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A STUDY ON THE PHYSIOLOGICAL ROLE OF SKELETAL MUSCLE RHO KINASE 2

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Dedication:

This work is dedicated to the memories of my best friend Emilee Dawn Gagnon. Thank you for being a constant inspiration.

This work is also dedicated to my parents Weibin Chen and Yanping Sun. Thank you for the sacrifices you have made that helped shape me into the person I am today.

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List of Abbreviations:

Abbreviation	Name
2DG	2 deoxy-glucose
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
BSA	Bovine Serum Albumin
ECAR	Extra cellular acidification rate
ETC	Electron transport Chain
GC	Gastrocnemius
GTT	Glucose tolerance test
HFD	High fat diet
IMLA	Intramuscular lipid accumulation
IOP	Intraocular pressure
ITT	Insulin tolerance test
LIMK	Lim domain kinase
MEF	Mouse embryonic fibroblast
MHC	Myosin heavy chain
Mito	Mitochondria
MLC	Myosin light chain
MYO CAROCK	Skeletal muscle specific constitutively active ROCK mouse strain
MYO R2KO	Skeletal muscle specific ROCK2 deficient mouse strain
MYPT1	Myosin phosphatase target subunit 1
NRF1	Nuclear respiratory factor 1
NCD	Normal chow diet
OCR	Oxygen consumption rate
PGC1α	Peroxisome Proliferator-activated receptor gamma coactivator 1 α
PH	Pleckstrin homology domain
PPARα	Peroxisome proliferator-activated receptor alpha
ROCK	Rho associated coiled coil kinase
TEM	Transmission electron microscopy

Abstract:

The aim of this dissertation project is to study the physiological role of Rho-associated coiled-coil kinase (ROCK) 2 in skeletal muscle. ROCK is a ubiquitous serine/threonine kinase with clinical implications in wide range of pathologies including hypertension, metabolic syndrome and Parkinson's Disease. Previous studies have attempted to investigate ROCK's physiological role, but outstanding conflicts exists due to absence of isoform-specific and tissue-specific tools. To study ROCK2's role in the skeletal muscle, the Liao lab has generated a novel genetic mouse model with a skeletal muscle-specific deletion of ROCK2 (hereby known as MYO R2KO). MYO R2KO demonstrate a decrease in endurance phenotype with no change in muscular strength. We linked the lower endurance with a lower proportion of oxidative fibers in MYO R2KO mice. Additional, ROCK2 deficient mice have an obese phenotype on a 43% high fat diet (HFD). Consequently, we measured skeletal muscle substrate utilization. There was no difference in glycolysis, but ROCK2 deficient muscles exhibited lower fatty acid oxidation. Next, we determined *in vitro* that the decrease in fatty acid oxidation is associated with mitochondrial dysfunction. Cellular and molecular studies demonstrated that mitochondrial dysfunction is caused by decreased mitochondrial fission potentially ROCK regulation of F-Actin formation. In conclusion, skeletal muscle ROCK2 regulates endurance performance through its impact on mitochondrial function. These findings suggest that skeletal muscle ROCK2 may be a viable target to treat mitochondria dysfunction with long term implications for multiple diseases including Type 2 Diabetes Mellitus and skeletal muscle atrophy.

Section A

Introduction

Chapter 1: The Basic Role of Rho Kinase

ROCK is a 160 kDa serine/threonine kinase that lies downstream of GTP-bound RhoA. ROCK was initially discovered as a GTP-bound RhoA binding protein through affinity column chromatography conducted on a GTP-bound RhoA matrix[1-3]. ROCK has two isoforms, ROCK1 and ROCK2. ROCK1 is also known as p160ROCK and ROKβ while ROCK 2 is also known as ROKα. There has been a strong interest in ROCK since its discovery in 1995 and over 10,000 papers have been published on the subject[4]. Additionally, over a dozen ROCK small molecule inhibitors are currently undergoing clinical trials[5]. Extensive reviews of ROCK in both basic and clinical context have been written[4-8]. In this chapter, we will introduce the basic role of ROCK including its structure, isoforms and role in regulating actin cytoskeleton.

Structure of ROCK

The structure of ROCK has been investigated through X-ray crystallography. There are 21 ROCK structures stored in the PDB database. ROCK's basic structure is as follows: an amino terminus is followed by a kinase domain then a coil-coil region then a pleckstrin homology (PH) domain and ends with the carboxyl terminus. A single Rho-GTP binding domain is positioned within the coiled-coil region[9, 10]. In its inactive state, the carboxyl terminus folds into the kinase domain and forms an auto-inhibitory loop. In the active state, RhoA binds to the Rho-GTP binding domain, ROCK is released from its autoinhibition and proceeds to phosphorylate downstream targets. Deletion of the carboxyl terminus portion of ROCK creates a constitutively active kinase[1, 11]. Thus, the effects of constitutively active ROCK (CA-ROCK) have been studied extensively *in vivo* and *in vitro*[12-14]. We will personally study the effect of constitutively active ROCK in the skeletal muscle in Chapter 10. It is important to note that CA-ROCK has the ability to phosphorylate all downstream targets, regardless of isoform specificity.

Crystal structures of ROCK differentiate ROCK from other members of AGC kinase superfamily. Traditionally, AGC kinases are activated when the carