- $\alpha 2$, $\beta 2$, and $\gamma 3$ protein expression [23].

The effect of HFD on LKB1 is not clear, with some studies showing that LKB1 protein didn't change after HFD [110, 111], and others showing that LKB1 mRNA or protein decreased after HFD [112-115]. Phosphorylation of LKB1, which likely regulates its localization in the cell [117] has been reported to increase or decrease [110, 111, 113] with HFD feeding, and there is a tendency for LKB1 to move into the nucleus instead the cytoplasm after HFD [116], which would decrease its ability to phosphorylate its targets in the cytoplasm.

Likewise, research shows that long-term HFD is able to change AMPK expression or activity or both, but the results are not consistent. Liu et al. showed that long-term HFD feeding decreases the expression (mRNA and protein) of AMPK- α , decreases AMPK phosphorylation and AMPK activity in rat skeletal muscle [118]. However, other studies contradict this, showing

that long-term HFD has the tendency to increase phosphorylated AMPK in rat skeletal muscle [119, 120]. Therefore, the conclusion about how HFD affects AMPK is not clear.

AMPK itself interacts with insulin signaling in the cell. AMPK can rapidly phosphorylate Irs1 on Ser-789 to inactivate it in culture as well as in mouse C2C12 myotubes incubated with AICAR [133]. In HEK293 cells, AMPK activation induced by energy depletion leads to its binding with Irs1 and phosphorylation of Irs1-Ser-794, and this was in a LKB1 dependent manner [134]. In vascular smooth muscle cells, AMPK activation induced Irs1-Ser-794 phosphorylation and inhibited IGF-I-stimulated PI3K pathway activation [135]. Glucose starvation enhanced tyrosine phosphorylation of Irs1 (to activate it) and the IR is AMPK dependent [136]. And Chopra et al. [136] proposed that AMPK first phosphorylates IR on Ser/Thr residue(s) to promote further phosphorylation on tyrosine of IR and Irs1. Akt seems not to be affected by AMPK activation, although Bolster et al. reported that its phosphorylation decreases with AICAR-induced AMPK activation [137]. The Akt substrate-AS160 and TBC1D1 can be phosphorylated by AMPK directly [138-142]. This phosphorylation can stop AS160 and TBC1D1's inhibitory effect on GLUT4 translocation, leading to its movement to the cell membrane to increase glucose uptake and decrease blood glucose. Taken together, the existing evidence suggests that LKB1-AMPK can directly affect and regulate the insulin signaling pathway.

Based on the potential interaction between LKB1/AMPK signaling and the insulin signaling pathway, therefore, it is interesting to determine how the knockout of LKB1 in skeletal muscle affects the development of HFD-induced insulin resistance. LKB1 knockout in adipocytes resulted in increased brown adipose tissue mass, improved glucose tolerance and insulin sensitivity, and resistance to HFD-induced obesity [121]. In another study [122] adult β

cell-specific LKB1 knockout mice were given high-fat chow and also had improved glucose tolerance. The adult islets had increased β cell mass and insulin secretion.

Skeletal muscle, as the organ that has the body's largest mass, uses a huge amount of energy every day. It is also the largest insulin-stimulated organ, but the role of skeletal muscle LKB1 in HFD-induced impairments in insulin signaling has not been reported. Therefore, the purpose of this study was to determine how skeletal muscle specific LKB1 knockout (skmLKB1-KO) affects short- and/or long-term HFD-induced body weight, glucose and insulin tolerance, and insulin signaling in skeletal muscle.

Materials and Methods

Animal Care

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University. All mice were housed in the pathogen-free animal facility in Life Science Building of Brigham Young University. The temperature in the animal facility was controlled at 21-23°C with a 12:12 hours light-dark cycle.

Mice

Skeletal muscle specific LKB1 knockout (skmLKB1-KO) mice were generated at Brigham Young University by crossing transgenic LKB1 “control KO” mice in which the LKB1 gene was homozygously replaced with the LKB1 gene flanked by LoxP sites (from R. DePinho and N. Bardeesy, Dana-Farber Cancer Institute, Boston, MA) with myf6-Cre-transgenic mice [143] that heterozygously express Cre recombinase under the skeletal muscle-specific Myf6 promoter (from M.R. Capecchi, University of Utah, Salt Lake City, UT). These offspring were backcrossed onto the FVB background as described previously [144, 145]. With the specific

expression of Cre in skeletal muscle, the LKB1 gene in the homozygously “floxed” LKB1 mice is deleted. Male skmLKB1-KO and littermate CON mice were used. Genotyping was determined by PCR, as described previously [146], and was also verified by western blotting for LKB1 (as described below). Prior to experimentation the mice were fed standard chow and water *ad libitum*.

Experimental Design

Table 2. 1: Nutrient Information for High-Fat Chow and Standard Chow.

ID.		Diet	
		TD. 8604 (St)	TD. 06414(HF)
% kcal from	Protein	32	18.4
	Carbohydrate	54	21.3
	Fat	14	60.3
Energy density (kcal/g)		3	5.1
Fatty acid profile, % total fat	% saturated	22	37
	% monounsaturated	27	47
	% polyunsaturated	51	16
Fat Sources, % by weight		0.7% C16:0 Palmitic 0.1% C18:0 Stearic 0.9% C18:1 ω 9 Oleic 1.9% C18:2 ω 6 Linoleic 0.2% C18:3 ω 3 Linolenic	31% lard 3% soybean oil

CON and skmLKB1-KO mice were placed on either high-fat chow (Envigo, TD. 06414) or continued on the standard chow (Envigo, TD. 8604) beginning at 5 weeks of age. Diet composition is shown in Table 2.1. There were five different diet treatments: 7 days HFD, 7 days standard diet (STD), 14 weeks HFD, 14 weeks STD, switch diet (14 weeks HFD followed by 7 days STD). Each group was comprised of 11-20 mice. Food consumption, water consumption and body weight were recorded weekly.

For the 14 weeks feeding groups, several additional tests were done before sacrificing the mice. Ambulatory activity was monitored for 3 days between day 74 and 88 of the experiment. Glucose tolerance testing was performed on day 90, and insulin tolerance testing was performed on day 94. (Figure 2.1)

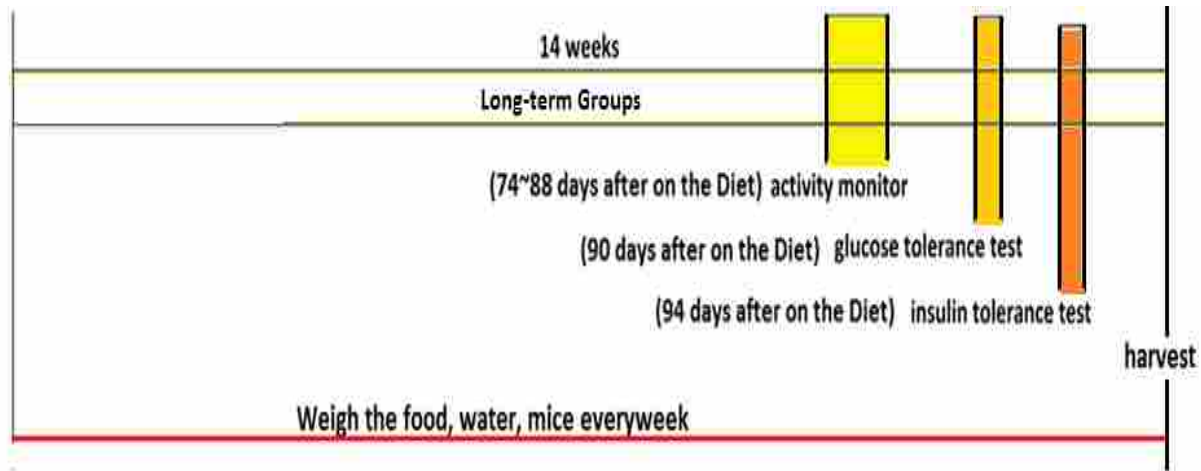


Figure 2. 1: Schedule for Long-Term Groups. For the long-term groups, activity monitor (74-88 days after on the diet), glucose tolerance test (90 days after on the diet), and insulin tolerance test (94 days after on the diet) had been done during the 14 weeks.

Activity Monitoring

In-cage ambulatory activity was measured for 3 consecutive days using an infrared beam activity monitoring system (Columbus Instruments Opto-M3 4-Channel Animal Activity Data Processing Monitor; Columbus, OH), and the number of beam-breaks were recorded by computer software. Mice were housed individually during the ambulatory activity monitor test.

Intraperitoneal Glucose Tolerance Test (IPGTT)

Mice were fasted for 6 hours, beginning between 8 and 10 am [147] in clean cages with no food, but free access to drinking water. After the fasting period, topical anesthetic (EMLA lidocaine/prilocaine 2.5%-2.5%; Hi-Tech Pharmacal) was applied to the tip of the tail. 20-30 minutes later, the distal tip (~1 mm) was cut from the tail with a scalpel blade. After discarding 3

small drops of blood, blood glucose concentration was measured using a glucometer (FreeStyle Lite Blood Glucose Monitoring System, Abbott Laboratories). The mice were then injected intraperitoneally with a sterile 20% glucose solution (2 mg glucose/g BW) that was freshly prepared each day. At 15, 30, 60, and 120 minutes after the injection, blood glucose was measured again by removing the clot from the first incision on the tail. EMLA was reapplied every 30 minutes during the 120-minute procedure. After the testing period, food was given back to the mice.

Intraperitoneal Insulin Tolerance Test (IPITT)

IPITT testing was performed as described above for IPGTT testing except for the mice being fasted for 4 hours and they were injected with insulin (0.5 U/kg) (Novolin R, Novo Nordisk A/S, Denmark), rather than glucose.

Tissue Collection

Mice were anesthetized with 2-2.5% isoflurane in supplemental oxygen. Serum was collected by withdrawing about 0.5 ml of blood from inferior vena cava and allowing it to coagulate at room temperature for 30 minutes before being centrifuged at 4°C and 1000g for 10 minutes. Quadriceps muscle (QUAD), retroperitoneal fat pad, and heart were harvested and clamp-frozen in liquid nitrogen. All harvest tissue and serum were stored at -90°C until further usage.

Whole Protein Homogenization

QUAD muscles were powdered in liquid nitrogen then about 30 mg of powdered tissue was 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 β -glycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 ug/ml soybean trypsin inhibitor). The homogenate was slowly freeze-thawed at -90°C three times to ensure cell lysis, then centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were kept as the whole protein homogenate.

Supernatants were analyzed for protein content using the DC Protein Assay (Biorad Laboratories, Hercules, CA, USA). Supernatants were stored in micro-centrifuge tubes at -90°C until further analysis.

Nuclear and Cytoplasm Protein Isolation

About 60 mg of powdered QUAD muscle tissue was ground-glass homogenized in 1 ml of lysis buffer A (10 mM Hepes, pH to 7.9 with KOH; 10mM KCl; 1.5 mM MgCl₂; 1 mM DTT; 0.5 mM EDTA; 0.1 mM EGTA; 1% NP-40; 1 mM sodium orthovanadate; 1 mM benzamidine; 200 mM phenylmethane sulfonyl fluoride; 5 ug/ μ l soybean trypsin inhibitor) and placed in microcentrifuge tubes. Homogenates were twice incubated on the ice for 10 minutes then vortexed for 5 seconds. Samples were then centrifuged at 16,000g for 10 min at 4°C. The supernatant was collected in a separate tube as the cytoplasmic protein homogenate and stored at -90°C for further use. The pellet was washed 2 times in 500 μ l lysis buffer A and centrifuged at 16000g for 1 minute at 4°C between each wash. The pellet was then resuspended in 500 μ l of lysis buffer B (20 mM Hepes, pH to 7.9 with KOH; 420 mM NaCl; 1.5 mM MgCl₂; 0.5 mM EDTA; 0.1 mM EGTA; 25% glycerol; 0.5 mM DTT; 1% NP-40; 1 mM sodium orthovanadate; 1 mM benzamidine; 200 mM phenylmethane sulfonyl fluoride; 5 ug/ μ l soybean trypsin inhibitor), vortexed for 15 seconds and incubated on the ice for 10 minutes. The vortex-incubation cycle was repeated 3 times, after which the samples were centrifuged at 16,000g for 10 min at 4°C. The

supernatant was collected in a separate tube as the nuclear protein homogenate and stored at -90°C for further use.

Western Blot and Immunodetection

Protein homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β -mercaptoethanol, and 0.01% bromophenol blue) and then loaded on 4-15% Tris-HCl gels (Bio-Rad Criterion System, Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) and visually inspected for equal protein loading. Membranes were then washed with TBST (Tris-buffered saline plus 0.1% Tween-20) for 2~5 minutes, blocked with 5% non-fat dry milk (dissolve 5 g non-fat dry milk in 100 ml 1X TBST, pH to 7.6) for 1 hour, and probed overnight at 4°C on a rotator with primary antibody diluted in 1% bovine serum albumin (BSA) (dissolve 5 g BSA in 500 ml 1X TBST, pH to 7.6).

Primary antibody manufacturers and dilutions were as follows: p-AMPK (Thr172) (#2535, 1:4000), t-AMPK (#2793, 1:2000), p-ACC (Ser79) (#3661, 1:4000), p-AS160 (Ser318) (#8619, 1:2000), p-AS160 (Thr642) (#8881, 1:2000), p-TBC1D1 (Thr590) (#6927, 1:2000), t-TBC1D1 (#66433, 1:2000), p-Glycogen Synthase (Ser641) (#3891, 1:2000), t-Glycogen Synthase (#3886, 1:2000), t-GSK-3 α/β (#5676, 1:2000), p-GSK-3 α/β (Ser21/9) (#9331, 1:2000), p-Akt (Thr308) (#2965, 1:4000), p-Akt (Ser473) (#4060, 1:5000), t-Akt (#9272, 1:4000), p-(Ser/Thr) Akt Substrate (#9611, 1:2000) from Cell Signaling Technology (Beverly, MA, USA); AMPK α 1 (A300-507A, 1:4000), AMPK α 2 (A300-508A, 1:20000) from Bethyl Laboratories, Inc. (Montgomery, TX, USA); and LKB1 (#07-694, 1:4000) from Upstate (Lake Placid, NY, USA); GLUT4 (AB1346-0703056190), t-AS160 (07-741, 1:10000), p-TBC1D1 (Ser237) (07-2268,

1:2000) from EMD Millipore (290 Concord Road, Billerica, MA 01821 USA); HXK II (sc-6521, 1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

After the overnight probing with primary antibody, membranes were washed with TBST for 5 minutes a time, three times. Then membranes were probed with appropriate secondary antibody diluted at 1:20000 in 2% non-fat dry milk for 1 hour at room temperature. Secondary antibody manufacturers were as follows: HRP-conjugated mouse anti-rabbit secondary antibody (#211-032-171, 1:20000) from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA, USA), HRP-conjugated goat anti-mouse secondary antibody (sc-2314, 1:20000) and HRP-conjugated donkey anti-goat IgG secondary antibody (sc-2020, 1:20000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

After incubation in secondary antibody, the membranes were washed 4 x 5 min with TBST. Clarity™ Western ECL Blotting Substrates (Bio-Rad Criterion System, Hercules, CA) was applied for 1~5 min. Chemiluminescent signals were detected with autoradiography film and quantified using Gel-Pro Analyzer 6.0 (Media Cybernetics, Inc. Bethesda, MD).

AMPK α 1 and α 2 Kinase Activity Assay

AMPK activity of the α 1/ α 2 subunits immunoprecipitated from QUAD whole protein homogenates was measured by counting how much radiolabeled phosphate from ATP was incorporated into the SAMS (HHMRSAMSGHLVKRR-OH) peptide using a scintillation counter.

Immunoprecipitates were prepared by adding 2.5 μ g of AMPK α 1 or α 2 (custom made by ABR) antibody to a microcentrifuge tubes containing 4 μ l of protein G sepharose (Sigma). 40 μ l of whole protein homogenate were added to the antibody and protein G sepharose pellet, mixed overnight at 4°C on a rotator. Next day, washed with ice-cold IP buffer (50 mM Tris-HCl, 150

mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1mM EGTA, 1mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml soybean trypsin inhibitor, pH 7.4) and 1 M NaCl. The pellet was then washed with lysate assay buffer (62.5 mM Na HEPES, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM sodium pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml soybean trypsin inhibitor, pH 7.4), centrifuged, and the supernatant aspirated. The pellet was resuspended in HEPES-Brij (HB) buffer (25 mM HEPES, 0.02% Brij, 1 mM DTT, pH 7.4) and transferred to microcentrifuge tubes. The reaction was started by adding 15 µl of the working assay cocktail (40 mM HEPES, 0.2 mM SAMS peptide (HHMRSAMSGHLVKRR-OH), 0.2 mM AMP, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl₂, 0.2 mM ATP, pH 7.0) at timed intervals of 30 sec. Samples were incubated at 150 rpm in a thermomixer at 30°C for 10 min. 15 µl of reaction mix was transferred to a quarter circle of P81 filter paper, washed five times in 1% phosphoric acid, and then washed with ddH₂O and acetone and allowed to dry. The filter paper was then added to a vial and placed in the scintillation counter along with a blank control vial and a positive control hot assay cocktail vial to calculate the specific activity. Activity was assessed and expressed as picomoles phosphate incorporated into SAMS peptide per gram tissue per minute.

LKB1 Kinase Activity Assay

LKB1 was immunoprecipitated from QUAD whole protein homogenates, and its activity was measured by counting how much radiolabeled phosphate from ATP was incorporated into the LKBtide (SNLYHQGKFLQTFCGSPLYRRR) peptide using a scintillation counter.

Starting with 600 µg of QUAD homogenate from each animal, LKB1 was immunoprecipitated, and activity was assessed as previously described in the AMPK activity protocol with a few modifications. 150 µl LKB1 antibody (Santa Cruz) was used in place of the

α 1- or α 2-AMPK antibodies and the LKB1- antibody-G-Sepharose complex was washed twice with 1 ml wash buffer A (homogenization buffer with 0.5 M NaCl) and then twice with 1 ml of wash buffer B (40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 5 mM MgCl₂, 0.8 mM DTT). LKB-tide (0.2 mM) was used in place of SAMS-peptide, and the incubation period was extended to 15 min. Finally, 40 μ l of the final mix was transferred to a half piece of Whatman P81 filter paper (2.5 cm) and allowed to absorb for 30 s before stopping the reaction in phosphoric acid.

Insulin Radioimmunoassay (RIA)

Fasting serum (6-8 hours fast) was diluted in PBS (1:50) and insulin was measured using a mouse insulin RIA kit (MP Biomedicals) following the manufacturer's directions, as described in previous publications. [148, 149].

Glycogen Concentration Assay

10 μ l cold 30% KOH per mg tissue was added to powdered QUAD muscle tissue and placed in the Thermomixer (100 °C and 1000 rpm) for 30 min. Samples were then neutralized with 10.7 M acetic acid until the pH reached 5~7. 0.1 ml of sample was transferred to a fresh microcentrifuge tube containing 0.9 ml amyloglucosidase buffer [amyloglucosidase, 50 mM acetate buffer (50 mM acetic acid, 50 mM sodium acetate), pH 4.7]. Samples were incubated at 55°C for 1 hour in the Thermomixer (1000 rpm), vortexed, and then centrifuged for 10 min at 13000 x g. 20 μ l of sample or standards or water (blank) was transferred to a 96-well plate. 176 μ l reaction buffer (25.4 mg MgCl₂, 0.4 mg DTT, 6.9 mg ATP, 7.7 mg NADP, 25 ml 100 mM Tris buffer, pH 8.8) was added to each well. Shake the plate for 10 second and do an initial absorbance read at 340 nm. Add 4 μ l of G6PDH-HK enzyme mixture (100 units GSPDH, Sigma

G-6378, 50 mM Tris, 80 units/mg solid Hexokinase, Sigma H5375, pH 7.6) to each well. Shake the plate for 1 minute and do one more absorbance read at 340 nm after 15 minutes, and then keep reading at every 10 minutes until the standards become stable.

Statistics

Statistical comparisons using Microsoft Excel or SPSS (Statistical Package for the Social Sciences) were made using *t*-test, one-way ANOVA (with Fisher's LSD post-hoc analysis), and two-way ANOVA to determine the statistical significance ($p \leq 0.05$). Values were reported as mean \pm SE.

Results

The Effect of Skmlkb1-KO and HFD on LKB1 and AMPK Content and AMPK Phosphorylation

LKB1-KO mice effectively decreased LKB1 protein expression in quadricep muscle tissue compared to CON muscles (Figure 2.2 A). LKB1-KO increased total AMPK alpha subunit expression in the adult mice (19 weeks old), but not the 6 week-old mice (Figure 2.2 E). The AMPK α subunit comes in 2 isoforms. LKB1-KO did not have any effect on AMPK $\alpha 2$ expression (Figure 2.2 C), but increased $\alpha 1$ expression only in the adult, but not 6 week-old mice (Figure 2.2 B).

As expected, given the role of LKB1 as the primary AMPK kinase in skeletal muscle, skmLKB1-KO greatly decreased AMPK phosphorylation (Figure 2.2 D,F).

HFD treatment did not affect LKB1 or AMPK $\alpha 1$ or $\alpha 2$ protein levels (Figure 2.2 A, B, C) but long-term HFD did increase total AMPK α subunit expression in LKB1-KO muscles (Figure 2.2 E). HFD had no effect on AMPK phosphorylation (Figure 2.2 D,F).

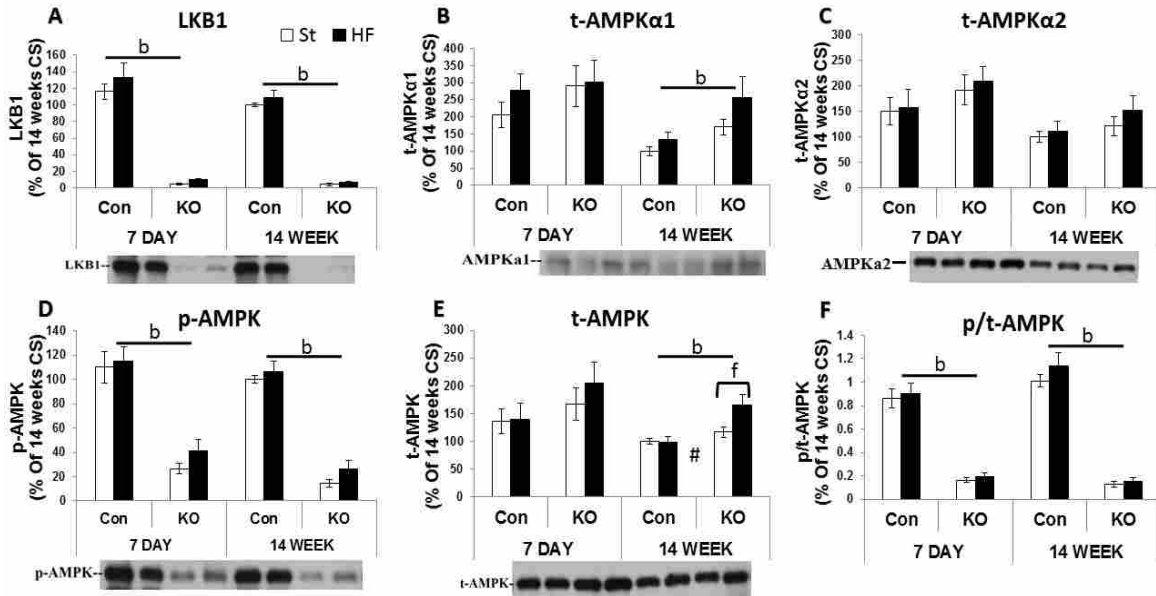


Figure 2. 2: The Protein Expression for LKB1-AMPK System. The protein expression for (A) LKB1, (B) AMPK α 1, (C) AMPK α 2, (D) p-AMPK (phospho-AMPK), (E) t-AMPK (total-AMPK), and (F) the p-AMPK/t-AMPK ratio in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. b=significant main effect for genotype. #=had genotype and diet interaction effect in two-way ANOVA. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

HFD and LKB1-KO Effects on the Cellular Location of LKB1 and AMPK

With LKB1-KO, LKB1 expression was dramatically decreased in both nuclear and cytoplasmic cell fractions, as expected (Figure 2.3 A). HFD had no effect on LKB1 localization (Figure 2.3 A).

Neither LKB1-KO nor HFD affected AMPK α expression in both nuclear and cytoplasmic fractions (Figure 2.3 E). On the other hand, p-AMPK was decreased in both nuclear and cytoplasm fractions from the LKB1-KO muscles, HFD also had an effect on p-AMPK levels. In the cytoplasmic fraction, long-term HFD decreased p-AMPK level for CON mice, not LKB1-KO mice. After the long-term diet treatment, diet and genotype had an interactive effect on cytoplasmic part of the p-AMPK level, which means that the effect that the diet caused on the cytoplasm part of the p-AMPK level in the CON mice was different from the one in the LKB1-

KO mice. HFD decreased p-AMPK level in the cytoplasm part of the CON mice, but didn't have effect on LKB1-KO mice. (Figure 2.3 D)

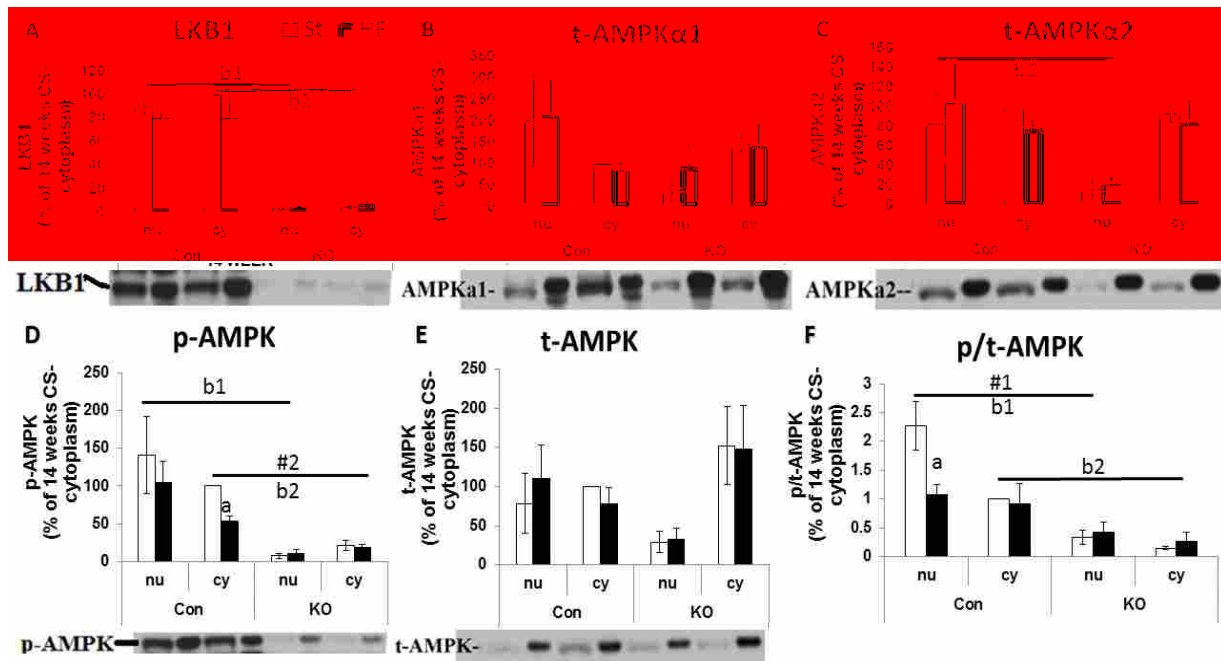


Figure 2. 3: Cellular Localization of the Proteins in LKB1-AMPK System. Cellular localization of (A) LKB1, (B) AMPK α 1, (C) AMPK α 2, (D) p-AMPK (phospho-AMPK), (E) t-AMPK (total-AMPK), and (F) the p-AMPK/t-AMPK ratio in nuclear (nu) and cytoplasmic (cy) fractions from quadriceps (QUAD) homogenates from LKB1-knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HFD) for 14 weeks. The western blot loading order was: 14CS-nu, 14CS-cy, 14CH-nu, 14CH-cy, 14KS-nu, 14KS-cy, 14KH-nu, 14KH-cy. a= significant main effect of diet. b1= significant main effect of genotype for the nuclear protein. b2= significant main effect of genotype for the cytoplasm protein. #1=had genotype and diet interaction effect in two-way ANOVA for the nuclear protein. #2=had genotype and diet interaction effect in two-way ANOVA for the cytoplasm protein. Data is presented as means \pm SEM (n=6/group).

For p/t-AMPK, the results were also interesting. LKB1-KO decreased the level of p/t-AMPK in both nuclear and cytoplasmic fractions. HFD also had effect on p/t-AMPK level. In the nuclear fraction, long-term HFD decreased p/t-AMPK level for CON mice, but not LKB1-KO mice (Figure 2.3 F). For AMPK α 1 expression in both nuclear and cytoplasmic fractions, neither LKB1-KO nor HFD affected it (Figure 2.3 B). For AMPK α 2, LKB1-KO decreased its expression level in nuclear part of the LKB1-KO mice. The cytoplasm fraction was not affected (Figure 2.3 C).

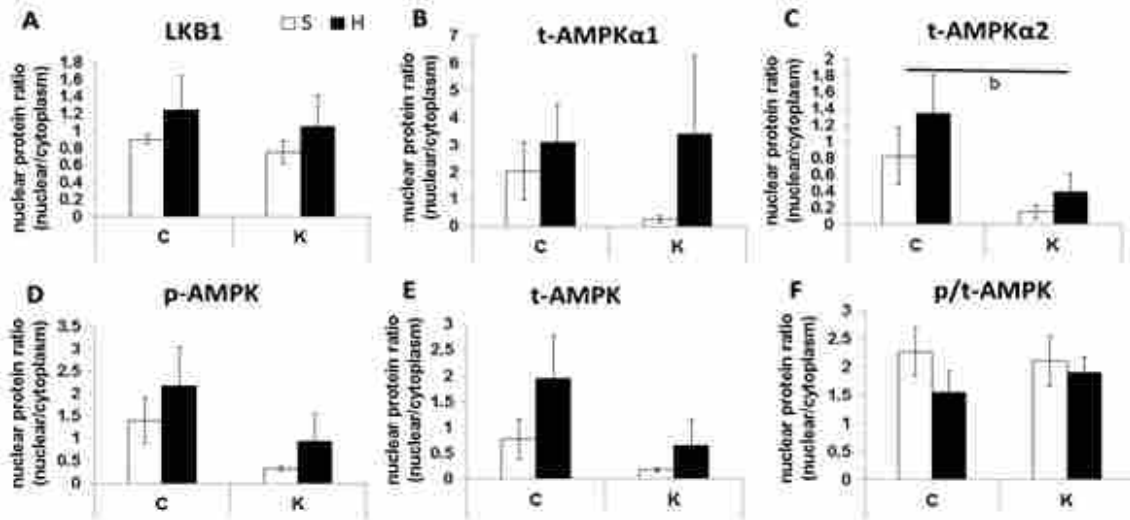


Figure 2. 4: The Nuclear Protein Ratio for the Proteins in LKB1-AMPK System. The nuclear protein ratio for (A) LKB1, (B) AMPK α 1, (C) AMPK α 2, (D) p-AMPK (phospho-AMPK), (E) t-AMPK (total-AMPK), and (F) the p-AMPK/t-AMPK ratio in the mice quadriceps (QUAD) homogenates from LKB1-knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HFD) for 14 weeks. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

In order to determine if the ratio of the protein that had been translocated into the nucleus changed or not, nuclear protein ratio was determined (nuclear protein/cytoplasmic protein). For AMPK α 2, LKB1-KO decreased the nuclear protein ratio for the adult mice (19 weeks old), but not the 6 weeks old mice, while diet composition had no effect. (Figure 2.4 C) That means that a smaller proportion of AMPK α 2 was in the nucleus in the LKB1-KO mice.

LKB1-KO Decreased AMPK Activity Through Decreasing AMPK α 2 Activity

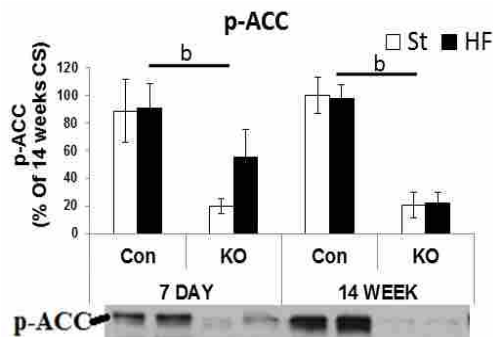


Figure 2. 5: The Protein Expression of p-ACC. The protein expression of p-ACC in whole protein homogenates for QUAD muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

LKB1 activates AMPK by phosphorylating it at Thr172. As discussed in the previous paragraph, LKB1-KO decreased both p-AMPK and p/t-AMPK, but there is no diet effect on for either measure (Figure 2.2 D and F). ACC is the downstream target of AMPK. AMPK is the main kinase regulator of ACC. Active AMPK phosphorylates ACC to inactivate it, so ACC phosphorylation is thought to reflect AMPK activity. ACC phosphorylation was decreased with LKB1-KO, but not HFD, although a trend was observed for increased p-ACC in LKB1-KO muscles on HFD (Figure 2.5).

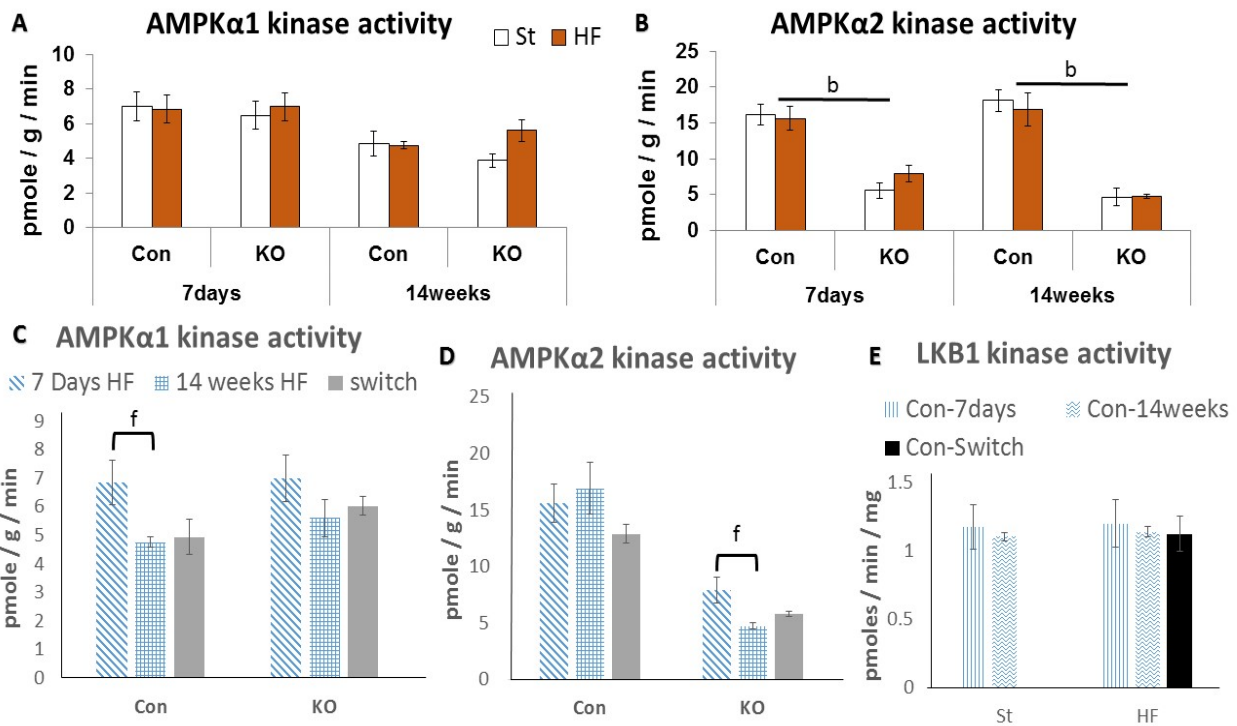


Figure 2. 6: The Kinase Activity for AMPK and LKB1. The kinase activity for (A,C) AMPK α 1, (B,D) AMPK α 2, and (D) LKB1 in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice that on standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks, or on 14 weeks high-fat diet then switched to standard diet for 1 week (switch). b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

Phosphorylation of ACC is more likely to estimate the *in vivo* AMPK activity, but it doesn't accurately measure the actual kinase activity for AMPK since it may be phosphorylated by other proteins as well. Therefore, *in vitro* AMPK activity was measured for both AMPK α 1

and AMPK α 2. AMPK α 1 activity didn't change with either LKB1-KO or HFD. (Figure 2.6 A) The AMPK α 1 activity decreased in long-term HF-CON mice compared with short-term HF-CON mice. Switched diet didn't effect AMPK α 1 activity. (Figure 2.6 C). AMPK α 2 activity decreased in the LKB1-KO mice, but HFD didn't affect it. (Figure 2.6 B) The AMPK α 2 activity decreased in long-term HF-KO mice compared with short-term HF-KO mice. Switched diet didn't effect AMPK α 2 activity either. (Figure 2.6 D) For LKB1 kinase activity, neither HFD nor switched diet had any effect on it (Figure 2.6 E).

Body and Tissue Weight Changes

Short-term HFD feeding did not increase absolute body weight more than STD feeding, and there was no difference for body weight between the CON and skmLKB1-knockout mice. (Figure 2.7 A) However, when expressed as percentage weight gain, HFD resulted in more growth than STD, but still no difference between CON and LKB1-KO. (Figure 2.7 B) During this one week, the retroperitoneal fat pad weight increased in both genotypes similarly with HFD (Figure 2.7 E), but heart weight was unchanged (Figure 2.7 G).

Body weight increased more on HFD than STD. And this is true for all mice that had been put on the long-term HFD including both the 14CH, 14KH, C-switched before switch and K-switched group before the switch back to STD chow. Even though the CON mice always tended to have higher body weight than the LKB1-KO mice with the high-fat feeding, there was no statistical difference between the two genotypes (Figure 2.7 C, D).

For the switched diet group, the mice had been switched to the STD chow after the 14 weeks HFD. One week after the switch, the body weight for the LKB1-KO mice, but not CON mice, decreased significantly (Figure 2.7 C). However, the percentage weight gain and percentage weight decrease were unchanged with switched diet (Figure 2.7 D, I). For the adipose

and heart weight, there was no difference between the long-term HFD groups and the switched groups (after the switch). (Figure 2.7 F, H)

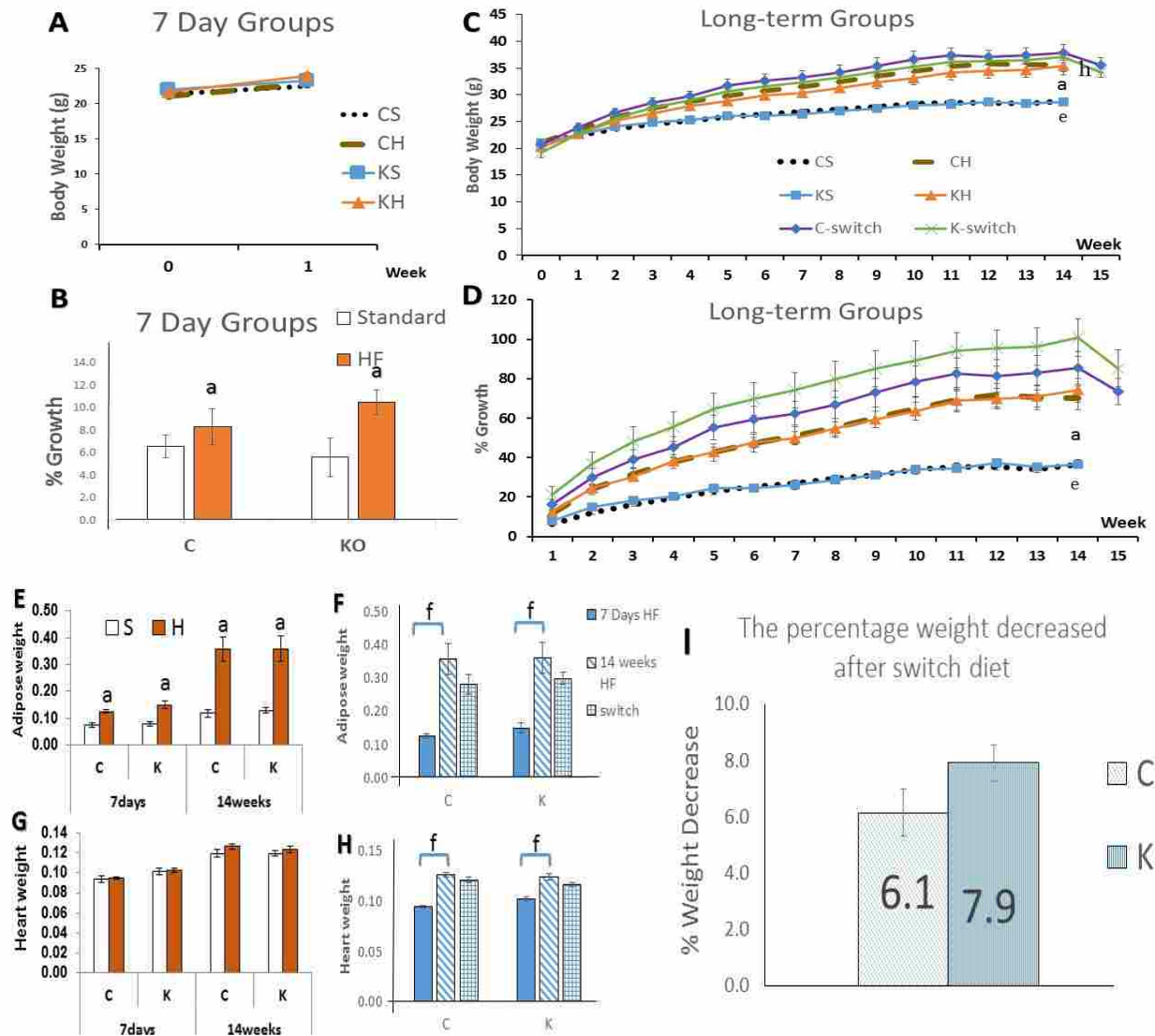


Figure 2. 7: Mouse Body Weight and Weight for Heart and Adipose. Mouse (A, B) body weight, (C, D) percentage growth (% Growth), (E, F) adipose weight, (G, H) heart weight, and (I) percentage weight decrease (% Weight Decrease) were checked for ten different groups [STD chow (S), high-fat (H) or 14 weeks high-fat diet then switched to standard diet for 1 week (switch) feeding in control (C) and skeletal muscle specific LKB1-KO (K) mice]. a= significant main effect of diet. h=significant between before and after switch diet. e=significant difference between CS and CH groups. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=11-20/group).

Effect of HFD on Food, Water and Calorie Consumption

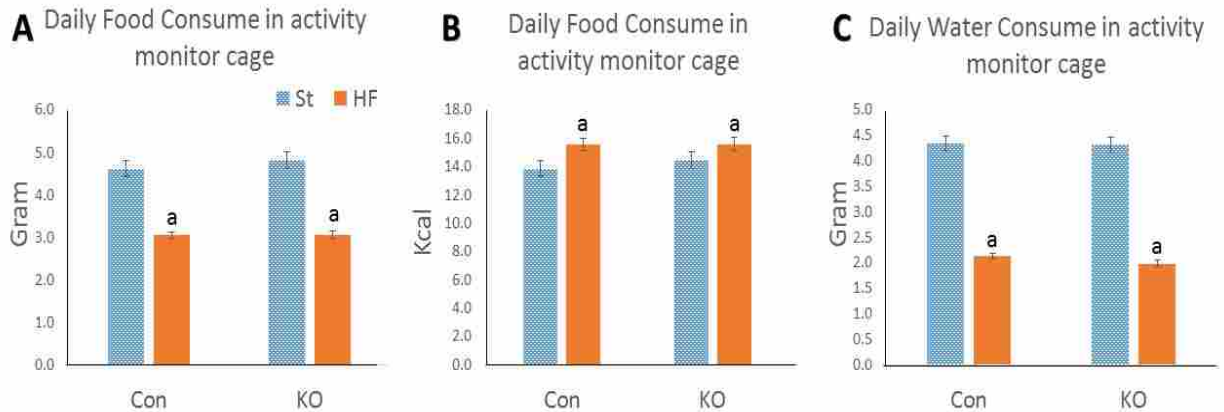


Figure 2. 8: Daily Food and Water Consumption. Daily (A, B) food and (C) water consume for long-term groups. Standard chow (St), high-fat diet (HF), LKB1 knockout mice (KO), and littermate control mice (Con). For the STD chow, one gram has 3 Kcal energy. For high-fat chow, one gram has 5.1 Kcal energy. a= significant main effect of diet. Data is presented as means \pm SEM (n=18-35/group).

Water, food and calorie consumption were measured while the mice were individually housed in the activity cages. HFD-fed mice ate more calories, but less amount of food (Figure 2.8 A,B). There was no difference between CON and LKB1-KO mice for water, food or calorie CONsumption (Figure 2.8).

Effect of LKB1-KO and HFD on Ambulatory Activity

After the mice had been on the long-term diets for 74~85 days, they were housed individually and in-cage activity was monitored in their using an infrared beam-based activity monitoring system. Activity during the light (sleeping) and dark (active) hours was similar between all treatment groups. The activity curves for the high-fat chow fed mice were more flat, while the curves for the STD chow fed mice were more irregular with many peaks and valleys (Figure 2.9A). When expressed as total activity over 24 hours, mice on the HFD had lower activity with no difference between the CON and LKB1-KO. (Figure 2.9B) Day time (6:00pm~6:00am) activity and dark time (6:00am~6:00pm) activity had also been calculated. Mice on the HFD had less activity in both dark and light time periods. (Figure 2.9C)

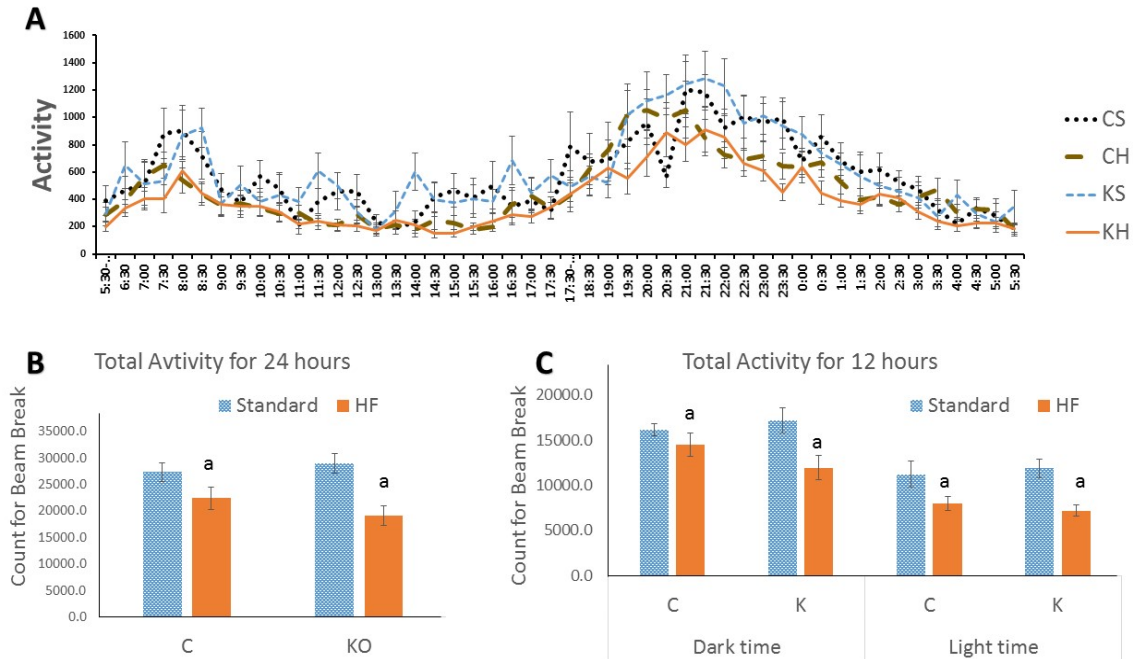


Figure 2. 9: Locomotor Activity for the Long Time Diet Treatment Groups. Mice were housed individually for three consecutive days in cages equipped with an infrared beam-based activity monitoring system that counts how many times the mice broke the beams every 30 minutes. (A) shows the count for every 30 minute interval. (B) shows the total count for 24 hours. (C) shows the total count for the dark time (6:00pm~6:00am) and the light time (6:00am~6:00pm). Standard chow (Standard/S), high-fat diet (HF/H), LKB1 knockout mice (KO/K), and littermate control mice (C). a= significant main effect of diet. Data is presented as means \pm SEM (n=14-29/group).

Effect of LKB1-KO and HFD on Glucose and Insulin Tolerance

For both glucose and insulin tolerance tests, only the long time treatment groups had been tested. After the mice had been on the different diet for 90 days, glucose tolerance test had been performed. The insulin tolerance test was done on the 94th day of the diet treatment. In both tests, mice were fasted before the test. The blood glucose was tested at five different time points during the test to see how it responded to glucose or insulin injection.

For the glucose tolerance test, the area under the curve (AUC) for the HFD groups were greater than for the STD chow groups, indicating decreased glucose tolerance. There was no difference between CON and LKB1-KO (Figure 2.10B). For the insulin tolerance test, the AUC for the high-fat groups were also greater than the STD chow groups, but the AUC for KH group

didn't increase as much as CH group. The AUC for the KS group was also not as high as the CS group (not significant here). The LKB1-KO mice were resistant to HFD induced insulin tolerance (Figure 2.10D).

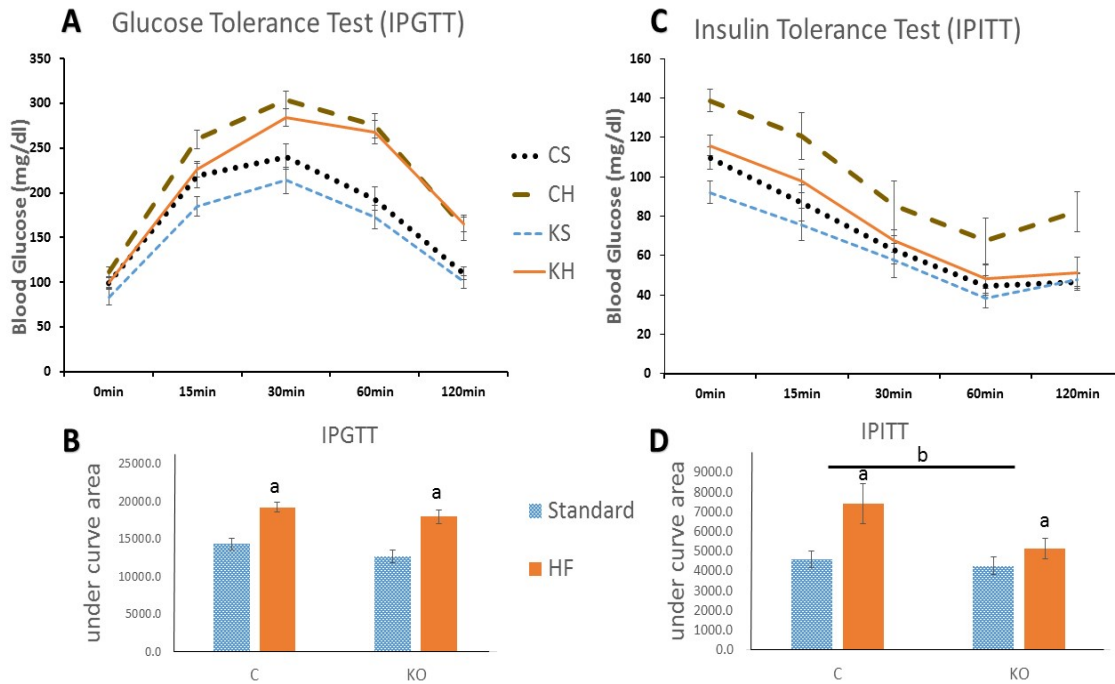


Figure 2. 10: Glucose Tolerance Test and Insulin Tolerance Test (Original Data). The results for (A, B) glucose tolerance test and (C, D) insulin tolerance test (original data) for long-term groups. Standard chow (S/Standard), high-fat diet (H/HF), LKB1 knockout mice (K/KO), and littermate control mice (C). The under curve areas were calculated by adding the area for the four trapezoids together. When calculating the area for each trapezoid, this equation was used: $\text{Area} = (\text{side W} + \text{side Y}) * \text{height} / 2$. Side W and side Y is the number for the glucose level. Height is the number that calculated from the time between each blood glucose checking point (15 minutes=10). a= significant main effect of diet. b=significant main effect for genotype. Data is presented as means \pm SEM (n=12-17/group).

When the data in figure 2.10 is normalized, or expressed relative to the initial fasting glucose concentration, as is often performed, the results are very different. In the case of the glucose tolerance test, both diet treatment and genotype had an effect on modified glucose tolerance test result. Mice on the HFD had a larger AUC than the STD diet mice, consistent with the original data. However, contrary to the un-normalized data, LKB1-KO mice had a greater AUC than the CON mice. This suggests that both HFD and LKB1-KO leads to glucose

intolerance (Figure 2.11B). For the normalized insulin tolerance test result, the AUC increased with HFD in CON, but not LKB1-KO mice (Figure 2.11D).

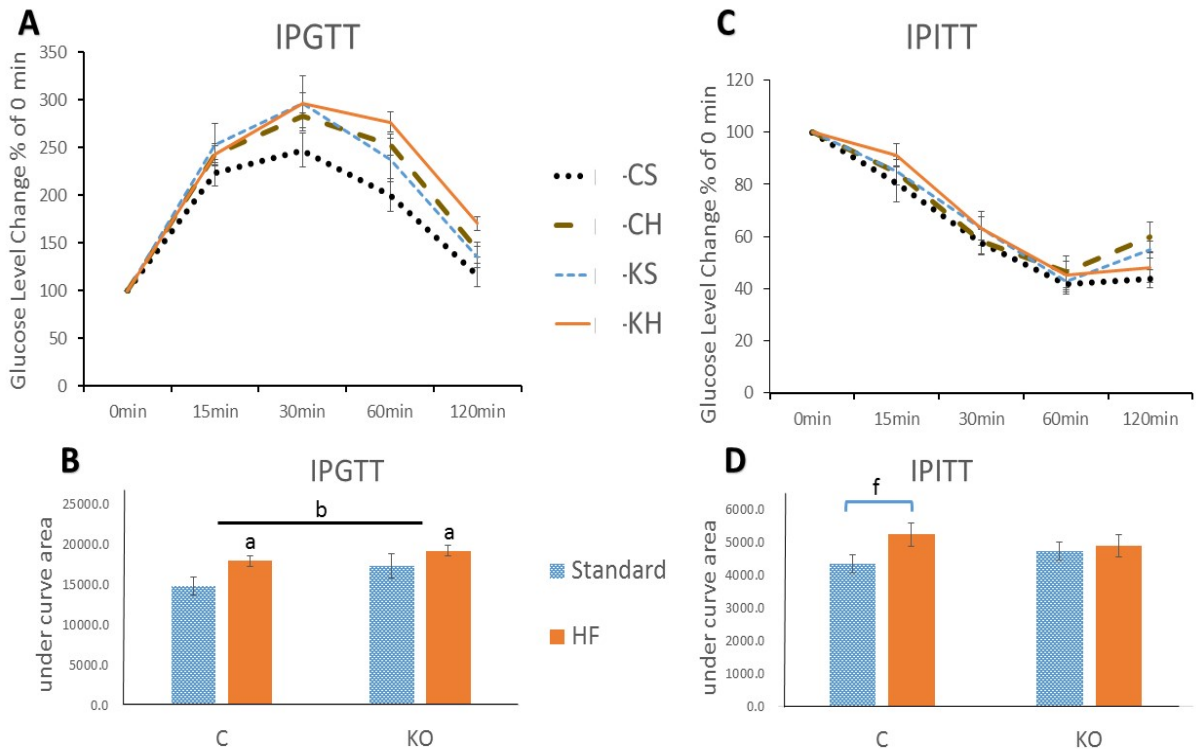


Figure 2. 11: Glucose Tolerance Test and Insulin Tolerance Test (Modified Data).The results for (A, B) glucose tolerance test and (C, D) insulin tolerance test (modified data) for long-term groups. Standard chow (S/Standard), high-fat diet (H/HF), LKB1 knockout mice (K/KO), and littermate control mice (C). “Glucose Level Change % of 0 min”=tested glucose level/0 min glucose level*100%. The under curve areas were calculated by adding the area for the four trapezoids together. When calculating the area for each trapezoid, this equation was used: Area= (side W + side Y)*height/2. Side W and side Y is the number for the glucose level. Height is the number that calculated from the time between each blood glucose checking point (15 minutes=10). a= significant main effect of diet. b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means ± SEM (n=12-17/group).

Effect of LKB1-KO and HFD on Fasting Glucose and Insulin Levels

Some of the mice were fasted for 6-8 hours prior to tissue harvesting (Figure 2.12 A, B). Serum glucose in these animals tended to be lower in LKB1-KO mice, but not significantly (Figure 2.12A). However, the insulin test in the same samples shows that the LKB1-KO mice have higher fasting insulin levels, which could help explain their lower blood glucose levels. But

the HFD fed mice of both genotypes had higher fasting insulin, consistent with insulin resistance (Figure 2.12 B).

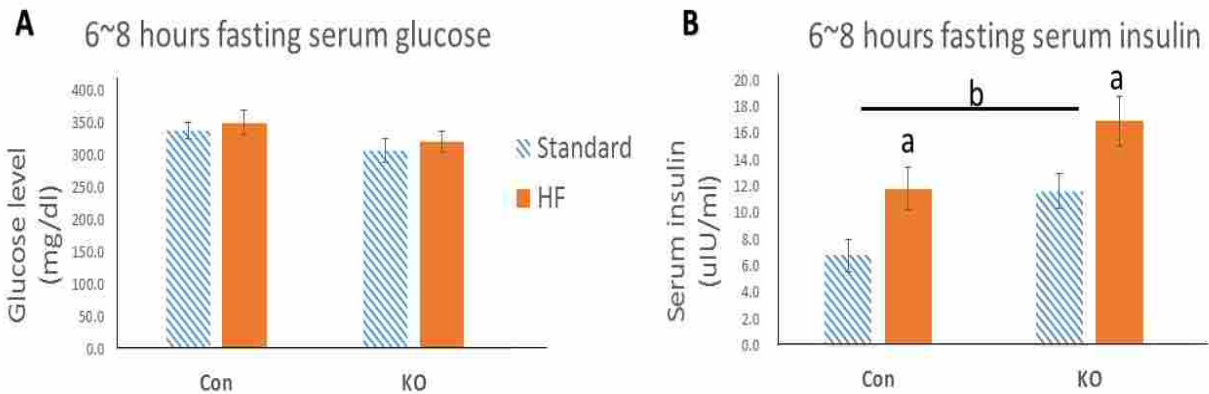


Figure 2. 12: Fasting Serum Glucose and Insulin. Fasting (A) serum glucose and (B) insulin for the long-term groups. Standard chow (Standard), high-fat diet (HF), LKB1 knockout mice (KO), and littermate control mice (Con). The 6~8 hours fasting data came from the serum that was collected during harvest. For the mice that will be harvested on the same day, their food had been taken away at around 4:00 am. The harvest began at around 10:00 am and ended around noon, or early if less mice were harvested on that day. So the fasting time period is different for different mice. a= significant main effect of diet. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

Effect of LKB1-KO and HFD on GLUT4 Expression

Glucose transporter type 4 (GLUT4) is the primary insulin-regulated glucose transporter in skeletal muscle [150]. It can be recycled into the cytoplasm (vesicle) or stay on the cell membrane. Once it gets onto the cell membrane, it can transport glucose into the cell by facilitated diffusion. Insulin stimulation usually stimulates movement of GLUT4 to the cell membrane.

Short-term HFD didn't change the total GLUT4 expression in the CON mice, but it increased the total GLUT4 expression in the LKB1-KO mice. (Figure 2.13A) For long-term diet groups, neither HFD nor LKB1-KO had a significant effect on total GLUT4 expression (Figure 2.13A). After 6~8 hours fasting, on the other hand, the total GLUT4 expression decreased in the HFD groups, and it increased only in the LKB1-KO mice that didn't eat HFD. When comparing

between the fasting and nonfasting 14 weeks groups, the total GLUT4 expression only increased in the KS group (Figure 2.13C).

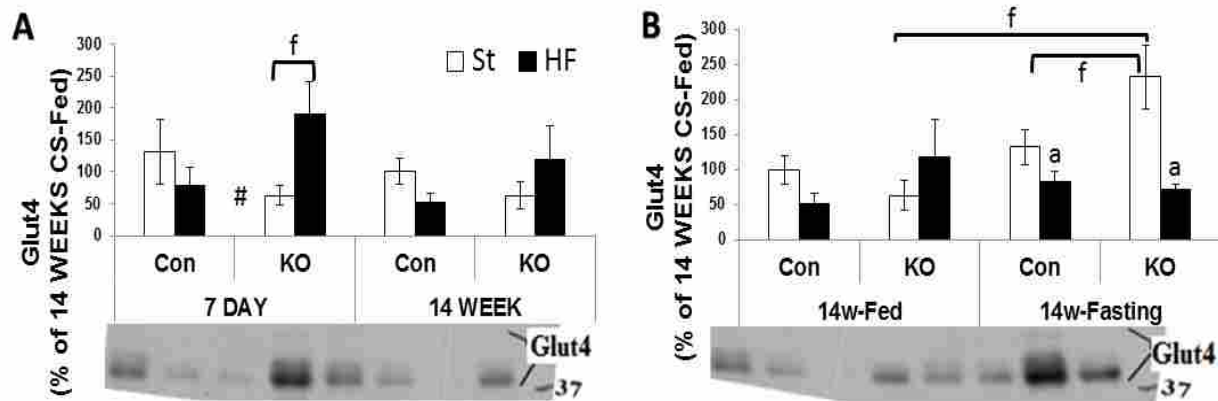


Figure 2. 13: The Total Protein Expression of GLUT4. The total protein expression of GLUT4 in the quadriceps (QUAD) homogenates for 7-day-groups vs 14-week-groups (A) and 14-week-fed-groups vs 14-week-fast-groups (B). The fasting data came from the 6~8 hours fasting mice (fast before harvest). Standard chow (St), high- fat diet (HF), LKB1 knockout mice (KO), and littermate control mice (Con). a= significant main effect of diet. #=had genotype and diet interaction effect in two-way ANOVA. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

Effect of LKB1-KO and HFD on AS160 and TBC1D1 Phosphorylation

AS160 (Akt substrate of 160 kDa) is also known as TBC1D4 (TBC1 domain family member 4). It is a Rab GTPase-activating protein. It can be phosphorylated both by Akt and AMPK. The non- phosphorylated AS160 prevents the GLUT4 vesicle from fusion into the cell membrane, but phosphorylation causes it to be sequestered by 14-3-3 protein and this allows GLUT4 exocytosis to the cell membrane to occur. AMPK phosphorylates AS160 at the Thr642 site [151]. Both Thr642 site and Ser318 site can be phosphorylated by Akt [152, 153].

TBC1 domain family member 1 (TBC1D1) is related to and regulated in the same way as AS160. It also can be phosphorylated by Akt and AMPK. AMPK phosphorylates TBC1D1 on Ser237 [151], while Akt phosphorylates it on Thr590 site [154].

For the short-term diet groups, LKB1-KO didn't affect p-AS160 on either Ser318 site or Thr642 site (Figure 2.14 A, B), but it increased total AS160 expression in skeletal muscle

(Figure 2.14 C). Consequently, the ratio for p318/t-AS160, p642/t-AS160, and all p/t AS160 all decreased in the short-term LKB1-KO mice (Figure 2.14 D, E, F). For the long-term groups, LKB1-KO only increased p-AS160 on Ser318 site (Figure 2.14 A). HFD didn't have effect on AS160 or its phosphorylation.

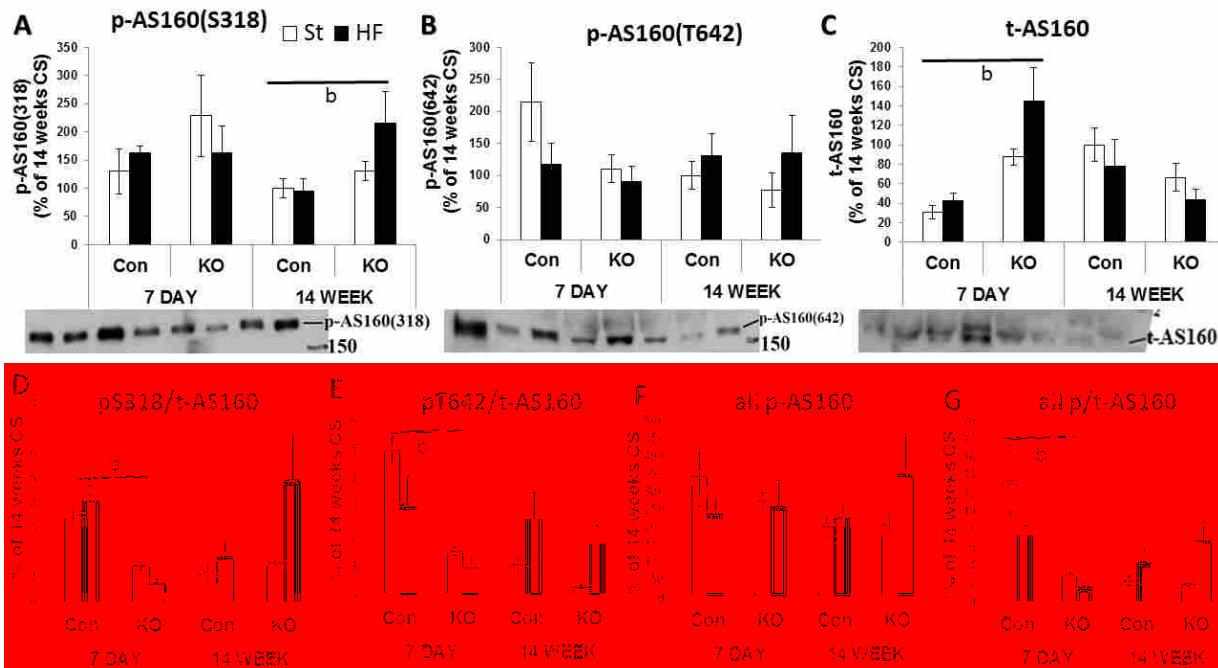


Figure 2. 14: The Protein Expression for AS160. The protein expression for (A) phosphor-AS160 (S318), (B) phosphor-AS160 (T642), (C) total AS160, (D) the pS318/t-AS160 ratio, (E) the pT642/t-AS160 ratio, (F) all p-AS160 ratio [all p-AS160=p-AS160(S318) + p-AS160(T642)], and (G) all p/t-AS160 ratio (all p/t-AS160=all p-AS160/t-AS160) in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

For the short-term groups, LKB1-KO decreased p-TBC1D1 on Ser237 site (Figure 2.15 A) and the combined p-TBC1D1 (all p-TBC1D1) (Figure 2.15 F). For the long-term groups, p-TBC1D1 on both the Ser237 site and Thr590 site decreased with LKB1-KO (Figure 2.15 A, B). And, of course, the combined p-TBC1D1 also decreased in LKB1-KO mice (Figure 2.15 F). For the p237/t-TBC1D1 ratio, it was affected by both genotype and diet. LKB1-KO decreased p237/t-TBC1D1 ratio. HFD also decreased the p237/t-TBC1D1 ratio, but this effect was more obvious in the CON mice than the LKB1-KO mice. The effect that the diet caused in the CON

mice is different from the one in the LKB1-KO mice. HFD decreased p237/t-TBC1D1 ratio in the CON mice, but the decrease was not significant in the LKB1-KO mice. (Figure 2.15D) For all p/t-TBC1D1 ratio, it had the same effect as p237/t-TBC1D1 ratio except the interaction effect was almost significant ($p=0.52$). LKB1-KO decreased the all p/t-TBC1D1 ratio. HFD also decreased all p/t-TBC1D1 ratio, the effect that the diet caused in the CON mice is different from the one in the LKB1-KO mice. HFD decreased all p/t-TBC1D1 ratio in the CON mice, but it didn't affect the LKB1-KO mice (Figure 2.15 G).

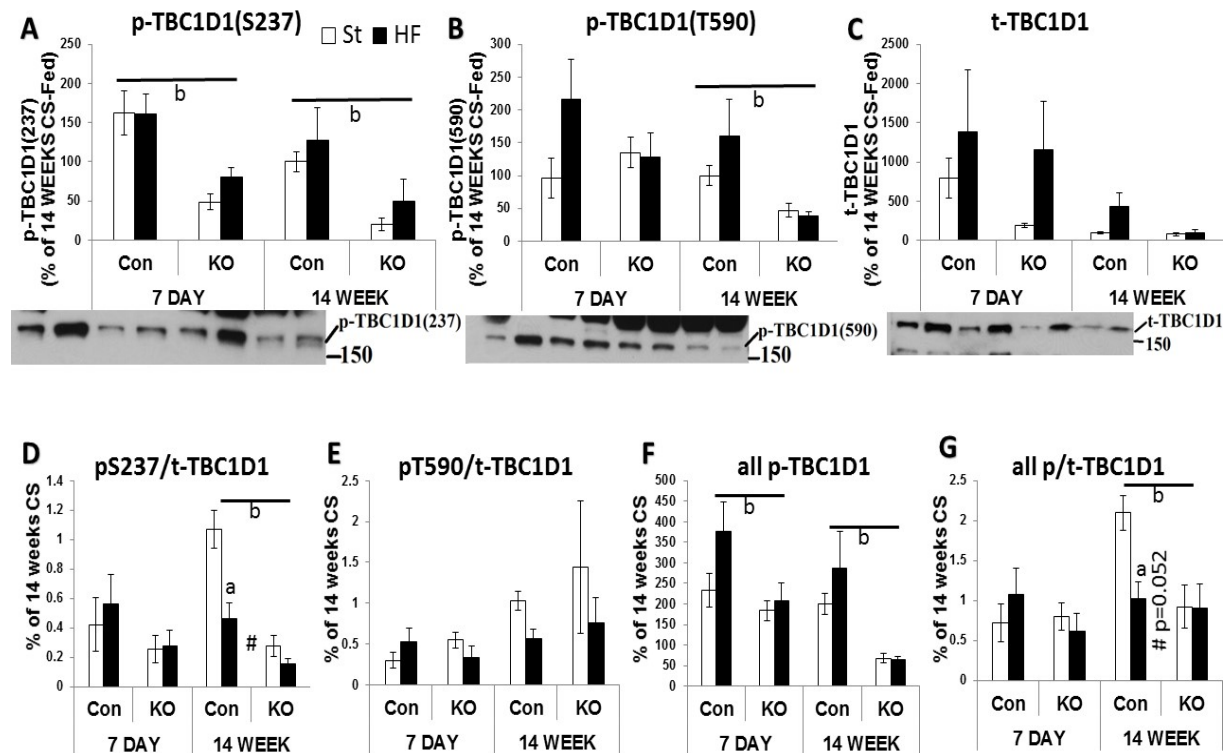


Figure 2. 15: The Protein Expression for TBC1D1. The protein expression for (A) phosphor- TBC1D1 (S237), (B) phosphor- TBC1D1 (T590), (C) total TBC1D1, (D) the pS237/t- TBC1D1 ratio, (E) the pT590/t- TBC1D1 ratio, (F) all p- TBC1D1 [all p- TBC1D1=p-TBC1D1 (S237) + p-TBC1D1 (T590)], and (G) all p/t-TBC1D1 ratio (all p/t-TBC1D1=all p-TBC1D1/t- TBC1D1) in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. a= significant main effect of diet. b=significant main effect for genotype. #=had genotype and diet interaction effect in two-way ANOVA. Data is presented as means \pm SEM (n=6/group).

Effect of LKB1-KO and HFD on HXKII Expression

Hexokinase II (HXKII) is an enzyme that phosphorylates glucose upon its entry into the cell to produce glucose-6-phosphate. This is the first obligatory step in glucose metabolism [155]. HXKII is the predominant HXK in the skeletal muscle [156]. In this study, HXKII expression wasn't affected by either HFD or LKB1-KO (Figure 2.16).

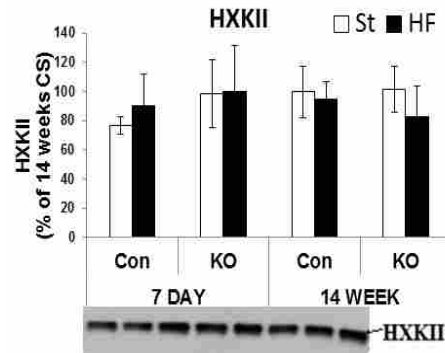


Figure 2. 16: The Total Protein Expression of HXKII. The total protein expression of HXKII in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks.

Effect of LKB1-KO, HFD and Fasting on Glycogen Content in Skeletal Muscle

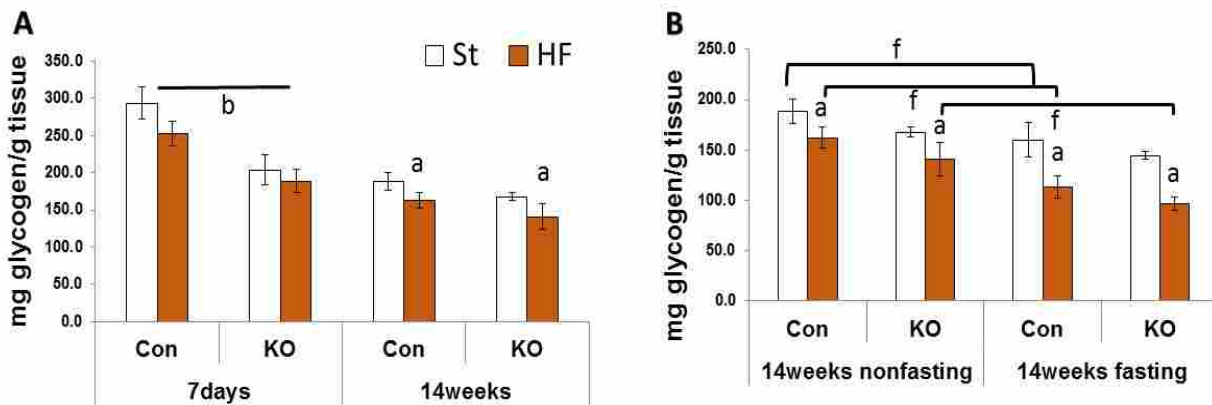


Figure 2. 17: The Glycogen Content. The glycogen content in the quadriceps (QUAD) homogenates for 7-day-groups vs 14-week-groups (A) and 14-week-fed-groups vs 14-week-fast-groups (B). The fasting data came from the 6~8 hours fasting mice (fast before harvest). Standard chow (St), high-fat diet (HF), LKB1 knockout mice (KO), and littermate control mice (Con). a= significant main effect of diet. b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=8/group).

Glycogen is a form of energy storage in liver and skeletal muscle. The glycogen in the liver can be converted to glucose and provide energy for the whole body when it is released into

the blood, but the glycogen in the muscle is not released into the blood and only provide energy for the muscle itself.

In short-term fed mice, LKB1-KO decreased glycogen storage in skeletal muscle (Figure 2.17 A). For the long-term groups, HFD decreased skeletal muscle glycogen content in both fasting and non-fasting mice. Fasting decreased glycogen content in all mice except for the LKB1-KO STD groups (Figure 2.17 B).

Effect of LKB1-KO and HFD on Glycogen Synthase

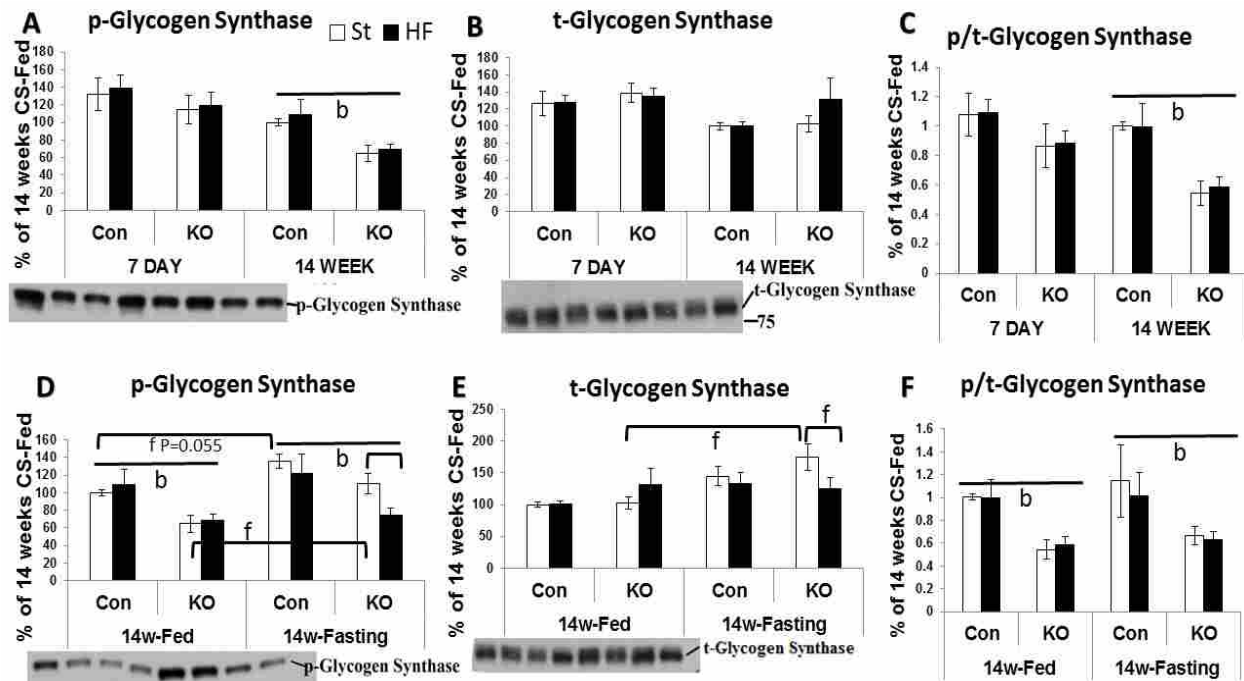


Figure 2. 18: The Protein Expression for Glycogen Synthase. The protein expression for (A) phosphor-glycogen synthase, (B) total glycogen synthase, and (C) p/t-glycogen synthase ratio (p-glycogen synthase/t-glycogen synthase) in whole protein homogenates for quadricep (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice that didn't fast (fed) before harvest, and the protein expression for (D) phosphor- glycogen synthase, (E) total glycogen synthase, (F) p/t-glycogen synthase ratio in whole protein homogenates for QUAD muscle for from LKB1 knockout (KO) and littermate control (Con) mice that fast before harvest. The fasting data came from the 6~8 hours fasting mice (fast before harvest). Standard chow (St), high-fat diet (HF). b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

Glycogen synthase (GS; UDP-glucose-glycogen glucosyltransferase) converts glucose into glycogen. It is a key enzyme in glycogen synthesis. GS can be phosphorylated by glycogen

synthase kinase 3 (GSK3) in addition to other kinases. Phosphorylation by GSK3 decreases GS activity.

The total GS level didn't change with either HFD or LKB1-KO (Figure 2.18B), but, after 6~8 hours fasting, its expression increased only in the KS group (Figure 2.18E). For the phospho-GS, it changed with both LKB1-KO and fasting. In the long term diet groups (fasting and non-fasting), both p-GS and p/t-GS ratio decreased with LKB1-KO (Figure 2.18A, C, D, F). With no change in total GS expression, that indicates that GS activity was greater in the LKB1-KO mice. But LKB1-KO skeletal muscle didn't have more glycogen storage. When comparing the data before and after fasting, only p-GS increased in the KS group after fasting.

As mentioned before, GSK3 can phosphorylate GS to inactive it. GSK3, in turn, is inactivated by phosphorylation by Akt in the insulin signaling pathway. GSK3 has two different types, both with similar functions: GSK3 α and GSK3 β . Akt can phosphorylate GSK3 α at Ser21 and GSK3 β at Ser9 to inactivate them [157, 158].

For GSK3 α , its phosphorylation increased with long-term HFD (Figure 2.19 A), and its protein expression increased with LKB1-KO in the long term diet groups (Figure 2.19 C).

When comparing the data before and after fasting, fasting increased total GSK3 α in muscles from STD diet group, but not the HFD-fed mice (Figure 2.19C). Fasting only increased p-GSK3 α in the CS group. For the KS group, it was increased insignificantly (Figure 2.19A). For the p/t-GSK3 α ratio, it didn't show any change except the ratio decreased in the KH group after fasting (Figure 2.19E).

For GSK3 β , its phosphorylation increased in the adult LKB1-KO mice, but not the young ones. After fasting, its phosphorylation only increased in the KS mice. (Figure 2.19B) The total GSK3 β increased in the STD chow fed mice after fasting, but was unchanged in the HFD group.

This resulted in the high-fat groups having lower t-GSK3 β in the fasting state (Figure 2.19D). For the p/t-GSK3 β ratio, it didn't show any change except the ratio in the 14-week-KH group was higher than the 14-week-CH group (Figure 2.19F).

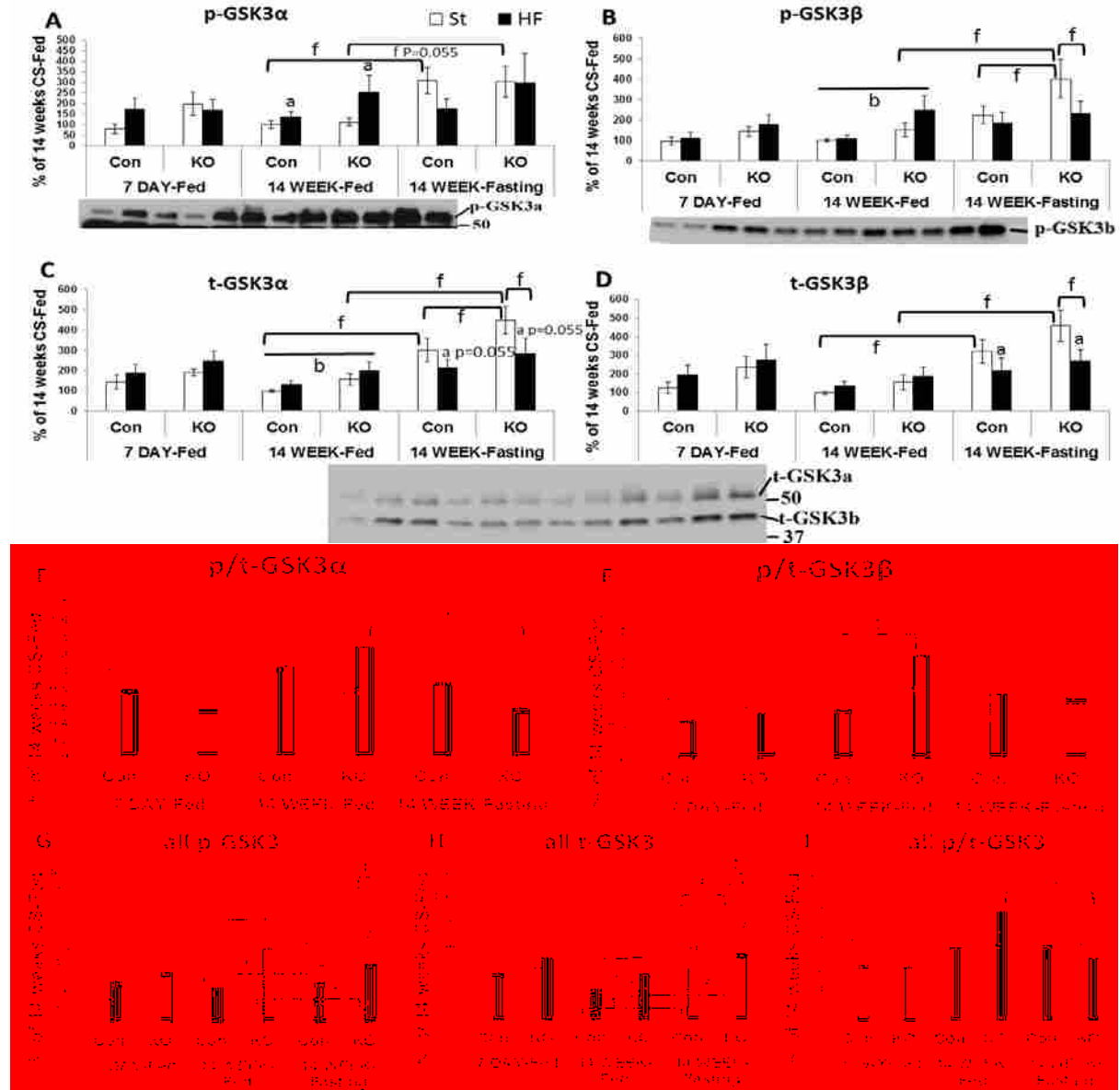


Figure 2. 19: The Protein Expression for GSK. The protein expression for (A) phosphor-GSK3 α , (B) phosphor-GSK3 β , (C) total GSK3 α , (D) total GSK3 β , (E) p/t-GSK3 α ratio (p-GSK3 α /t-GSK3 α), (F) p/t-GSK3 β ratio (p-GSK3 β /t-GSK3 β), (G) all p-GSK3 (all p-GSK3=p-GSK3 α +p-GSK3 β), (H) all t-GSK3 (all t-GSK3=t-GSK3 α +t-GSK3 β), and (I) all p/t-GSK3 ratio (all p/t-GSK3=all p-GSK3/all t-GSK3) in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks at fed or fast state. The fasting data came from the 6~8 hours fasting mice (fast before harvest). b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

For combined p-GSK3, it increased in the STD chow fed mice during fasting. In the fasting state, the HFD groups had lower p-GSK3 than the STD chow fed mice. (Figure 2.19G) The combined t-GSK3 had the same effect as the combined p-GSK3 (Figure 2.19H). This resulted in no difference between groups for the p/t-GSK3 ratio (Figure 2.19I).

Effect of LKB1-KO and HFD on Akt Activity

As mentioned before, Akt is the “hub” of the insulin pathway. It can phosphorylate AS160 and TBC1D1 to regulate GLUT4 exocytosis. It also phosphorylates GSK3 α and GSK3 β to regulate glycogen synthesis. Akt also can be phosphorylated by other kinases, like PDK1. Akt is activated by phosphorylation at Thr308 and Ser473 [159].

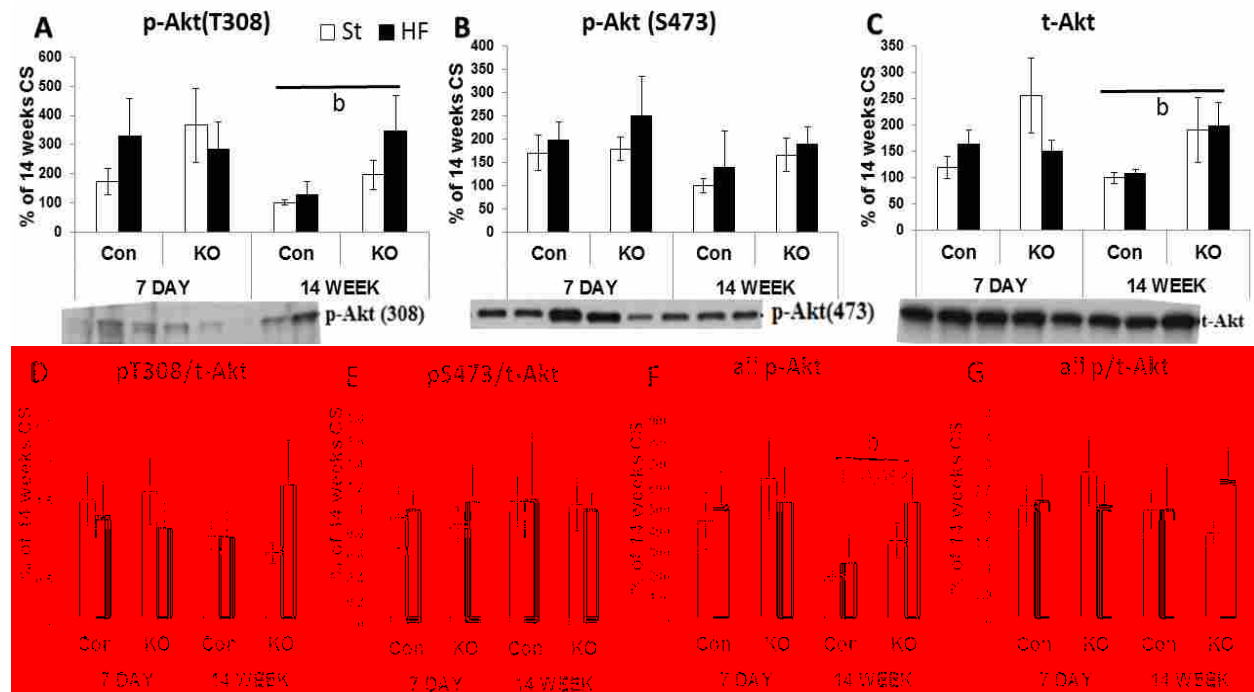


Figure 2. 20: The Protein Expression for Akt. The protein expression for (A) phosphor- Akt(T308), (B) phosphor- Akt (S473), (C) total Akt, (D) the pT308/t-Akt ratio, (E) the pS473/t-Akt ratio, (F) all p-Akt [all p-Akt=p-Akt (T308) + p-Akt (S473)], and (G) all p/t-Akt ratio (all p/t-Akt= all p-Akt/t-Akt) in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

Only the Akt phosphorylation at Thr308 and total Akt were affected by LKB1-KO in the long-term diet groups. They both increased in the LKB1-KO skeletal muscle. (Figure 2.20A, C) Neither Akt expression nor its phosphorylation were affected by HFD.

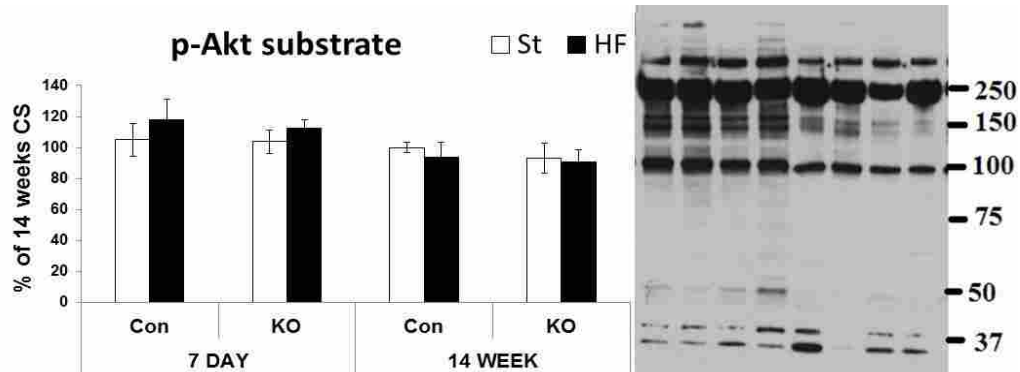


Figure 2. 21: The Total Protein Expression of P-Akt Substrate. The total protein expression of p-Akt substrate in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks.

Western blotting for Akt substrate detects all the proteins that can be phosphorylated by Akt, so its phosphorylation reflects overall Akt activity. Akt substrate phosphorylation wasn't affected by either HFD or LKB1-KO (Figure 2.21). This means the whole Akt activity didn't change with either HFD or LKB1-KO in the skeletal muscle.

Discussion

The above results show that long-term *ad libitum* HFD can increase body weight and adipose tissue in mice. This agrees with most previous studies [45-49]. Also as some studies have shown [53, 60], the heart weight didn't change with HFD. For the HFD groups, mice ate more calories and had less daily activity. This may have contributed to why they gained more body weight.

However, the reason for the decreased activity of HFD mice is not clear. Some previous work was shown results opposite to our findings. López-Soldado et al., for instance, showed that total locomotor activity and total ambulation activity didn't change after HFD, but the

ambulation activity in the light phases tended to decrease (author didn't compare the data between different diets) [81, 160]. Akira et al. [161] showed that HFD lead to changes in the period of the locomotor activity rhythm: the free running period was lengthened, diurnal rhythm of locomotor activity was dampened, and they showed an overall diurnal rhythm in feeding. Another paper [162] also showed that the mice preferred to eat high fat food during the light phase, not the dark one. Maybe this altered eating habit affected the activity level of the mice. HFD can attenuate the amplitude of clock gene expression in hypothalamus, liver, and adipose. Besides that, HFD also changed the day-night expression pattern and the total amount of serum leptin and corticosterone levels, and some neuropeptides in hypothalamus. [161] These changes may play a role in HFD-induced activity changes.

Another interesting thing is the mice on the HFD had less water consumption. There isn't any clear explanation for this, but previous work has shown the same effect [163-165]. However, there are some papers shown the inconsistent effects: one showed HFD didn't change the water intake [166], while another one showed water intake increased with HFD [167]. Maybe because they didn't put mice on the HFD for enough time (only 6 weeks) [167] or because their mice were too young (9 weeks) [167] or too old (23 weeks) [166] to show the same effect. In the current study, the mice were about 16 weeks old (equivalent to about 23 years old in humans) when their water and food consumption was tested. One review paper showed that when the drinking water was removed, the young adult will increase energy intake, while the senior adult (55+) will decrease energy intake [168]. Therefore, the amount of water consumption is related to the amount of calorie intake, and this relationship is affected by age.

The lack of LKB1 in skeletal muscle did not affect weight gain or food/water consumption or ambulatory activity. The lack of difference between genotypes agrees with previous work

from our lab [145]. Therefore, any differences between LKB1-KO and CON mice wasn't caused by different body weights, different calories consumption or different activity levels between genotypes.

Perplexingly, the % growth for the K-switch mice (before switch) was higher than the long-term LKB1-KO HFD mice since the first week on the HFD. The reason for this difference is not clear, but it may be due to slightly different environmental conditions during the periods that they were housed in the animal facility. The long-term (14-week)-fed mouse cohorts were fed prior to the HFD-switched cohorts. Since they were not treated at the same time, it is assumed that minor variations in the lots of chow or the automatic watering system may have had some effect.

LKB1 expression was performed to verify the genotypes of the mice but also to determine how HFD can affect LKB1 expression. The results confirmed that the “myf6-Cre-” method successfully knocked out LKB1 in skeletal muscle. Total LKB1 expression in skeletal muscle was not affected by HFD feeding. This is consistent with some articles [110, 111], but not the same as other papers in which LKB1 mRNA or protein decreased after HFD [112-115]. Others have reported that there was a tendency for LKB1 to move into the nucleus instead the cytoplasm after HFD [116], but here LKB1 content in the nuclear and cytoplasmic fractions were about the same, although HFD tended to decrease (not significantly) the localization of LKB1 to the nucleus, so it is opposite from the result that was shown in the liver in a previous study [116]. HFD did not affect LKB1 activity.

After LKB1-KO, p-AMPK decreased and AMPK's kinase activity also decreased. This makes sense because LKB1 phosphorylates and activates AMPK. Less LKB1 should lead to less p-AMPK/AMPK activity. This result was supported by all the previous studies [19-21, 23].

AMPK is a heterotrimeric protein formed by α , β , and γ subunits, and the α subunit only has two isoforms: $\alpha 1$ and $\alpha 2$. We found that AMPK $\alpha 1$ activity didn't get affected by LKB1-KO, while AMPK $\alpha 2$ activity decreased when no LKB1 existed. That means that the LKB1-KO induced decrease in AMPK activity was caused by decreased activity for AMPK $\alpha 2$, not $\alpha 1$. This is also supported by previous research [23], and indicates that AMPK $\alpha 1$ activity is not fully LKB1 dependent. AMPK also can be phosphorylated by Tak1 [169] and CaMKK [170, 171]. In some conditions, AMPK $\alpha 1$ can be selectively activated by Tak1 [172] or CaMKK [173]. Maybe in the LKB1-KO mice, Tak1 and/or CaMKK activity are somehow increased to rescue the decreased AMPK activity.

With all the results in the above paragraph, it was expected that the expression for AMPK $\alpha 2$ would increase, but not $\alpha 1$, as was found in a previous study [23]. However, expression for AMPK $\alpha 1$ increased, but not $\alpha 2$. And this increase only happened in the adult mice (19 weeks old). For the young mice (6 weeks old), it tended to increase, but was not significant. With the AMPK $\alpha 1$ increasing and AMPK $\alpha 2$ tending to increase in the LKB1-KO mice, the total AMPK expression increased in the adult LKB1-KO mice, suggesting that LKB1-KO induced increased expression of AMPK $\alpha 1$ is age-dependent. What controls or triggers this needs further research. Perhaps the muscle adapts to the decreased AMPK activity in LKB1-KO by increasing AMPK expression. While LKB1-KO induces increased expression of AMPK $\alpha 1$, this wasn't associated with increased AMPK $\alpha 1$ activity.

After the long-term HFD, the AMPK $\alpha 1$ activity decreased in CON mice compared with short-term HFD and the AMPK $\alpha 2$ activity decreased in LKB1-KO mice compared with short-term HFD. This may be caused by long-term HFD or maturation (from adolescent stage to adult stage). Comparing short-term STD and long-term STD groups, it seems that the change should

be caused by maturation instead of long-term HFD. For the AMPK $\alpha 1$, LKB1-KO improves the maturation caused decrease in AMPK activity. For AMPK $\alpha 2$, skeletal muscle needs LKB1 to be there in order to prevent the maturation-caused decrease in activity.

The part of the LKB1 that went into nuclear and the part that stayed in cytoplasm were about the same. LKB1 requires MO25 α and STRAD α to anchor it in the cytoplasm, and for full kinase activity [3]. Therefore, there should be more p-AMPK in the cytoplasm than the nucleus. However, we found that there is at least the same amount of, or even more, p-AMPK in the nucleus as in the cytoplasm. This is likely caused by the translocation of p-AMPK after phosphorylation since AMPK is phosphorylated in the cytoplasm and then transferred into nucleus [174].

The nuclear distribution for AMPK $\alpha 2$ decreased with LKB1-KO in the adult mice, not the young mice. A tendency for this decrease was also observed for AMPK $\alpha 1$, but was not significant. Combined with the data that AMPK $\alpha 1$ activity didn't get affected by LKB1-KO, while AMPK $\alpha 2$ activity decreased with LKB1-KO, this suggests that it's possible that AMPK needs to be phosphorylated in order to be able to be transferred in active form into nucleus. This needs further research.

Insulin resistance is one of the most detrimental effects caused by HFD-induced obesity [59]. This was also shown by this study. However, the extent of the insulin resistance is unclear. When expressed using absolute glucose concentrations, IPGTT and IPITT testing showed that the HFD mice became both glucose and insulin intolerant, but when the data is normalized and expressed as a percent of the initial, fasting blood glucose level, HFD mice only became glucose intolerant. Furthermore, when expressed in absolute values, LKB1-KO improved the insulin

tolerance, but not the glucose tolerance, but using normalized data, LKB1-KO made glucose tolerance worse, but not the insulin resistance.

There are two ways to present the glucose and insulin tolerance test results: using the absolute data [175-179] and using data normalized to the fasting glucose level [175, 176, 178]. Both ways of analyzing the data are widely used. Previous reports [175, 176, 178] even used both analysis in the same paper, but to show the different experiments data. They all used original data for glucose tolerance test results, and modified data for insulin tolerance test results. Also by using two different analysis methods, the results can be quite different. This happened in both this study and previous papers [175, 176, 178].

Insulin tolerance and glucose tolerance testing is often performed to determine whether insulin resistance occurs. Actually, however, these two tests are testing different physiological responses. For the insulin tolerance test, exogenous insulin is injected into the mice. The test measures how well the body responds to the extra insulin to bring down blood glucose levels, and how well the body can bring the blood glucose back before it's really low. For the glucose tolerance test, glucose is injected into the mice. The test measures how well the body responds to the high blood glucose to not let it go very high and bring it down to normal. Upon examination of the AUC for the original data of insulin tolerance test, LKB1-KO mice may have better insulin sensitivity. But if we also consider the normalized data, we can tell the the size of the area under the curve depends on the level of blood glucose at 0 minutes. The slopes for the curve were similar between different groups. Therefore, LKB1-KO muscle may not have better insulin sensitivity but they just had lower fasting blood glucose. HFD may have simply increased fasting blood glucose levels, but not necessary induced the insulin resistance. Therefore, with

non-physiological level of insulin (super high insulin, higher than physiological level), neither HFD nor LKB1-KO can affect blood glucose uptake ability.

The normalized data suggest that LKB1-KO can make glucose tolerance worse because the blood glucose rose faster in the LKB1-KO mice than the CON mice. Blood glucose also rose faster during the first 30 minutes of the test in the CH mice than the CS, but was not different between KS and KH mice. During this time, the insulin level should increase from low to peak levels [180, 181]. During this time, two things happen: the high blood glucose induces insulin secretion and then the increased insulin induces glucose uptake by insulin sensitive tissue such as skeletal muscle. In the original data, the blood glucose levels declined more slowly in the LKB1-KO mice (KS slower than CS, KH slower than CH). No difference was observed between CH and CS, or KS and KH mice. During this time, the insulin level should drop from the peak back to normal. Those two things also happened here, but insulin induced glucose uptake has a greater effect. Therefore, with the physiological insulin level, LKB1-KO can make glucose uptake ability worse, while HFD made high blood glucose induced insulin secretion worse only in the CON mice.

After both 4 hours and 6 hours fasting, no matter which diet they were given, LKB-KO mice had lower fasting blood glucose. What caused this lower fasting glucose level for the LKB1-KO mice is not certain, but the following are some possible reasons: 1) LKB1-KO mice have increased insulin levels in the blood. 2) LKB1-KO in the skeletal muscle leads to less glycogen storage in muscle. In response to this, perhaps the muscle takes up more blood glucose to provide enough energy for the muscle which could cause lower fasting blood glucose levels. 3) LKB1-KO in skeletal muscle makes the muscle more insulin-sensitive. Even though the

insulin level is the same as in CON mice, the increased insulin sensitivity might lead to lower blood glucose. Each of these possibilities will be discussed further below.

Fasting insulin was indeed higher in the LKB1-KO mice. Glucose concentration was tested in the same serum, and the glucose level was expected to be lower in the LKB1-KO mice. However, for the 6~8 hours fasting mice, the LKB1-KO mice's fasting serum glucose wasn't lower than the CON mice, even it did show a trend for such. This may be because it's serum that had been tested, not blood. The blood from some mice had slight hemolysis before or during the centrifuge process. Since blood cells can have glucose inside of the cell, but not insulin, the glucose that was released from those broken cells may have eliminated the significant difference for fasting glucose. Or it is possible that their fasting time was different (6-8 hours vs 4 hours), and this difference is enough to eliminate the significant difference for fasting glucose, but not fasting insulin. The simplest and possible reason for these abnormal results is that somehow the β -cells of the pancreas that secrete insulin were changed so that lower blood glucose still induced abundant insulin release in the LKB1-KO mice. Since LKB1-KO is specific to skeletal muscle in these mice, altered β -cell function would suggest that skeletal muscle LKB1 is important in muscle cross-talk with β -cells. It is also possible that the Myf6 promoter (which is thought to be skeletal muscle specific) controls genes expressed in the β -cells. All these inferences need further research.

The second potential reason for decreased fasting glucose content in LKB1-KO mice relates to glycogen storage. Skeletal muscle glycogen content only decreased in the young LKB1-KO mice (6 weeks old), not adult ones (19 weeks old), but the mice that had lower fasting blood glucose were 19 weeks old. Therefore, lower fasting blood glucose here was not caused by decreased glycogen storage. However, the glycogen content was lower in the young LKB1-KO

vs. CON mice. For the adult mice, the ones on the HFD had lower glycogen content in skeletal muscle. In order to find out the reason for this change, GS and GSK3 were measured. Neither GS expression nor its activity (phosphorylation) changed in the young LKB1-KO mice. The same was true for GSK3. For the HFD did not change GS expression or its activity. Therefore, none of the glycogen content changes can be explained by altered GS and GSK3. Another interesting phenomenon is the GS activity increased in the adult LKB1-KO mice, and, as mentioned above, glycogen content in these mice didn't change, so that suggests that glycogen usage may be increased in the adult LKB1-KO mice. This was not tested here and further research is needed in order to prove this.

The third potential explanation for decreased fasting blood glucose in LKB1-KO mice is increased insulin-induced glucose uptake effect in LKB1-KO mice. Several important proteins in the insulin pathway were measured in this study. The adult LKB1-KO mice did have a higher Akt expression and phosphorylation on Thr308 site (associated with increased Akt activity), consistent with elevated insulin signaling or sensitivity. AS160 can be phosphorylated by Akt at Ser318 [152, 153], leading to its sequestration away from GLUT4 vesicles and increased GLUT4 translocation to the cell membrane. Consistent with this, AS160 phosphorylation at Ser318 also increased in the adult LKB1-KO mice, which should result in more AS160 controlled GLUT4 vesicle exocytosis, more GLUT 4 on the cell membrane and increased glucose uptake. However, AS160/TBC1D4 works in concert with TBC1D1 which plays the same role as AS160. The phosphorylation for both Ser237 and Thr590 on TBC1D1 decreased in the adult LKB1-KO mice, which is opposite to the change in AS160. The combined phosphorylation amount and ratio also decreased in these mice. These means there are less TBC1D1 controlled GLUT4 vesicles for exocytosis. The phosphorylation for Ser237 site can be done by AMPK [151], which should

decrease with LKB1-KO. But since the Thr590 site can be phosphorylated by Akt [154], a decrease in its phosphorylation was not expected. Thus, it is not clear how the glucose uptake effect changed in the LKB1-KO mouse skeletal muscle, because the AS160's inhibition effect (inhibit GLUT4 vesicle exocytosis) decreased but the TBC1D1's inhibition effect increased. With the conflicting results for AS160 and TBC1D1, total GLUT4 expression was measured, but didn't change with LKB1-KO. Further studies should determine the glucose uptake rate in LKB1-KO muscles.

In summary, the lower fasting blood glucose that we observed here may have been caused by increased fasting insulin and/or increased p-Ser318-AS160 induced GLUT4 vesicle exocytosis.

In contrast to the decreased fasting glucose level in LKB1-KO mice, HFD fed mice had increased blood glucose levels. This expected increase in fasting glucose combined with increased fasting insulin suggests that HFD cause insulin resistance in these mice. To prove this, several important proteins in the insulin pathway were checked. HFD didn't have any effect on Akt and AS160 expression or its phosphorylation. For TBC1D1, however, HFD decreased p237/t-TBC1D1 ratio and almost decreased the total p/t-TBC1D1 ratio ($p=0.052$), which should be associated with an increased inhibition of GLUT4 translocation by TBC1D1. The HFD made p-237-TBC1D1 induced GLUT4 vesicle exocytosis decrease. For GLUT4, HFD decreased its total expression and cytoplasmic fraction expression in the adult mice (19 weeks old). Therefore, by decreasing TBC1D1 phosphorylation and GLUT4 expression, HFD may have caused insulin resistance in adult mice.

Can LKB1-KO make physiological insulin-induced glucose uptake worse? The changes in several important proteins (Akt, AS160, TBC1D1, GLUT4) in the insulin pathway in LKB1-KO

mice has already been discussed above. Only the change for TBC1D1 can support this hypothesis. The phosphorylation for both Ser237 and Thr590 on TBC1D1 decreased in the adult LKB1-KO mice. The combined phosphorylation amount and ratio also decreased in these mice. These means there are less TBC1D1 controlled GLUT4 vesicle available for exocytosis, which can lead to less glucose uptake. Therefore, by decreasing TBC1D1 phosphorylation, LKB1-KO may cause impaired physiological insulin induced glucose uptake, although this was not measured in this study.

Koh et al. [23] showed that combined skeletal and cardiac muscle specific LKB1 knockout improved *in vivo* and *in vitro* glucose uptake. However, fasting insulin was decreased in those mice which is opposite to the result in the current study. Sakamoto et al. [182] showed in similar mice that AICAR or muscle contraction induced-, but not insulin-induced, glucose uptake was inhibited in their LKB1-KO mice. In their *in situ* muscle contraction experiment, when comparing the glucose uptake rate for the non-contracting leg between LKB1-KO and control mice, it seems that the glucose uptake rate tended to decrease in the LKB1-KO mice. However, their mice were not skeletal muscle specific knock out mice. The LKB1 in kidney, heart, testis, and liver had also been decreased by their knockout model. Therefore, in order to solve the conflict, the *in vivo* glucose uptake rate needs to be tested in the future.

In conclusion, 1) HFD increased body weight at least in part by increasing fat mass; 2) increased caloric consumption and less daily activity likely contributed to HFD induced body weight and fat gain; 3) LKB1-KO lowers fasting blood glucose levels, while HFD increases fasting blood glucose; 4) With supra-physiological levels of insulin, neither HFD nor LKB1-KO affects blood glucose uptake ability, but with physiological insulin levels, LKB1-KO can make glucose uptake ability worse, while HFD made high blood glucose induced insulin secretion

worse only in the CON mice; 5) HFD didn't affect LKB1 expression, cellular location or activity; 6) LKB1-KO decreased AMPK activity; 7) LKB1-KO increased AMPK α 1 expression and increased total AMPK expression; 8) LKB1-KO decreased nuclear distribution of AMPK α 2; 9) long-term HFD increased t-AMPK expression in LKB1-KO mice; 10) HFD decreased the AMPK activity in the cytoplasmic fraction from adult CON mice; 11) HFD also decreased the AMPK activation percentage in the nuclear fraction of the adult CON mice; 12) the lower fasting blood glucose may be caused by increased fasting insulin which was associated with increased p-Ser318-AS160 induced GLUT4 vesicle exocytosis.

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CHAPTER 3: The Effect of High-Fat Diet-induced Obesity and/or Skeletal Muscle Specific LKB1 Knockout on the Transcription of Target Genes

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Abstract

Ad libitum high-fat diet (HFD) feeding results in weight gain and is associated with many metabolic disease conditions. Liver kinase B1 (LKB1) is an important regulator of skeletal muscle metabolism through its regulation of the AMP-activated protein kinase (AMPK) family. The purpose of this study was to determine potential genes that play an important role in HFD-induced obesity and how those genes are affected by skeletal muscle specific LKB1 knockout (LKB1-KO). To determine this, control and skm LKB1 knock-out (LKB1-KO) mice were put on either standard diet (STD) or HFD for 1 week or 14 weeks, or put on the HFD for 14 weeks and then switched to STD for 1 additional week (switched diet). Skeletal muscle target genes were then identified based on the data from previously published microarray testing in LKB1-KO mice, microarray data from mice on a HFD, and data from no-replicate mRNA-sequencing of genes from LKB1-Ko mice on STD and HFD. These genes were quantified by RT-PCR and grouped according to changes in their patterns of expression among the different. Among several other interesting changes in gene expression, the muscle growth-related protein, Ky was not affected by short-term HFD, but increased after long-term HFD, and did not decrease after switched diet, showing that its expression may be an important long-term adaptation to HFD. Ky protein expression and location was also determined. The results show that long-term HFD increased Ky protein level in cytoplasm, while the total Ky expression wasn't affected by either diet or genotype.

Introduction

It's been known for a long time that *ad libitum* high-fat diet (HFD) feeding can be detrimental to health. It can either cause or increase the risk of obesity, chronic inflammation, [40] hypertriglyceridemia, insulin resistance, non-alcoholic fatty liver disease, [41] diabetes, Alzheimer's disease, stroke, schizophrenia, some types of cancer, [42] kidney disease [43], atherosclerosis, and osteopenia [44].

Many studies had been done that use the microarray technique to assess the effects of HFD on whole genome expression profile in liver [183-186], adipose [184, 186, 187], skeletal muscle [109, 188], and brain [189]. Understanding how these changes in gene expression are regulated will be crucial in developing novel therapies for obesity and obesity-related disorders.

Liver kinase B1 (LKB1), also known as Serine/threonine kinase 11 (STK11), is a conserved protein kinase. LKB1 only has poor intrinsic kinase activity, but this is dramatically improved when bound to STE-20 related adaptor (STRAD) and mouse protein 25 (MO25) [3]. LKB1 is an important metabolic regulator through its activation of AMP-activated protein kinase (AMPK) and other AMPK related kinase (ARK) family members [16]. AMPK is a well-characterized energy-sensing protein in the cell. LKB1 plays a master role to AMPK. Activation of AMPK in skeletal muscle, by phenformin or AICAR, requires LKB1 [19-21]. LKB1 knockout decreases phosphorylated AMPK, AMPK- $\alpha 2$ activity (not $\alpha 1$), but increases AMPK- $\alpha 2$, $\beta 2$, and $\gamma 3$ protein expression [23].

While LKB1's effects on skeletal muscle metabolism through AMPK family members is well-known, its role in HFD-induced adaptations in skeletal muscle has not been determined. Therefore, the purpose of this study was to identify changes in the expression of specific genes by HFD feeding and the effect of LKB1-KO in those expression changes. To do this, a list of

potentially important candidate genes were selected based on analysis of microarray data performed previously by our laboratory in LKB1-KO [144] along with a previously published set of data comparing gene expression in skeletal muscle from HFD and STD-fed mice. Expression of these genes was then assessed in muscle from skeletal muscle specific LKB1-knockout (LKB1-KO) and littermate control mice that were fed a HFD for 14 weeks.

Materials and Methods

Animal Care

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University. All mice were housed in the pathogen-free animal facility in Life Science Building of Brigham Young University. The temperature in the animal facility was controlled at 21-23°C with a 12:12 hours light-dark cycle.

Mice

Skeletal muscle specific LKB1 knockout (skmLKB1-KO) mice were generated at Brigham Young University by crossing transgenic LKB1 “control KO” mice in which the LKB1 gene has been homozygously replaced with the LKB1 gene flanked by LoxP sites (from R. DePinho and N. Bardeesy, Dana-Farber Cancer Institute, Boston, MA) with myf6-Cre-transgenic mice [143] that heterozygously express Cre recombinase under the skeletal muscle-specific Myf6 promoter (from M.R. Capecchi, University of Utah, Salt Lake City, UT), and backcrossed onto the FVB background as described previously [144, 145]. With the specific expression of Cre in skeletal muscle, the LKB1 gene in the homozygously “floxed” LKB1 mice is deleted. Male skmLKB1-KO and littermate CON mice were used in this study. Genotyping was determined by PCR, as

described previously [146], and was also verified by western blotting for LKB1 (as described below). Prior to experimentation the mice were fed standard chow and water *ad libitum*.

Experimental Design

CON and skmLKB1-KO mice were placed on either high-fat chow (Envigo, TD. 06414) or continued on the standard chow (Envigo, TD. 8604) beginning at 5 weeks of age. There were five different diet treatments: 7 days HFD, 7 days STD, 14 weeks HFD, 14 weeks STD, switch diet (14 weeks HFD followed by 7 days STD). Each group was comprised of 11-20 mice. Food consumption, water consumption and body weight were recorded weekly.

Tissue Collection

Mice were anesthetized with 2-2.5% isoflurane in supplemental oxygen. Quadricep muscle (QUAD) were harvested and clamp-frozen in liquid nitrogen. All harvested tissue and serum were stored at -90°C until further usage.

Whole Protein Homogenization

QUAD muscles were powdered in liquid nitrogen then about 30 mg of powdered tissue was 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 Bglycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 ug/ml soybean trypsin inhibitor). The homogenate was slowly freeze-thawed at -90°C three times to ensure cell lysis, then centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were kept as the whole protein homogenate. Supernatants were analyzed for protein content using the DC Protein Assay (Biorad

Laboratories, Hercules, CA, USA). Supernatants were stored in micro-centrifuge tubes at -90°C until further analysis.

RNA Isolation and Reverse Transcription

RNA was isolated from the powdered QUAD muscle using Trizol (Life Technologies, Carlsbad, CA), then cleaned up with DIRECT-ZOL™ RNA MINIPREP Kit (Zymo Research) following the manufacturer's directions. RNA concentration and purity (260:280 ratio >1.9) was assessed by NanoDrop® spectrophotometer ND1000.

Synthesis of cDNA was performed from 1 µg RNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA) following the manufacturer's directions.

Determination of Target Genes

Our original intent was to perform gene sequencing on RNA from our CON and HFD-fed mice to determine genes of interest for further study. 12 samples were sent for analysis (3 mice per treatment condition) but, unfortunately, only the data for two samples (one KS mouse and one KH mouse) were successfully obtained and the rest of samples all failed for unknown reasons. Therefore, determination of potential target genes was accomplished using microarray or mRNA-sequencing data from three different sources:

1) The data from one KS mouse and one KH mouse. mRNA samples were sent to the sequencing center in the Huntsman Cancer Institute (University of Utah, Salt Lake City, UT 84112). “Ribo-Zero” rRNA removal was performed on the mRNA samples, and then used “Illumina Sequencing” to do the RNA-sequencing on the 14-week groups.

Custom script analysis was performed between the two samples (one KS mouse and one KH mouse) in the R programming language (R Foundation). The “edgeR” package [190, 191]

was used in the analysis, which provided a list of genes that were significantly changed between the KS and KH mouse with the p value for each gene. These genes responded to long-term HFD in LKB1 knockout skeletal muscle.

2) Microarray data from a previous publication from our laboratory was assessed for the determination of potential candidate genes for analysis in the muscle samples from these mice [144]. In that study, we did *in situ* muscle contraction on one leg of the mice and microarray testing was done to compare the gene expression differences between LKB1-KO and CON muscles, and between resting and contracted muscles. The genes that were different between CON and LKB1-KO resting muscles were used here to as a list of genes that respond to knock out LKB1 from skeletal muscle.

3) Published microarray data from Li et al.[192] was collected from skeletal muscles from CON and HFD-fed C57Bl6 mice. Although this paper didn't use FVB mice and didn't report what kind of HFD they used, it provided all the genes that changed between the HFD fed control group and STD fed control group (they used exercise as another treatment) with the p value. These data indicate how the skeletal muscle respond to HFD.

Based on all the above three lists of genes, 16 genes that were significantly changed in all three analyses were determined: kyphoscoliosis peptidase (Ky) , Cystatin-B (Cstb) , cardiac muscle alpha actin (Actc1), Keratin 18 (Krt18), Ryanodine receptor 1 (Ryr1), Neuroplastin (Nptn), Galectin-3 (Lgals3), Beta-glucuronidase (Gusb), Protein phosphatase 1 regulatory subunit 3C (Ppp1r3c), DNA-damage-inducible transcript 4 protein (Ddit4), retinol saturase (Retsat), p21/ cyclin-dependent kinase inhibitor 1 (Cdkn1a), Oxct1, Mitochondrial uncoupling protein 2 (Ucp2), Pyruvate dehydrogenase lipoamide kinase isozyme 4 (Pdk4), Nicotinamide nucleotide transhydrogenase (Nnt). Based on these genes expression level in our lab's

microarray data, 10 genes were chosen that had higher a relatively high expression level (Ky, Ryr1, Lgals3, Ppp1r3c, Ddit4, Cdkn1a, Oxct1, Ucp2, Pdk4, and Nnt).

6 inflammation related genes were also added to the analysis: IL-6, SOCS3, IER3, Bcl3, Fos, Zfp36, because inflammation is a key biological response to HFD feeding, and LKB1 is important for the inflammation response. A previous paper from our laboratory [144] showed that the skeletal muscle specific LKB1-KO mice had increased inflammation in muscle, and all the above 6 genes were the ones that showed significant change in that paper.

Primer Design

Primer sequences were designed for Pdk4, Ucp2, Nnt, Oxct1, Cdkn1a, Ddit4, Ppp1r3c, Lgals3, Ryr1, Ky, IL-6, SOCS3, IER3, Bcl3, Fos, Zfp36 (Table 3.1) using “Primer Blast” (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and NetPrimer (<http://www.premierbiosoft.com>)

Table 3. 1: Primer Sequence for the Tested Genes.

Gene Symble	Gene name	Primer (5'->3')	
Pdk4	Pyruvate dehydrogenase lipoamide kinase isozyme 4	Forward	GGATTACTGACCGCCTCTTTAGTT
		Reverse	ACACCAGTCATCAGCTTCGG
Ucp2	Mitochondrial uncoupling protein 2	Forward	CCTTCTGCACTCCTGTGTTCT
		Reverse	AAACGGGGACCTTCAATCGG
Nnt	Nicotinamide nucleotide transhydrogenase	Forward	CCTCAAAGTTGGGGCTGTTCT
		Reverse	CGCCATGCTGCTTTATGCTC
Oxct1	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	Forward	AGATGGCGGCTCTCAAACCTC
		Reverse	CACAGCCCAAACCACCAAC
Cdkn1a	Cyclin-dependent kinase inhibitor 1 or p21 ^{Cip1}	Forward	TTGCACTCTGGTGTCTGAGC
		Reverse	GGGCACTTCAGGGTTTTCTCT
Ddit4	DNA-damage-inducible transcript 4 protein	Forward	TCTTGTCGCAATCTTCGCT
		Reverse	GTCAAGGCCCTCTTCTCTGG

Ppp1r3c	Protein phosphatase 1 regulatory subunit 3C	Forward	AATGAGCTGCACCAGAATGAT
		Reverse	ACTCACTCTGCGATTTGGCT
Lgals3	Lectin, Galactoside Binding Soluble 3, Galectin-3	Forward	AATCAGGAAAATGGCAGACAGC
		Reverse	GTAGGCCCCAGGATAAGCAG
Ryr1	Ryanodine receptor 1	Forward	GCTTCCCTAGAGGTCTCCGAC
		Reverse	ACCACTTCATCATCCGTCCG
Ky	Kyphoscoliosis Peptidase	Forward	CCATCGACATGCTGCTCATC
		Reverse	TCCA ACTCTTGCCACTTCCG
Bcl3	B-cell lymphoma 3	Forward	CCGGAGGCCCTTTACTACCA
		Reverse	GGAGTAGGGGTGAGTAGGCAG
IER3	Immediate early response 3	Forward	GCCGAAGGGTGCTCTAC
		Reverse	CATAAATGGGCTCAGGTGT
Fos	Finkel-Biskis-Jinkins osteosarcoma/ FBJ murine osteosarcoma	Forward	CGGGTTTCAACGCCGACTA
		Reverse	TTGGCACTAGAGACGGACAGA
IL-6	Interleukin 6	Forward	CCAATTTCCAATGCTCTCCT
		Reverse	ACCACAGTGAGGAATGTCCA
SOCS3	Suppressor of cytokine signaling 3	Forward	ATGGTCACCCACAGCAAGTTT
		Reverse	TCCAGTAGAATCCGCTCTCCT
ZFP36	zinc finger protein 36 homolog	Forward	TCTCTGCCATCTACGAGAGCC
		Reverse	CCAGTCAGGCGAGAGGTGA

Real-Time PCR

Real-time PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad), according to the manufacturer's instructions using a CFX96 real-time detection system (Bio-Rad). Amplification efficiency was verified prior to experimentation and was between 87-109% for all primer sets. Melt curves analysis was also performed to verify the generation of a single transcript. The following conditions were used: an initial denaturation at 95°C for 30 seconds, followed by 60 cycles of 5 seconds denaturation at 95°C, 5 seconds annealing and extension at

optimal T_m (50~60°C). Three replicates for each samples. Gene expression relative to 14-week-CS group was performed using the $2^{-\Delta\Delta C_t}$ method using β -actin for normalization.

Nuclear and Cytoplasm Protein Isolation

About 60 mg of powdered QUAD muscle tissue was ground-glass homogenized in 1 ml of lysis buffer A (10 mM Hepes, pH to 7.9 with KOH; 10mM KCl; 1.5 mM $MgCl_2$; 1 mM DTT; 0.5 mM EDTA; 0.1 mM EGTA; 1% NP-40; 1 mM sodium orthovanadate; 1 mM benzamidine; 200 mM phenylmethane sulfonyl fluoride; 5 ug/ μ l soybean trypsin inhibitor) and placed in microcentrifuge tubes. Homogenates were twice incubated on the ice for 10 minutes then vortexed for 5 seconds. Samples were then centrifuged at 16,000g for 10 min at 4°C. The supernatant was collected in a separate tube as the cytoplasmic protein homogenate and stored at -90°C for further use. The pellet was washed 2 times in 500 μ l lysis buffer A and centrifuged at 16000g for 1 minute at 4°C between each wash. The pellet was then resuspended in 500 μ l of lysis buffer B (20 mM Hepes, pH to 7.9 with KOH; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.5 mM EDTA; 0.1 mM EGTA; 25% glycerol; 0.5 mM DTT; 1% NP-40; 1 mM sodium orthovanadate; 1 mM benzamidine; 200 mM phenylmethane sulfonyl fluoride; 5 ug/ μ l soybean trypsin inhibitor), vortexed for 15 seconds and incubated on the ice for 10 minutes. The vortex-incubation cycle was repeated 3 times, after which the samples were centrifuged at 16,000g for 10 min at 4°C. The supernatant was collected in a separate tube as the nuclear protein homogenate and stored at -90°C for further use.

Western Blot and Immunodetection

Protein homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β -mercaptoethanol, and 0.01% bromophenol blue) and then loaded

on 4-15% Tris·HCl gels (Bio-Rad Criterion System, Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) and visually inspected for equal protein loading. Membranes were then washed with TBST (Tris-buffered saline plus 0.1% Tween-20) for 2~5 minutes, blocked with 5% non-fat dry milk (dissolve 5 g non-fat dry milk in 100 ml 1X TBST, pH to 7.6) for 1 hour, and probed overnight at 4°C on a rotator with primary antibody diluted in 1% bovine serum albumin (BSA) (dissolve 5 g BSA in 500 ml 1X TBST, pH to 7.6).

Primary antibody manufacturers and dilutions were as follows: Ky (sc-99961, 1:500), FLNC (sc-48496, 1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

After the overnight probing with primary antibody, membranes were washed three times with TBST for 5 minutes a time. Then the membranes were probed with appropriate secondary antibody diluted at 1:20000 in 2% non-fat dry milk for 1 hour at room temperature. Membranes were washed 4 x 5 min with TBST. Clarity™ Western ECL Blotting Substrates (Bio-Rad Criterion System, Hercules, CA) was applied for 1~5 min. Chemiluminescent signals were detected with autoradiography film and quantified using Gel-Pro Analyzer 6.0 (Media Cybernetics, Inc. Bethesda, MD).

Secondary antibody was HRP-conjugated donkey anti-goat IgG secondary antibody (sc-2020, 1:20000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Statistics

Statistical comparisons using SPSS (Statistical Package for the Social Sciences) were made using one-way ANOVA (with Fisher's LSD post-hoc analysis) and two-way ANOVA to determine the statistical significance ($p \leq 0.05$). Values were reported as mean \pm SE.

Results

Effect of HFD and LKB1-KO on Inflammation-related Gene Expression

IER3 (immediate early response 3) is an inflammation-related gene. It functions in the protection of cells from Fos or TNF α induced apoptosis. HFD increased IER3 expression in both CON and LKB1-KO muscles. This effect was appears much stronger in CON vs. LKB1-KO mice, but this was not significant. LKB1-KO itself didn't affect IER3 expression as its levels were similar between genotypes fed STD. Switched diet also did not affect IER3 mRNA level (Figure 3.1 A, C).

SOCS3 (suppressor of cytokine signaling 3) is also an inflammation related gene. It can be induced by cytokines and then provides negative feedback on cytokine signaling through IL-6, IL-10 and IFN- γ . HFD didn't have effect on SOCS3 mRNA level. In the short term diet mice, LKB1-KO tended to decrease SOCS3 transcription, but not significantly, due to the too much variability for the CON mice. For the long-term diet mice, LKB1-KO in skeletal muscle increased SOCS3 mRNA level. After switch diet, SOCS3 mRNA level tended to decrease in KO mice, but this was not significant. (Figure 3.1 B, E)

Bcl3 (B-cell lymphoma 3) is an inflammation related gene. It is a transcriptional coactivator that binds with NF-kappa B homodimers to exert its function. It is increased in the CON but not LKB1-KO mice after the short-term HFD, but the effect disappeared in the long-term treatment. LKB1-KO itself didn't affect Bcl3 transcription. Switched diet only had an effect on the LKB1-KO mice in which it decreased Bcl3 transcription (Figure 3.1 G, D).

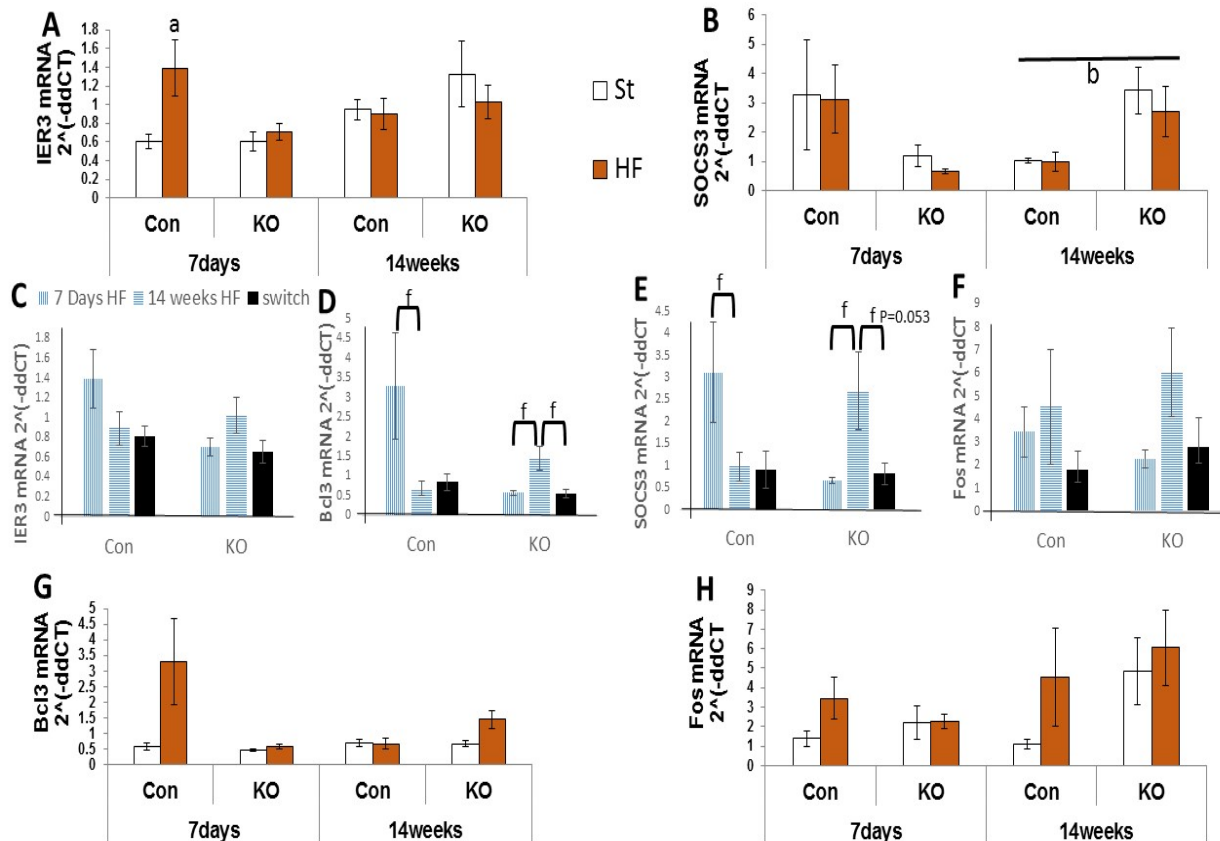


Figure 3. 1: mRNA Expression Level for IER3, SOCS3, Bcl3, and Fos. mRNA expression level for (A) IER3, (B) SOCS3, (G) Bcl3, and (H) Fos in control (Con) and LKB1-knockout (KO) mice that were fed standard (St) chow or high-fat (HF) chow for 7 days or 14 weeks. Comparison of IER3 (C), Bcl3 (D), SOCS3 (E), and Fos (F) expression in muscles from 7-day and 14-week HF-fed and switched diet (switch) mice. a= significant main effect of diet. b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

Fos (Finkel-Biskis-Jinkins osteosarcoma/ FBJ murine osteosarcoma) is an inflammation-related gene. Both long-term HFD and LKB1-KO for the long-term diet mice tended to increase Fos mRNA level, but none of them showed significance. After switched diet, Fos mRNA level tended to decrease in both CON and KO mice, but was also not significant. (Figure 3.1 H, F)

IL-6 (Interleukin 6) is an inflammation related gene. It's a cytokine and also a myokine. It plays a role in both pro-inflammation and anti-inflammation. HFD didn't have any effect on IL-6 mRNA level. LKB1-KO in skeletal muscle increased IL-6 transcription only in the long-term diet mice, not the short term diet mice, although it was elevated in the short-term diet LKB1-KO

vs. CON muscles, but non-significantly. The switched diet tended to have an opposite effect on CON and KO mice, but this was not significant. (Figure 3.2 A, C).

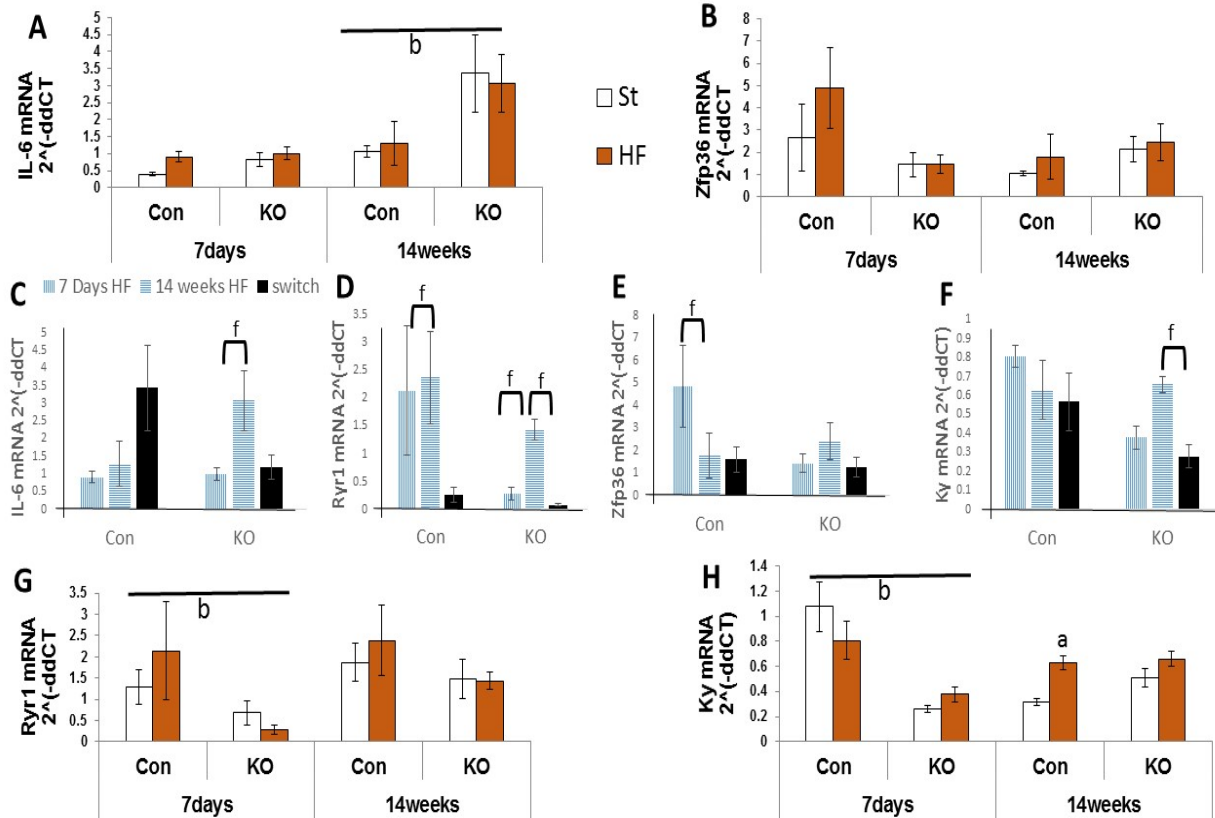


Figure 3. 2: mRNA Expression Level for IL-6, Zfp36, Ryr1, and Ky. mRNA expression level for (A) IL-6, (B) Zfp36, (G) Ryr1, and (H) Ky in control (Con) and LKB1-knockout (KO) mice that were fed standard (St) chow or high-fat (HF) chow for 7 days or 14 weeks. Comparison of IL-6 (C), Ryr1 (D), Zfp36 (E), and Ky (F) expression in muscles from 7-day and 14-week HF-fed and switched diet (switch) mice. a= significant main effect of diet. b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

Zfp36 (zinc finger protein 36) is an inflammation related gene. Neither HFD nor LKB1-KO affect Zfp36 mRNA level. Switched diet didn't affect Zfp36 transcription (Figure 3.2 B, E)

Ryr1 Transcription can be Affected by LKB1-KO and Switch Diet

Ryr1 (ryanodine receptor 1) is skeletal muscle-specific calcium release channel. It releases calcium in response to muscle stimulation to allow muscle contraction to occur. HFD didn't have any effect on the Ryr1 mRNA level. For the short term diet mice, LKB1-KO in skeletal muscle

decreased Ryr1 mRNA level, this effect decreased after 13 weeks (in the long-term diet mice). For switch diet effect, Ryr1 mRNA level significantly decreased in both CON and KO mice after switch food from high-fat to standard chow (Figure 3.2 G, D).

Effect of HFD and LKB1-KO on Ky mRNA Level

Ky (kyphoscoliosis peptidase) plays an important role in muscle growth [193]. In Ky mutated mice, muscles were smaller, slower contracting and weaker, and dramatic shifts to the expression of contractile protein isoforms typical of slow muscle, including myosin heavy chains and myosin light chains [194-197]. Ky binds with many important structural proteins including FLNC, GFN1, MYBPC1, KYIP1, Xin and titin [198-202], and FLNC is an Akt substrate [199, 200]. Ky also influences neuromuscular junctions [203]. LKB1-KO decreased Ky transcription only in short term diet mice. Long-term HFD increased Ky transcription in both CON and KO mice. Switched diet decreased Ky transcription only in KO mice. (Figure 3.2 H, F)

Both HFD and LKB1-KO can Affect Lgals3 Transcription

Lgals3/Galectin-3 is expressed throughout the cell; in the nucleus, cytoplasm, mitochondrion, cell surface, and even extracellular space. It plays an important role in cell-cell adhesion, cell-matrix interactions, macrophage activation, angiogenesis, metastasis and apoptosis. [204-208]. For the short term diet mice, short-term HFD increased Lgals3 mRNA level. For the long-term diet mice, LKB1-KO increased Lgals3 mRNA level. However, switch diet didn't affect IER3 transcription. (Figure 3.3 B, E)

Effect of HFD and LKB1-KO on Mitochondrial Gene Expression

Ucp2 (uncoupling protein 2) is a mitochondrial anion carrier protein and its expression helps control mitochondria-derived reactive oxygen species generation [209]. Short-term HFD

increased Ucp2 mRNA level, but this effect disappeared after long-term HFD diet mice. For the long-term diet mice, LKB1-KO increased Ucp2 mRNA level. After switched diet, Ucp2 mRNA level significantly decreased only in KO mice after switch food from high-fat to standard chow (Figure 3.3 A, C).

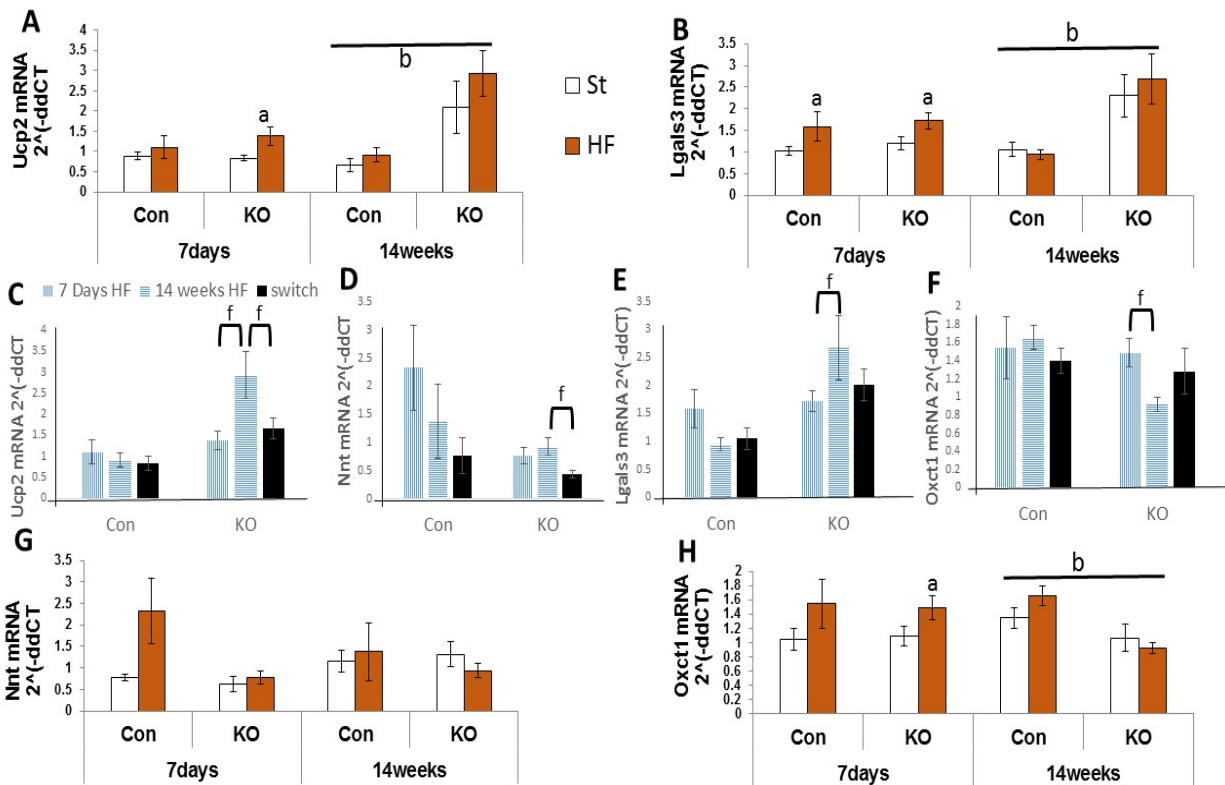


Figure 3.3: mRNA Expression Level for Ucp2, Lgals3, Nnt, and Oxct1. mRNA expression level for (A) Ucp2, (B) Lgals3, (G) Nnt, and (H) Oxct1 in control (Con) and LKB1-knockout (KO) mice that were fed standard (St) chow or high-fat (HF) chow for 7 days or 14 weeks. Comparison of Ucp2 (C), Nnt (D), Lgals3 (E), and Oxct1 (F) expression in muscles from 7-day and 14-week HF-fed and switched diet (switch) mice. a= significant main effect of diet. b=significant main effect for genotype. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

Nnt (nicotinamide nucleotide transhydrogenase) is an enzyme in the mitochondria. It can help with antioxidant defense. Nnt knockout leads to defective insulin secretion from β -cells and inappropriate glucose homeostasis [210]. Neither HFD nor LKB1-KO had effect on Nnt transcription, except Nnt mRNA level increased after short-term HFD only in the CON mice. For

the switched diet effect, *Nnt* mRNA level significantly decreased only in KO mice after switch food from high-fat to standard chow (Figure 3.3 G, D)

Oxct1 (succinyl-CoA:3-ketoacid-coenzyme A transferase 1) is a mitochondrial matrix enzyme. It controls the first rate-limiting step in ketone body breakdown [211]. For the short term diet mice, short-term HFD increased Oxct1 mRNA level. For the long-term diet mice, LKB1-KO decreased Oxct1 mRNA level. However, the switched diet didn't affect Oxct1 transcription. (Figure 3.3 H, F)

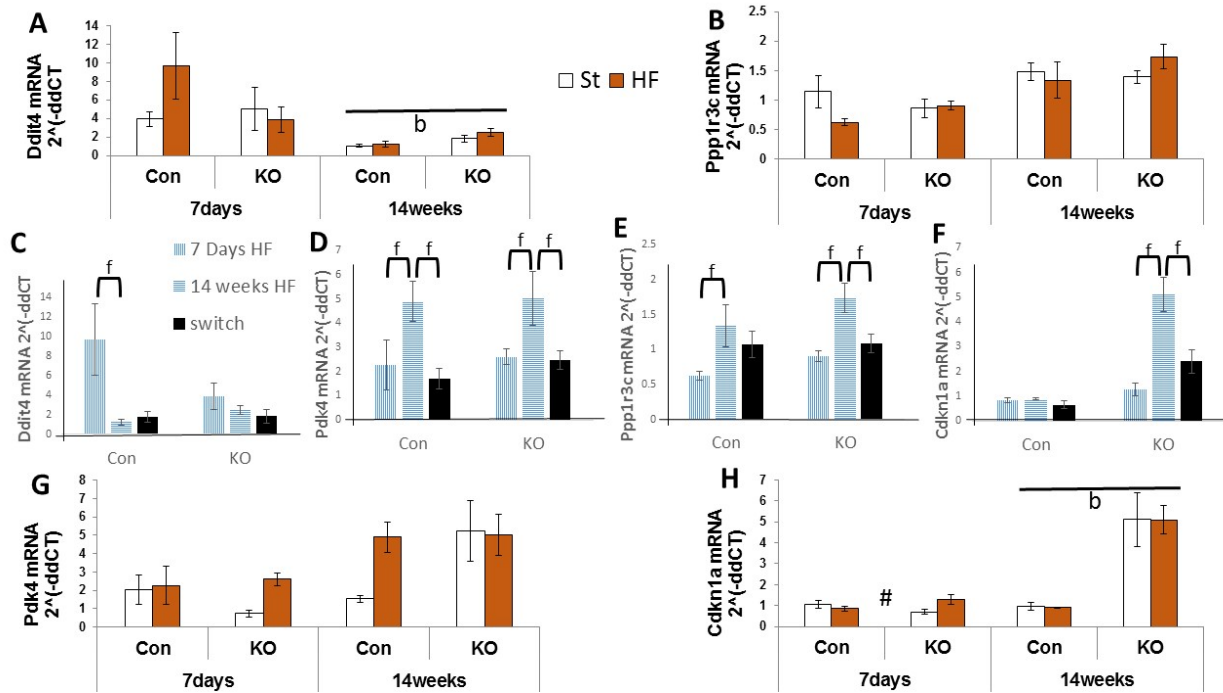


Figure 3. 4: mRNA Expression Level for Ddit4, Ppp1r3c, Pdk4, and Cdkn1a. mRNA expression level for (A) Ddit4, (B) Ppp1r3c, (G) Pdk4, and (H) Cdkn1a in control (Con) and LKB1-knockout (KO) mice that were fed standard (St) chow or high-fat (HF) chow for 7 days or 14 weeks. Comparison of Ddit4 (C), Pdk4 (D), Ppp1r3c (E), and Cdkn1a (F) expression in muscles from 7-day and 14-week HF-fed and switched diet (switch) mice. b=significant main effect for genotype. #=had genotype and diet interaction effect in two-way ANOVA. f=significant difference between the bracketed groups. Data is presented as means \pm SEM (n=6/group).

LKB1-KO Increased Ddit4 mRNA Level Only in Long-Term Diet Mice

Ddit4 (DNA-damage-inducible transcript 4) can negatively regulate mTOR [212]. mTOR is an important kinase that can regulate many cell functions, like growth, proliferation and

autophagy. HFD didn't have effect on Ddit4 mRNA level. For the long-term diet mice, LKB1-KO increased Ddit4 mRNA level. This was not the case in the short term diet mice. Switch diet didn't affect Ddit4 transcription (Figure 3.4 A, C).

Switch Diet can Affect Ppp1r3c Transcription Only in KO Mice

Ppp1r3c (protein phosphatase 1 regulatory subunit 3C) is PP1's regulatory subunit. PP1 is a phosphatase, so it reverses protein phosphorylation. PP1 can enhance insulin induced glycogen storage, and regulate RNA processing and cell cycle [213, 214]. LKB1-KO in skeletal muscle didn't affect Ppp1r3c transcription. But in HFD-fed mice, Ppp1r3c's mRNA level increased as the time goes. For the switched diet effect, Ppp1r3c mRNA level only significantly decreased in the KO mice after switched diet from high-fat to standard chow. (Figure 3.4 B, E).

Switch Diet can Affect Pdk4 Transcription in Both CON And KO Mice

Pdk4 (pyruvate dehydrogenase lipoamide kinase isozyme 4) is an isozyme for pyruvate dehydrogenase kinase. It can phosphorylate pyruvate dehydrogenase to inactivate it. Pyruvate dehydrogenase is an enzyme that links glycolysis to the citric acid cycle and generates NADH for use in oxidative phosphorylation. LKB1-KO didn't affect Pdk4 transcription. But the HFD-fed mice had increased Pdk4 mRNA levels. For the switched diet effect, Pdk4 mRNA level significantly decreased in both CON and KO mice after switch food from high-fat to standard chow (Figure 3.4 G, D).

Cdkn1a Transcription was Affected by HFD, LKB1-KO and Switch Diet

Cdkn1a (cyclin-dependent kinase inhibitor 1) is also known as p21^{Cip1} or p21^{Waf1}. It can bind and inhibit the activity for CDK2 and CDK1. Both CDK2 and CKD1 can regulate and control cell cycle. For the short term diet mice, diet and genotype had a interaction effect on

Cdkn1a mRNA level, which means that the effect that the diet caused on the CON mice was different from the one caused on the KO mice. HFD increased Cdkn1a mRNA level in the KO mice, but didn't have effect on CON mice. For the long-term diet mice, LKB1-KO in skeletal muscle dramatically increased Cdkn1a mRNA level. For the switched diet effect, Cdkn1a mRNA level only significantly decreased in the KO mice after switch food from high-fat to standard chow (Figure 3.4 H, F).

HFD Affected Cytoplasm Part of the Ky Protein Level

Ky (kyphoscoliosis peptidase) is important for normal muscle growth, and its expression can influence neuromuscular junctions. The total Ky expression didn't change with either HFD treatment or LKB1-KO (Figure 3.5A), but the cytoplasm part was different. For the long-term group, HFD increased Ky protein level in cytoplasm part. Ky protein showed here in the nuclear part, but it didn't necessary mean Ky protein was expressed in the nucleus. LKB1-KO didn't affect Ky protein level. (Figure 3.5B)

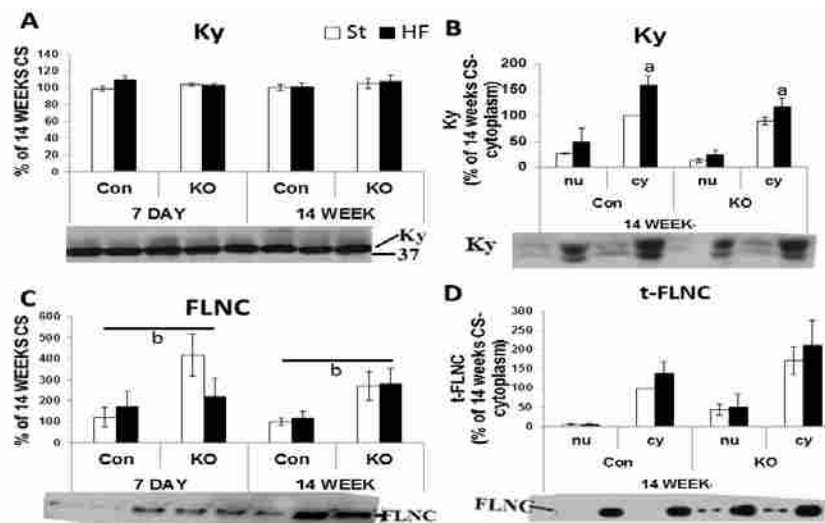


Figure 3. 5: The Protein Expression and Cellular Localization for Ky and FLNC. The protein expression for (A) Ky and (C) FLNC, and cellular localization of (B) Ky and (D) FLNC in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. a= significant main effect of diet. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

LKB1-KO Increased Total FLNC Expression

FLNC (filamin C) is cytoskeleton protein. FLNC is the only isoform expressed in mature myotubes and in adult muscle fibers. It mainly located in myotendinous junctions, sarcolemma, and Z-lines. FLNC can bind with Ky. Ky mutation can cause FLNC re-arrangement in both human and mouse skeletal muscle [198, 200]. Ky can work as protease to degrade FLNC in transfected cells [200]. For both short term diet (6 weeks old) and long-term diet (19 weeks old) mice, LKB1-KO increased total FLNC. HFD didn't have effect on it. (Figure 3.5 C) For the cytoplasmic fraction of the FLNC protein level, neither HFD nor LKB1-KO affected it (Figure 3.5 D).

Discussion

The purpose of this study was to discover potential genes that play a role in HFD-induced adaptation and determine the effect of LKB1-KO on the expression of those genes.

The initial plan was to use mRNA-sequencing to determine potential target genes from HFD and STD-fed LKB1-KO muscles. Because of the failed mRNA-sequencing, data from three different sources was used to identify potential key genes: 1) The RNA sequencing data from two LKB1-KO muscle samples (one HFD-fed and the other STD-fed) that were successfully sequenced; 2) The data from a previously published microarray test from our laboratory that examined gene expression in LKB-KO vs. CON mice [144]; 3) Previously published microarray data from Li et al [192] comparing HFD vs. STD diet in mouse skeletal muscle. The genes that changed significantly in all three places were the “target genes”. Because inflammation is regulated by HFD and LKB, 6 inflammation-related genes were also assessed. Thus, 16 target genes in total were analyzed: Ky, Ryr1, Lgals3, Ppp1r3c, Ddit4, Cdkn1a, Oxct1, Ucp2, Pdk4, Nnt, IL-6, SOCS3, IER3, Bcl3, Fos, Zfp36.

The second thing was the group design. This dissertation had ten different groups of mice: 7 days control mice HFD (7CH), 7 days control mice STD (7CS), 7 days LKB1-knockout mice HFD (7KH), 7 days LKB1-knockout mice STD (7KS), 14 weeks control mice HFD (14CH), 14 weeks control mice STD (14CS), 14 weeks LKB1-knockout mice HFD (14KH), 14 weeks LKB1-knockout mice STD (14KS), control mice switch diet (C-switch), LKB1-knockout mice switch diet (K-switch). 11~20 mice were assigned to each groups. The purpose for this 10-group-design was to determine the genes that only significantly changed after long-term HFD, not short-term or change less for the short-term, and also the change won't disappear after switch back to the normal diet. The critical effects that have long-lasting impact on health are not likely to occur with just one meal or several meals, but will result from long-term feeding. The genes that respond only in the earlier stages (in this case the short-term feeding groups) also can be important, but these gene should more play a defense role or guiding role, not the role to be reflective of the real critical effect of having high-fat diet as a prolonged lifestyle. And also the critical effect would not be expected to disappear quickly after reversing from HFD back to STD diet.

Based on the changes that were observed in those ten different groups, those target genes had been grouped into the following categories:

- 1) Genes for which long term HFD had an effect on the gene transcription, but not the short term, and for which the effect doesn't disappear after switched diet, at least in the CON mice. This group is the one I was looking for in this study. Only Ky belonged to this group.

- 2) Genes for which short term HFD had an effect on the gene transcription, but not the long term diet. IER3, Lgals3, Oxct1, and Ucp2 belonged to this group. This group seems play a role in HFD induced acute response, but not the chronic response.

3) Genes for which the gene transcription level was different between two different genotypes after long-term HFD (long-term diet), but not short term diet. *Lgals3*, *Ucp2*, *Cdkn1a*, *SOCS3*, *Oxct1*, *Ddit4*, and *IL-6* belonged to this group. This group may contribute to the previously defined phenotype for the *LKB1*-KO skeletal muscle, like poor exercise capacity, early-onset aging phenotype, or increased muscle damage after exercise or contraction [144, 215].

4) Genes for which expression was different between the two genotypes after short-term, but not long-term HFD feeding. *Ky* and *Ryr1* belonged to this group. The short-term group mice were 6 weeks, while the long-term group mice were 19 weeks. They were in the different life stages. The mice in the short-term group were still in the late development stage, but the long-term group mice were in the mature adult stage. These genes that only showed genotype effect in the young mice are more likely to play a role in muscle development.

5) Genes for which the expression changed after switched diet. For the switched diet group, it's not just switch diet for them, these mice are also under voluntary calorie restriction. *Pdk4* and *Ryr1* belonged to this group.

Only *Ky* was induced by long-term HF feeding, but not short-term, and was not decreased by switched diet. Further studies will be needed in order to find out whether *Ky* played a decisive role in long-term HFD induced effects. But for the other genes, each group can answer different questions, and those genes that belonged to each group can be further studied to answer that question.

To see if the protein level also showed the same change as mRNA, western blot was performed to check *Ky* protein level. Unexpectedly, the results show that the total *Ky* protein didn't change at all, but the content of *Ky* in the cytoplasmic fraction increased with long-term

HFD, suggesting that its cellular location is important for its activity. The Ky band in the Western blot was not a single thin band, like most of proteins, but diffuse with many bands shifting upward and downward. This suggests that it may be associated with transport vesicles or perhaps it can have different post-translational modifications. All these need further research.

For FLNC protein level, HFD didn't have effect on it, but LKB1-KO increased it, and this was the case for both short term diet and long-term diet mice. FLNC is a cytoskeleton protein that can be phosphorylated by Akt in response to insulin stimulation [216, 217]. Since LKB1-KO mice had higher fasting insulin it is reasonable to hypothesize that may have contributed to the altered handling that we observed in the LKB1-KO mice. This needs further research.

Previous work from our lab showed that knocking-out LKB1 increased Bcl3 in skeletal muscle [144], but in this study, knocking-out LKB1 increased SOCS3 and IL-6 in the adult skeletal muscle. That old study used mice from 3~5 months old. Even though it is within the mature adult stage, it is still a very broad age range. In this study, all 14-week groups mice were harvested at the age of 19 weeks \pm 2days, so the all the mice were in a very similar development or maturation stage. This may have contributed to the different results.

Some studies showed that skeletal muscle IL-6 increased after 3~16 weeks HFD, but not 1 week [86, 218, 219]. In the current study, high-fat diet didn't have any effect on IL-6 expression in skeletal muscle. However, one study [220] showed that HFD-induced obesity and glucose homeostasis was mouse strain-dependent. HFD even caused opposite responses for the expression of some genes between different mouse strains. Many studies use C57BL/6 mice, but this study used FVB mice. This maybe the reason why the skeletal muscle IL-6 wasn't affect by HFD in this study.

Previous research showed that SOCS3 mRNA increased with 12 weeks of HFD, and skeletal muscle specific SOCS3 knock out can also prevent HFD-induced insulin resistance [221], suggesting that SOCS3 is a critical gene in HFD-induced adaptation. In this study, HFD didn't seem to influence SOCS3 mRNA level, but LKB1-KO increased SOCS3 in skeletal muscle. Therefore, LKB1-KO should make HFD-induced insulin resistance worse, but the results from Chapter 2 were opposite. Maybe because the increased SOCS3 mRNA didn't turn into protein in the LKB1-KO mice, which was not assessed in this study.

Short-term HFD increased Bcl2, Fos, and IL-6 only in the Con mice, not the LKB1-KO mice. IER3 gene expression was similarly altered. Even though there was a main effect of diet on its transcription for 7 day groups, *t*-test didn't show significance between 7 days KO-St and 7 days KO-HF groups. These are all inflammation-related genes. But if the change between 7 days Con-St and 7 days KO-St groups are also considered, then these four genes can be grouped into two categories: IER3/Bcl2 and Fos /IL-6. For IER3 and Bcl2, LKB1-KO didn't increase their mRNA level, while only short-term HFD in Con mice increased their mRNA level. That means LKB1-KO can prevent short-term HFD-induced increases in gene expression for IER3 and Bcl2. However, for Fos and IL-6, LKB1-KO increased their mRNA level, while short-term HFD also can increase their mRNA level in Con mice. That means LKB1-KO itself can increase their gene transcription, and short-term HFD only induce these gene transcription increasing in Con mice. Therefore, even they are all inflammation-related genes, their role in short-term HFD adaptation and relationship with LKB1 are different.

Oxct1 is a mitochondrial matrix enzyme. It controls the first rate-limiting step in ketone body breakdown [211]. Short-term HFD increased Oxct1 mRNA level. For the long-term diet mice, LKB1-KO decreased Oxct1 mRNA level. This result means that the capacity for ketolysis

may be decreased in the adult LKB1-KO mice. We observed that the LKB1-KO mice seemed to be prone to dying during insulin tolerance testing (in Chapter 2). This could be partly explained by decreased Oxct1 in the LKB1-KO mice.

In conclusion, Ky is a potentially important gene for long-term HFD-induced effect. Even though the whole protein level didn't show the same change, the cytoplasm part of the Ky increased only with long-term HFD. Therefore, Ky cellular location may be important for its function.

Ky can bind with structure proteins in skeletal muscle, like FLNC, GFN1, MYBPC1, KYIP1, Xin and titin. FLNC is the downstream target of Akt. Ky is important for normal muscle growth. In Ky mutated mice, muscles were smaller, slower contracting and weaker, and dramatic shifts to the expression of contractile protein isoforms typical of slow muscle, including myosin heavy chains and myosin light chains. Ky also can influence neuromuscular junctions. Considering Ky's important function in skeletal muscle, further research is needed to find out if long-term HFD can affect muscle growth, or muscle structure stability, or insulin-induced glucose uptake, through decreased Ky.

CHAPTER 4: Effect of High-fat Diet and LKB1 Knockout on Anabolic Signaling in Skeletal Muscle

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Abstract

High-fat diet (HFD) feeding influences both skeletal muscle protein synthesis and degradation, while liver kinase B1 (LKB1) is known to regulate cell anabolism as well. The purpose of this study was to determine if HFD and LKB1 knockout (LKB1-KO) affects skeletal muscle growth and anabolic (growth) signaling in skeletal muscle. In order to do this, control (CON) and LKB1-KO mice were put on either HFD or STD for 1 week or 14 weeks. The results show that long-term (14 week) HFD-feeding decreased quadricep (QUAD) weight/body weight ratio, but not absolute quadriceps weight, suggesting that it does not adversely affect muscle anabolism. However, LKB1-KO resulted in decreased absolute QUAD weight in long-term fed diet mice regardless of diet. LKB1-KO promoted increased t-eIF2 α and eIF4E expression, and increased ubiquitination (suggesting increased protein degradation) in adult mice. Long-term HFD increase 4EBP1 which should promote a general inhibitory effect on anabolic signaling in skeletal muscle, but also had rescue effect only in LKB1-KO mice by increasing total eIF2 α . Together the data showed that increase protein degradation in LKB1-KO skeletal muscle may cause the associated decrease in QUAD weight.

Introduction

High-fat diet (HFD)-induced obesity results in the loss muscle mass, strength, sensorimotor coordination and muscle fiber peak force [93-96]. Furthermore, the growth of

regenerating myofiber and the myofiber maturation is delayed after HFD [97]. Myofiber area, satellite cells, and myonuclei of the muscle were decreased, while collagen deposition increased after HFD [93, 97]. However, one study showed HFD didn't impaired muscle growth after injury [222]. HFD also can alter the expression of some myokines: IL-6, IL-15, and apelin expression are all increased [98, 99].

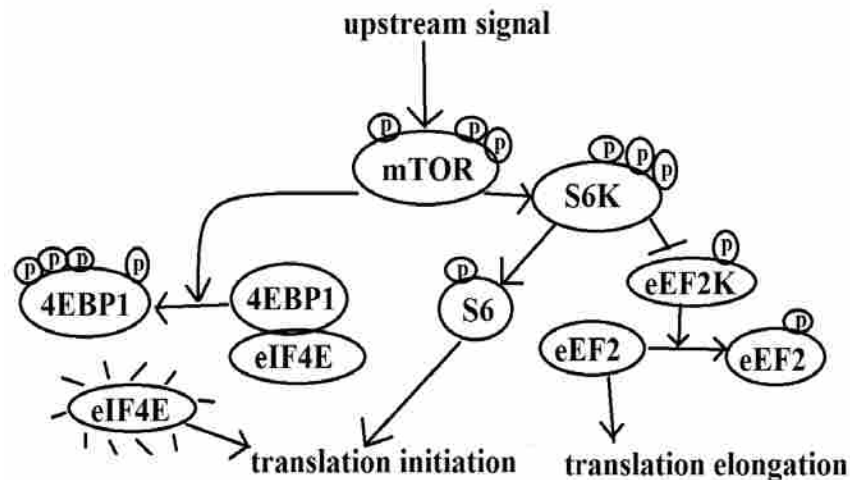


Figure 4. 1: mTOR Signaling Pathways Regulate Protein Synthesis. eIF4E=eukaryotic translation initiation factor 4E; S6= ribosomal protein s6; S6K=s6 kinase; eEF2=eukaryotic translation elongation factor 2; eEF2K= eEF2 kinase.

mTOR is a critical anabolic kinase that can regulate many cell functions, such as growth, proliferation and autophagy. It can sense ATP and amino acid in order to balance nutrient availability and cell growth [223, 224]. mTOR can be phosphorylated/activated by upstream signaling from growth factors and then phosphorylates 4EBP1 and ribosomal protein S6 kinase (S6K), among other targets. Hyper-phosphorylated 4EBP1 won't interact with eukaryotic translation initiation factor 4E (eIF4E), so eIF4E can participate in protein translation. Phosphorylated/activated S6K can phosphorylate ribosomal protein S6 and eEF2 kinase (eEF2K). Phosphorylated/activated S6 works as part of the translation machine. Phosphorylated eEF2K will phosphorylate and inactivate eukaryotic translation elongation factor 2 (eEF2). eEF2

is a GTP-binding translation elongation factor and it's essential for protein synthesis (Figure 4.1). Through these and other mechanisms, mTOR stimulates protein synthesis.

The protein degradation pathway can also regulate muscle size. Ubiquitin is a small regulator protein that can be added to other proteins through a process called ubiquitination. After ubiquitination, the protein is targeted for degradation by proteasomes. Muscle atrophy F-box (MAFbx), also known as FBXO32 (F-box only protein 32), is a muscle-specific E3 ubiquitin ligase. Ubiquitin ligases bind with target proteins and E2 ubiquitin-conjugating enzyme that binds with ubiquitin, and then transfers the ubiquitin from E2 to the protein.

Previous work has reported the effect of HFD on proteins that are important for anabolic and catabolic signaling. Louise et al. [225] showed that HFD tended to increase phosphorylation of S6K on Thr389 and Thr421/Ser424, but not significantly. Gordon et al. [226] showed that omega-3 fatty acids stimulate muscle protein synthesis in older adults with increased phosphorylation of the mechanistic target of rapamycin (mTOR) at Ser2448 and ribosomal protein S6 kinase (S6K) at Thr389. Larysa et al. [227] showed that HFD increased protein ubiquitination in HFD-fed mice. Ju et al. [228] showed total mTOR decreased, while mTORC1, mTORC2, and S6K1 increased with HFD, but they didn't check the phosphorylation state of these proteins. Many studies have assessed how mTOR levels changed in the obesity animal or HFD fed animal, few of them really focus on anabolic and catabolic signaling pathway.

Liver kinase B1 (LKB1), also known as Serine/threonine kinase 11 (STK11), is conserved throughout evolution from worms to mammals 1. It has two isoforms long (50 kDa) and short (48 kDa) form, but the short one appears restricted to testis 2. LKB1 has been involved in cell cycle arrest 45, apoptosis 6, autophagia 7, energy metabolism 8as well as in T cell maturation 9, liver glucose homaeostasis 10, tumor growth 11 and epithelial apicobasal polarity 12. But LKB1

only has poor intrinsic kinase activity. It needs a binding partner, pseudo kinase STRAD (STE-20 Related Adaptor), to dramatically improve its kinase activity and MO25 (mouse protein25) stabilizes the interaction between STRAD and LKB13. Active LKB1 seems to exert these cellular effects by activating AMP-activated protein kinase (AMPK) and its family members (ARCs) through phosphorylation at a conserved N-terminal residue [16]. AMPK is a well-characterized energy-sensing protein in the cell.

AMPK downstream of LKB1 inhibits mTOR, so it makes sense to see if LKB1-KO can affect muscle size. A previous paper from our laboratory shows that LKB1-KO in skeletal muscle leads to weight loss and atrophy of type II skeletal muscle [215], but we don't know if LKB1 affect protein synthesis or degradation in skeletal muscle, or whether it is involved in the effect of HFD on these processes.

Therefore, the purpose of this study was to determine the effect of LKB1-KO on HFD-induced alterations in skeletal muscle anabolic and catabolic signaling.

Materials and Methods

Animal Care

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Brigham Young University. All mice were housed in the pathogen-free animal facility in Life Science Building of Brigham Young University. The temperature in the animal facility was controlled at 21-23°C with a 12:12 hours light-dark cycle.

Mice

Skeletal muscle specific LKB1 knockout (skmLKB1-KO) mice were generated at Brigham Young University by crossing transgenic LKB1 “control KO” mice in which the LKB1 gene has

been homozygously replaced with the LKB1 gene flanked by LoxP sites (from R. DePinho and N. Bardeesy, Dana-Farber Cancer Institute, Boston, MA) with myf6-Cre-transgenic mice [143] that heterozygously express Cre recombinase under the skeletal muscle-specific Myf6 promoter (from M.R. Capecchi, University of Utah, Salt Lake City, UT), and backcrossed onto the FVB background as described previously [144, 145]. With the specific expression of Cre in skeletal muscle, the LKB1 gene in the homozygously “floxed” LKB1 mice is deleted. Male skmLKB1-KO and littermate CON mice were used in this study. Genotyping was determined by PCR, as described previously [146], and was also verified by western blotting for LKB1 (as described below). Prior to experimentation the mice were fed standard chow and water *ad libitum*.

Experimental Design

CON and skmLKB1-KO mice were placed on either high-fat chow (Envigo, TD. 06414) or continued on the standard chow (Envigo, TD. 8604) beginning at 5 weeks of age. There were five different diet treatments: 7 days HFD, 7 days STD, 14 weeks HFD, 14 weeks STD, switch diet (14 weeks HFD followed by 7 days STD). Each group was comprised of 11-20 mice. Food consumption, water consumption and body weight were recorded weekly.

Tissue Collection

Mice were anesthetized with 2-2.5% isoflurane in supplemental oxygen. Quadriceps muscle (QUAD) were harvested and clamp-frozen in liquid nitrogen. All harvested tissue and serum were stored at -90°C until further usage.

Whole Protein Homogenization

QUAD muscles were powdered in liquid nitrogen then about 30 mg of powdered tissue was 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 Bglycerophosphate; 1 mM sodium orthovanadate; 1 mM DTT; 1 mM benzamidine; 0.1 mM phenylmethane sulfonyl fluoride; 5 ug/ml soybean trypsin inhibitor). The homogenate was slowly freeze-thawed at -90°C three times to ensure cell lysis, then centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were kept as the whole protein homogenate. Supernatants were analyzed for protein content using the DC Protein Assay (Biorad Laboratories, Hercules, CA, USA). Supernatants were stored in micro-centrifuge tubes at -90°C until further analysis.

Western Blot and Immunodetection

Protein homogenates were diluted in sample loading buffer (125 mM Tris HCl, pH 6.8, 20% glycerol, 4% SDS, 5% β -mercaptoethanol, and 0.01% bromophenol blue) and then loaded on 4-15% Tris-HCl gels (Bio-Rad Criterion System, Hercules, CA). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) and visually inspected for equal protein loading. Membranes were then washed with TBST (Tris-buffered saline plus 0.1% Tween-20) for 2~5 minutes, blocked with 5% non-fat dry milk (dissolve 5 g non-fat dry milk in 100 ml 1X TBST, pH to 7.6) for 1 hour, and probed overnight at 4°C on a rotator with primary antibody diluted in 1% bovine serum albumin (BSA) (dissolve 5 g BSA in 500 ml 1X TBST, pH to 7.6).

Primary antibody manufacturers and dilutions were as follows: Ub/ubiquitin (sc-8017, 1:1000), MAFbx (sc-33782, 1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p-mTOR (Ser2448) (#2971, 1:4000), t-mTOR (#2983, 1:2000), p-4E-BP1 (Thr37/46) (#2855, 1:8000), t-4E-BP1 (#9644, 1:2000), t-eEF2 (#2332, 1:5000), p-eEF2 (Thr56) (#2331, 1:5000), t-

eIF2 α (#9722, 1:1000), p-eIF2 α (Ser51) (#9721, 1:2000), t-S6 Ribosomal Protein (#2217, 1:2000), p-S6 Ribosomal Protein (Ser235/236) (#4858, 1:4000), eIF4E (#2067, 1:2000) from Cell Signaling Technology (Beverly, MA, USA).

After the overnight probing with primary antibody, membranes were washed with TBST for 5 minutes a time, three times. Then membranes were probed with appropriate secondary antibody diluted at 1:20000 in 2% non-fat dry milk for 1 hour at room temperature. Membranes were washed 4 x 5 min with TBST. Clarity™ Western ECL Blotting Substrates (Bio-Rad Criterion System, Hercules, CA) was applied for 1~5 min. Chemiluminescent signals were detected with autoradiography film and quantified using Gel-Pro Analyzer 6.0 (Media Cybernetics, Inc. Bethesda, MD).

Secondary antibody manufacturers were as follows: HRP-conjugated mouse anti-rabbit secondary antibody (#211-032-171) from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA), HRP-conjugated goat anti-mouse secondary antibody (sc-2314) and HRP-conjugated donkey anti-goat IgG secondary antibody (sc-2020) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Specific Growth Rate Calculations

Specific growth rate was calculated as follows: $SGR = (\text{current body weight} - \text{previous week's body weight}) / \text{previous week's body weight} * 100\%$ [57].

Statistics

Statistical comparisons using SPSS (Statistical Package for the Social Sciences) were made using two-way ANOVA to determine the statistical significance ($p \leq 0.05$). Values were reported as mean \pm SE.

Results

Effect of HFD and LKB1-KO on General Growth Rate

The specific growth rate had been calculated as an index of growth of the mice [57]. As the mice became more and more mature, the SGR decreased from 7~13 to around zero. For the first 4 weeks, especially the first two weeks, the SGR was higher for the HFD fed mice than the STD fed mice. There was no difference between CON and LKB1-KO. (Figure 4.2 A)

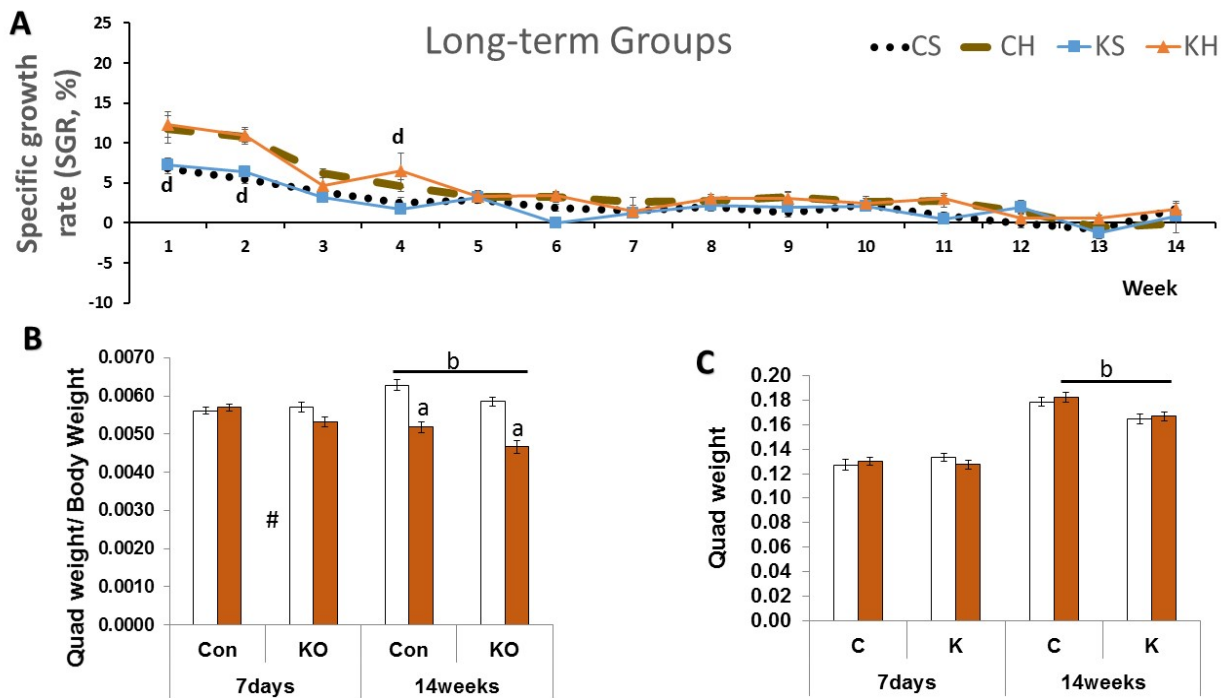


Figure 4. 2: The Mice Specific Growth Rate (SGR) and Quadriceps (QUAD) Muscle Weight. The mice specific growth rate (SGR) (A) [57], and quadriceps (QUAD) muscle actual weight (B) and weight/body weight (C). The unite for weight is gram. a= significant main effect of diet. b=significant main effect for genotype. #=had genotype and diet interaction in two-way ANOVA. Data is presented as means \pm SEM (n=11-20/group).

Effect of HFD and LKB1-KO on QUAD Weight

The long-term HFD decreased muscle weight/body weight for QUAD muscles, but this was due entirely to the increase in body weight since the absolute muscle weights were not decreased by HFD (Figure 4.2B). In the long-term diet mice, LKB1-KO further decreased

muscle weight/body weight for QUAD (Figure 4.2 B). This effect was likely driven by decreased absolute QUAD muscle weight in the LKB1-KO mice (Figure 4.2 C). Short-term HFD decreased QUAD weight/body weight in LKB1-KO, but not CON mice (Figure 4.2 B).

Effect of HFD and LKB1-KO on Anabolic Signaling Pathways

mTOR is a critical kinase in the regulation of many cell functions, like growth, proliferation and autophagy. It can sense ATP and amino acid in order to balance nutrient availability and cell growth [223, 224]. Like Akt, mTOR is also a “hub” for many pathways. Interestingly, Akt and mTOR can phosphorylate each other [229, 230]. When phosphorylated at Ser2448, mTOR is activated. Neither HFD nor LKB1-KO had an effect on p-mTOR (Figure 4.3A), but both short-term and long-term HFD affect total mTOR protein level. Short-term HFD increased t-mTOR only in LKB1-KO mice and long-term HFD, increased t-mTOR protein level in both LKB1-KO and CON mice. There was a genotype and diet interaction effect for the long-term groups where long-term HFD increased t-mTOR protein level in LKB1-KO mice, but decreased mTOR in CON mice (Figure 4.3B). HFD increased the p/t-mTOR ratio in long-term but not short-term diet mice. (Figure 4.3C)

4EBP1 is a translation repressor protein. It can bind with eIF4E (translation initiation factor) to inhibit cap-dependent translation. Both Akt and mTOR can phosphate 4EBP1. Hyper-phosphorylated 4EBP1 releases eIF4E [231-234], so the translation event can happen. In this study, short-term HFD didn't have effect on either total 4EBP1 protein level or its phosphorylation (Figure 4.3 D, E, F). Long-term HFD increased total 4EBP1 protein level, and almost had an effect on the 4EBP1 phosphorylation ($p=0.055$; Figure 4.3 D, E). Together, this resulted in no difference in the p/t-4EBP1 ratio with HFD. (Figure 4.3 F) LKB1-KO didn't affect 4EBP1 content or phosphorylation at all (Figure 4.3 D, E, F).

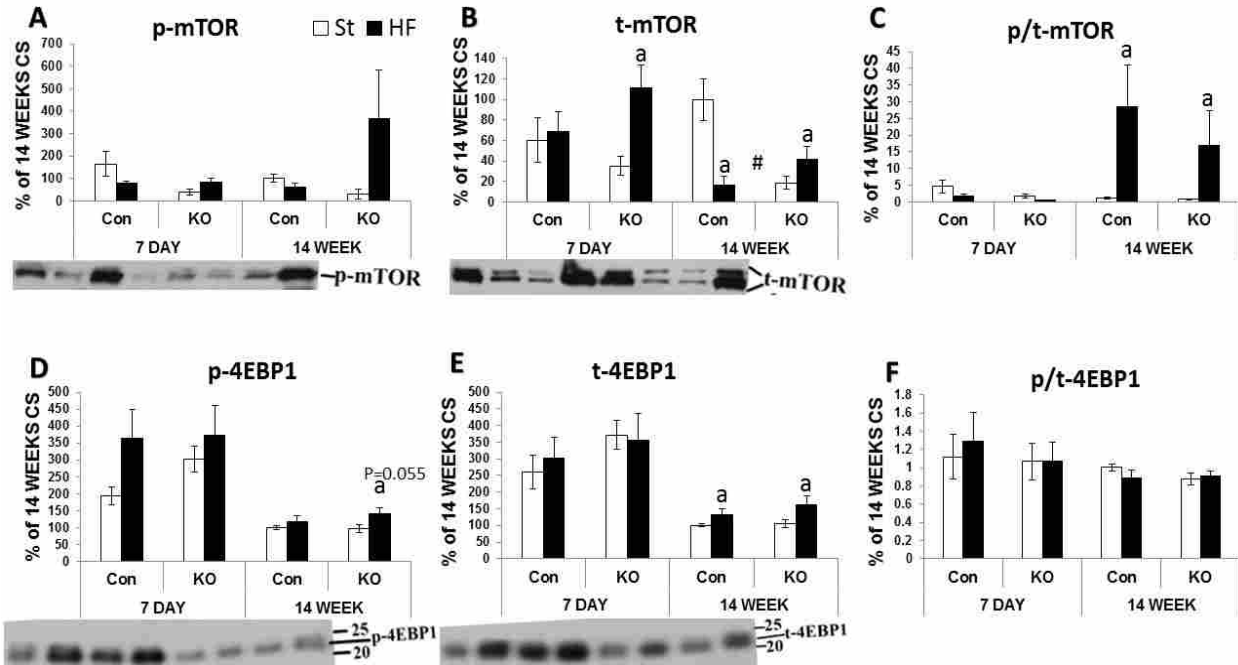


Figure 4. 3: The Protein Expression for mTOR and 4EBP1. The protein expression for (A) phosphor-mTOR, (B) total mTOR, (C) p/t-mTOR ratio (D) phosphor-4EBP1, (E) total 4EBP1, and (F) the p/t-4EBP1 ratio in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. a= significant main effect of diet. #=had genotype and diet interaction effect in two-way ANOVA. Data is presented as means \pm SEM (n=6/group).

Eukaryotic translation elongation factor (eEF2) is a GTP-binding translation elongation factor and it's essential for protein synthesis. It is inactivated by phosphorylation by eEF2 kinase, which, in turn, is regulated by S6k, downstream of mTOR. Phosphorylation of eEF2 was not affected by HFD or LKB1-KO (Figure 4.4 A,C). The total eEF2 level tended to increase in the short term diet LKB1-KO muscles, but not significantly (Figure 4.4 B).

eIF2 α is the α subunit of eIF2 (eukaryotic initiation factor 2) is required in the initiation of translation machine. Besides α subunit, eIF2 also has β and γ subunit, but α subunit is the main phosphorylation target. Many kinases, like GCN2, PERK, PKR, and HRI, can phosphorylate α subunit to inactivate eIF2, so protein synthesis can be down-regulated. LKB1-KO, only in the long-term diet mice, increased total eIF2 α protein level. HFD also had effect on these mice, but it increased total eIF2 α protein expression only in the LKB1-KO mice. Neither short-term HFD

nor LKB1-KO in the short term diet mice had effect on total eIF2 α protein expression. (Figure 4.4 E) LKB1-KO in skeletal muscle tended to decrease eIF2 α phosphorylation, but not significant. HFD didn't have effect on p-eIF2 α or p/t-eIF2 α ratio. (Figure 4.4 D, F)

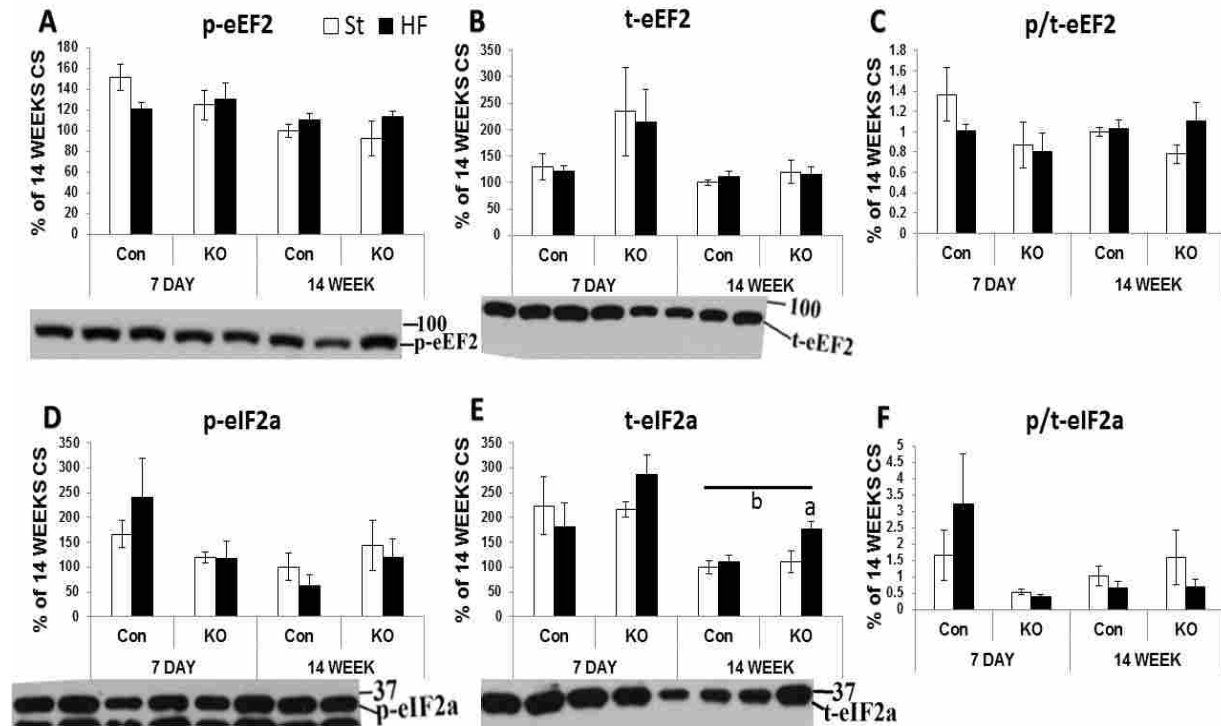


Figure 4. 4: The Protein Expression for eEF2 and eIF2 α . The protein expression for (A) phosphor-eEF2, (B) total eEF2, (C) p/t-eEF2 ratio (D) phosphor-eIF2 α , (E) total eIF2 α , and (F) the p/t-eIF2 α ratio in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. a= significant main effect of diet. b=significant main effect for genotype. Data is presented as means \pm SEM (n=6/group).

S6/rpS6 (ribosomal protein s6) a component of the 40S ribosomal, and it is activated by phosphorylation. S6k, downstream of mTOR, can phosphorylate S6 [235]. LKB1-KO in skeletal muscle tended to increased S6 phosphorylation, but not significant due to too much variability. (Figure 4.5 A, C) Generally speaking, neither HFD nor LKB1-KO affected S6 total protein expression and its phosphorylation (Figure 4.5 A, B, C).

eIF4E (eukaryotic translation initiation factor 4E) is required for almost all cellular mRNA translation. It helps directing ribosomes to the cap structure of mRNA. HFD didn't affect eIF4E

protein expression, but LKB1-KO can. eIF4E protein level increased in the long-term diet LKB1-KO mice, not short term diet ones (Figure 4.5 D).

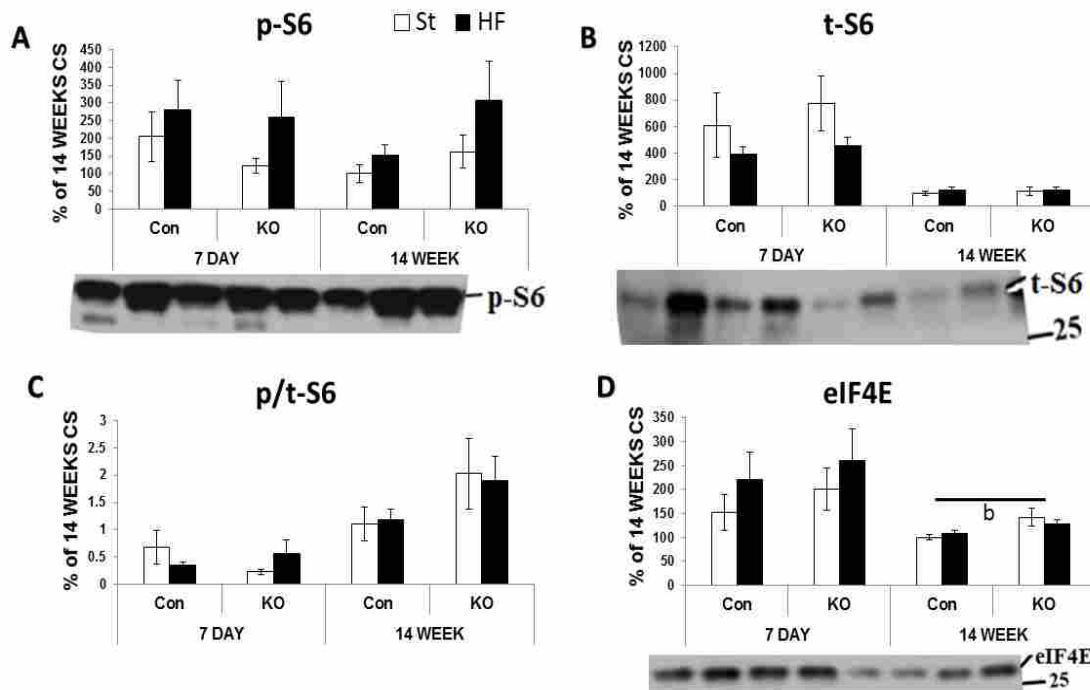


Figure 4. 5: The Protein Expression for S6 and eIF4E. The protein expression for (A) phosphor-S6, (B) total S6, (C) p/t-S6 ratio, and (D) eIF4E in whole protein homogenates for quadricep (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. b=significant main effect for genotype. #=had genotype and diet interaction effect in two-way ANOVA. Data is presented as means \pm SEM (n=6/group).

Effect of HFD and LKB1-KO on Catabolic Pathways

Ubiquitin is a small regulator protein that can be added to other proteins, and this is called ubiquitination. After ubiquitination, the protein is targeted for degraded by proteasomes. The ubiquitination also can affect the protein's location, activity and protein-protein interaction [236-238]. Ubiquitin in western blots reflects the protein degradation level [239-241]. HFD didn't have any effect on the ubiquitin level, but LKB1-KO in skeletal muscle affect protein ubiquitination level. Protein ubiquitin level increased in LKB1-KO long-term diet mice, not the short term diet ones (Figure 4.6 A).

Muscle atrophy F-box (MAFbx), also known as FBXO32 (F-box only protein 32), is a muscle-specific E3 ubiquitin ligase. Ubiquitin ligases bind with target proteins and E2 ubiquitin-conjugating enzyme that binds with ubiquitin, and then transfers the ubiquitin from E2 to the protein. Increased MAFbx expression is a marker for muscle atrophy. [242, 243] Short-term HFD and LKB1-KO almost ($p=0.055$) had an interaction effect on MAFbx protein expression in the short term diet mice. For the long-term diet mice, LKB1-KO in skeletal muscle increased MAFbx protein expression (Figure 4.6 B).

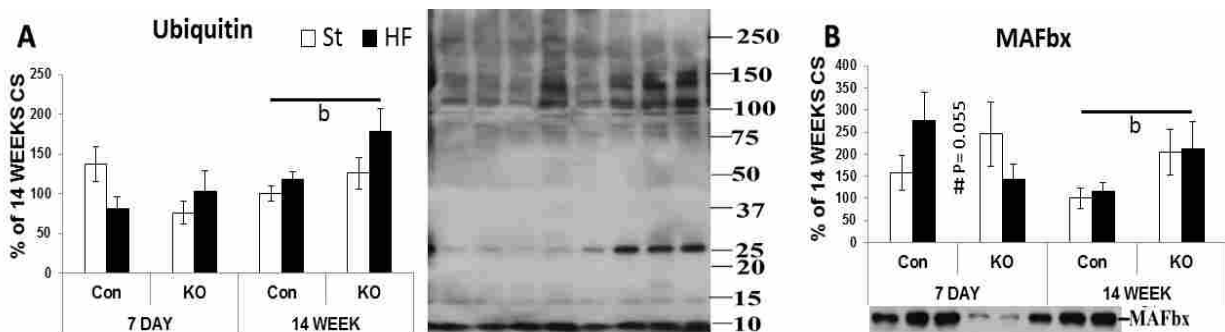


Figure 4. 6: The Protein Expression for Ubiquitin and MAFbx. The protein expression for (A) ubiquitin, and (B) MAFbx in whole protein homogenates for quadriceps (QUAD) muscle from LKB1 knockout (KO) and littermate control (Con) mice fed standard chow (St) or high-fat diet (HF) for 7 days or 14 weeks. b=significant main effect for genotype. #=had genotype and diet interaction effect in two-way ANOVA. Data is presented as means \pm SEM ($n=6$ /group).

Discussion

The purpose of this study was to determine if HFD and/or LKB1-KO affect muscle growth and anabolic/catabolic signaling in skeletal muscle. The above results show that only LKB1-KO can affect QUAD muscle size. For first several weeks of the experiment, the mice on the HFD grew much faster than the standard chow fed mice, but this was due to increased fat weight instead of muscle weight. Long-term HFD didn't increase or decrease QUAD weight. Because the QUAD growth rate was much slower than the whole body weight growth, the QUAD weight/body weight ratio decreased after long-term HFD. LKB1-KO decreased muscle weight for QUAD in the long-term diet mice. After LKB1-KO, the muscle growth rate for QUAD were

even slower than the whole body weight growth. Therefore, LKB1-KO can influence QUAD muscle size.

For protein synthesis related proteins, total mTOR, p/t-mTOR ratio, total 4EBP1, and total eIF2 α all changed with HFD. Only t-mTOR changed with both short-term and long-term HFD. The rest of them all only changed with long-term HFD. Phosphorylation of mTOR itself was not affected by HFD, and p-mTOR is the activate form, so how much p-mTOR was in the muscle is more important. But p-mTOR level did not change with either HFD or LKB1-KO. The total 4EBP1 changed with long-term HFD. 4EBP1 is a translation repressor, and its phosphorylation prevents this inhibitory effect. This means the p/t-4EBP1 ratio is more important, but it didn't change with long-term HFD. Increased t-4EBP1 inhibition effect hadn't been eliminated. Therefore, long-term HFD increased the capacity for 4EBP1 inhibition effect by increasing its total protein expression. The total eIF2 α increased only in LKB1-KO mice after long-term HFD, not short-term. eIF2 α is the α subunit of eIF2 (eukaryotic initiation factor 2) is required in the initiation of translation, so more eIF2 α can allow the cell to assemble more translation initiation machinery. Phosphorylation of eIF2 α inactivates it to down-regulate protein synthesis, but the p-eIF2 α didn't change with long-term HFD in the LKB1-KO mice. Therefore, LKB1-KO in skeletal muscle allows long-term HFD to induce increased total eIF2 α . This gives muscle more materials to build more translation initiation machine. Long-term HFD, then, had a general inhibition effect on protein synthesis in skeletal muscle through increase 4EBP1, but had a kind of rescue effect only in LKB1-KO mice by increasing total eIF2 α .

For the protein synthesis related proteins, t-eIF2 α and eIF4E changed with LKB1-KO. LKB1-KO increased both t-eIF2 α and eIF4E protein level only in long-term diet mice. eIF2 α is required for almost all cellular mRNA translation. eIF4E helps directing ribosomes to the cap

structure of mRNA, so more eIF4E can allow more ribosome bind with the cap structure of mRNA. Therefore, LKB1-KO would be expected to have a general stimulatory effect on protein synthesis in long-term diet skeletal muscle by increasing t-eIF2 α and eIF4E expression.

For the protein degradation related markers, none of them changed with HFD, while both ubiquitin and MAFbx increased with LKB1-KO. LKB1-KO increased ubiquitin level only for the long-term diet mice. Ubiquitin content reflects the protein degradation level [239-241], so more ubiquitin level suggests that protein degradation was increased. MAFbx also increased with LKB1-KO in long-term diet mice. MAFbx (muscle atrophy F-box) is a muscle-specific E3 ubiquitin ligase that leads to the addition of ubiquitin to targeted proteins through ubiquitin-conjugating enzyme E2. Therefore, more MAFbx will promote ubiquitination of more proteins, which was consistent with the ubiquitin results. Therefore, LKB1-KO had a general stimulatory effect on protein degradation in long-term diet skeletal muscle through increasing protein ubiquitination.

The long-term HFD only affected protein synthesis, not degradation. It may decrease protein synthesis by increasing total 4EBP1 (translation repressor protein) expression. This is consistent with decreased muscle weight/bodyweight for QUAD.

In summary, LKB1-KO appears to have two opposite effects: promotion of increased protein synthesis in long-term diet skeletal muscle through increasing t-eIF2 α and eIF4E expression, and promotion of increased protein degradation in long-term diet skeletal muscle through increased protein ubiquitination. Together this indicates that LKB1 may increase protein turnover. Previous research from our lab showed that LKB1-KO in skeletal muscle leads to weight loss and atrophy of type II skeletal muscle [215]. Here in this study, QUAD weight was lower in the long-term diet LKB1-KO mice. This appears to be due primarily to increased

protein degradation in the LKB1-KO muscles, but how null LKB1 leads to more MAFbx expression and protein ubiquitination needs further research.

CHAPTER 5: Summary

This purpose of this dissertation was to find out how HFD affects mouse skeletal muscle and how LKB1-KO in skeletal muscle can affect the HFD induced changes.

In Chapter 2, the effect of short- and long-term *ad libitum* HFD feeding was determined for CON and LKB1-KO mice. Weight gain was similar for both genotypes, which appeared to be due primarily to adipose weight, as expected, and this was associated with similar calorie intake and in-cage mouse activity between genotypes. However, glucose and insulin tolerance testing showed the most interesting results. HFD increased the fasting blood glucose levels in both mice as expected, consistent with impaired insulin sensitivity. However, fasting blood glucose was lower in LKB1-KO vs. CON mice regardless of which diet they were fed. This was likely due at least in part to elevated blood insulin levels in the LKB1-KO mice. The cause of elevated blood insulin levels cannot be determined from this study, but we speculate that it may be due to cross-talk between the skeletal muscle and the pancreatic beta cells. Further research is needed in order to confirm this speculation. After checking the insulin pathway, some interesting results showed up. The lower fasting blood glucose should be caused by increased fasting insulin, may be caused by increased p-Ser318-AS160 induced GLUT4 vesicle exocytosis. By decreasing TBC1D1 phosphorylation and GLUT4 expression, long-term HFD causes insulin resistance in mice. The pSer318-AS160 increased in the LKB1-KO adult mice, but both pSer237-TBC1D1 and pThr590-TBC1D1 decreased in the LKB1-KO adult mice. AS160 and TBC1D1 play the same role in glucose uptake: increased phosphorylation will increase glucose uptake. This leads to one conflict. This conflict maybe somehow can explain another conflict that raised from glucose/insulin tolerance test: LKB1-KO improve insulin tolerance test result in the original

data, but made the glucose tolerance test result worse in the modified data. Further research is needed to solve the conflicts.

Chapter 2 also checked how HFD and/or LKB1-KO affect LKB1 and its downstream target AMPK. The results showed HFD didn't affect LKB1 expression, cellular location or activity. Long-term HFD increased t-AMPK expression in LKB1-KO mice. Long-term HFD also decreased the AMPK activity in the cytoplasm part of the CON mice and the AMPK activation percentage in the nuclear part of CON mice. LKB1-KO decreased AMPK activity, which should always come with low LKB1 expression. Nuclear localization for AMPK $\alpha 2$ also decreased in the LKB1-KO mice. But, like a compensation mechanism, LKB1-KO increased AMPK $\alpha 1$ expression and then increased total AMPK expression. In conclusion, LKB1-KO affect AMPK expression, cellular location and activity. HFD only can affect AMPK cellular location. HFD had a null-LKB1 dependent effect on AMPK expression and cellular distribution.

In Chapter 3, in order to find out the potential genes that play a decisive role in long-term HFD induced effects in skeletal muscle, there are two specific aspects designed into this study. The first approach was to group the target genes and to determine whether they were affected by HFD or LKB1-KO. Combining data from three different places, 16 target gene had been found. After using RT-PCR to check all these genes in 10 different mice groups, those target genes had been grouped into different groups. The most important group (genes that were affected by long-term HFD, but not short-term HFD and/or switched diet) for the purpose of this study only included one gene: Ky. Ky increased only after long-term HFD. Since Ky is an important gene for normal muscle growth, and muscle weights were affected by LKB1-KO, Chapter 4 tried to see if anabolic and catabolic pathway changed. The results show that LKB1-KO decreased QUAD weight in long-term diet mice. LKB1-KO increased t-eIF2 α and eIF4E expression

suggestive of anabolic enhancement, but on the other hand, promoted increased MAFbx expression and protein ubiquitination suggestive of increased protein degradation. Long-term HFD had a general inhibitory effect on anabolic signaling in skeletal muscle through increase 4EBP1, but had a kind of rescue effect only in LKB1-KO mice by increasing total eIF2 α .

Conclusion

In conclusion, long-term HFD increased mice body weight by increasing adipose tissue content. HFD made mice become insulin resistance by decreasing TBC1D1 phosphorylation and GLUT4 expression. LKB1-KO caused mice to react differently in both glucose and insulin tolerance tests. LKB1-KO lowered the fasting blood glucose level, and increased the fasting insulin level. With the physiological insulin level, LKB1-KO can make glucose uptake ability worse, by decreasing TBC1D1 phosphorylation, but increased p-Ser318-AS160 and cytoplasmic GLUT4 expression at the same time. Further research is needed in order to solve this conflict. Many genes, including *Lgals3*, *Ucp2*, *Cdkn1a*, *SOCS3*, *Oxct1*, *Ddit4*, and *IL-6*, changed with LKB1-KO, but these genes' transcription were not affected by HFD. Only *Ky* increased after long-term HFD, but was unaffected by short-term and switched diets. Since *Ky* is an important gene for normal muscle growth, protein synthesis and degradation were determined. Long-term HFD had a general inhibition effect on protein synthesis in skeletal muscle through increase 4EBP1, but had a kind of rescue effect only in LKB1-KO mice by increasing total eIF2 α . LKB1-KO increased FLNC expression, promoted effect on protein synthesis in long-term diet skeletal muscle through increasing t-eIF2 α and eIF4E expression, and promoted effect on protein degradation in long-term diet skeletal muscle through increasing protein ubiquitination. LKB1-KO also can affect AMPK expression, cellular location and activity, while HFD only can affect AMPK cellular location. HFD had a LKB1-KO dependent effect on AMPK expression and

cellular distribution. Together, these findings illustrate an important role for LKB1 in the adaptations of skeletal muscle to long-term HFD feeding.

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Education

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Major: Exercise Biochemistry. Mentors: Yang Hu and Ying Zhang
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GPA: 3.92
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Major: Exercise Rehabilitation and Health. Mentors: ShanShan Mao
Honors Thesis: Effect of Sling Exercise Training on the Anti-Falling Ability of the Elder
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GPA: 3.83

Awards and Honors

2015 Full tuition Scholarship (Two years)

2015 Teaching Assistantship- PDBIO Department, BYU, Provo, UT
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2015 Research Assistantship- PDBIO Department, BYU, Provo, UT
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2015 Brigham Young University's Graduate Research Fellowship Award
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2012 High Impact Doctoral Research Assistantship (HIDRA) Award
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2009 Excellent Honors Thesis

2006 Beijing Sport University Fellowship

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Publications

Timothy M. Moore, Xavier Mortenson, Conrad Ashby, Alex Harris, Karson Kump, David Laird, Aaron Adams, Jeremy Bray, Ting Chen, and David M. Thomson. The effect of caffeine on skeletal muscle anabolic signaling and hypertrophy. *Applied Physiology, Nutrition, and Metabolism*. Published on the web 26 January 2017.10.1139/apnm-2016-0547.

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Manuscripts in Preparation

Ting Chen, Venkataswamy Sorna, Susie Choi, Lee Call, Jared Bearss, Kent Carpenter, Steven L. Warner, Sunil Sharma, David J. Bearss, Hariprasad Vankayalapati. Fragment-Based Design, Synthesis, Biological Evaluation and SAR of 1H-benzo[d]imidazol-2-yl)-1H-indazol Derivatives as Potent PDK1 Inhibitors (Submitted).

Ting Chen, Marcus Matsumura, Jeffery S. Tessem, David M. Thomson. Does knocking out LKB1 in skeletal muscle protect from high-fat diet induced insulin resistance? (Writing the paper)

Ting Chen, Marcus Matsumura, JJ Tsukamoto, David M. Thomson. How high-fat diet and/or knocking out LKB1 influence the skeletal muscle growth? (Writing the paper)

Ting Chen, Jonathan K. Alder, David M. Thomson. What does overexpress LKB1 complex do in C2C12 cell? (Writing the paper)

Conference Activity

Marc S. Matsumura, Ting Chen, David M. Thomson. High-fat diet induces LKB1-dependent attenuation of skeletal muscle p70S6k phosphorylation by insulin. *INTERSOCIETY MEETING: the integrative Biology of Exercise VII*. November 2-4, 2016

Ting Chen, Aaron Adams, Morgan Jensen, Tim M. Moore, and David M. Thomson. LKB1 Knock-out Increases Exercise-induced Cell Membrane Damage in Skeletal Muscle. *ACSM CONFERENCE ON INTEGRATIVE PHYSIOLOGY OF EXERCISE (IPE)*. September 17-20, 2014.

Natalie McVey, Ting Chen, Tim M. Moore, Mark T. W. Ebbert, Steven R. Madsen, David M. Hallowell, John S.K. Kauwe and David M. Thomson. LKB1 Regulates Skeletal Muscle Inflammation After Contractions. *SWACSM ANNUAL MEETING*. October 18-19, 2013.

Ting Chen, ShanShan Mao. Effect of Sling Exercise Training on the Anti-Falling Ability of the Elder. *XXXI FIMS SPORTS MEDICINE WORLD CONGRESS*. May 19-22, 2010.

Research Experience

RA Physiology & Developmental Biology Department, Dr. David Thomson's Lab
Brigham Young University
(August 2012~Present)

New Lab Skills: Radiation certified, nuclear protein isolation, kinase activity assay, gene cloning, build plasmid, E.coil culture and selection, bacteria transformation, plasmid isolation, cell transfection and transduction, electroporation, in vivo muscle fatigue test, animal dissections, mouse colony maintenance, rodent handling and surgeries, tissue sectioning, immunohistochemistry, immunoprecipitation, glycogen assay, satellite cell isolation.

Responsibilities: Develop, adapt, and implement protocols new protocol when needed, design, administrate and lead the whole high-fat project and the LKB1 overexpression project, help out with other projects, design primers, training undergraduate students, help Dr. David Thomson to manage the lab (I'm the only graduate student in this lab).

Lab Rotation Physiology & Developmental Biology Department, Dr. David Bearss's Lab
(January 2013~May 2013)

New Lab Skills: siRNA, ATP lite, drug treatment for cancer cell lines.

Responsibilities: Keep the "PDK1" project going on, finish writing the article for this project.

RA Exercise Biochemistry Department, Dr. Ying Zhang's Lab
Beijing Sport University, Beijing, China
(January 2012~ June 2012)

New Lab Skills: RNA isolation, reverse transcription, RT-PCR, chromatin immunoprecipitation (ChIP), western blot, protein assay.

Responsibilities: Develop, adapt, and implement protocols for RNA isolation, reverse transcription, RT-PCR, and chromatin immunoprecipitation (ChIP); training undergraduate students.

RA Sport Science Research Center (cell culture lab, histochemistry lab, molecule biochemistry lab)
Beijing Sport University, Beijing, China
(March 2010~ June 2012)

New Lab Skills: Cell culture, immortalize human white blood cells and maintain the cell lines, DNA isolation, traditional PCR, design primer, running DNA gel, R software, data analysis, human blood test.

Responsibilities: Manage lab; keep the “Elite athlete white blood cell bank” project going on; do blood test for elite athletes.

Teaching Experience

TA PDBio 363 Adv Physiology Lab
Brigham Young University
(Sep 2015~ Dec 2016)
Taught lab lecture, set up and managed the lab, administered grades.

Languages

Chinese: native

English: fluent reading, writing and speaking

Japanese: beginner

Community Service

Volunteer 2016 BYU SUGAR RUSH 5K

Judge 2016 Central Utah STEM Fair, Provo, UT, USA

Volunteer 2008 Beijing Olympics, Beijing, China

President 2007~2008 Green Heart Environment Protection Association, Beijing Sport
University, Beijing, China

Founder 2007 Green Heart Environment Protection Association, Beijing Sport University,
Beijing, China

Volunteer 2005~2007 Beijing Marathon, Beijing, China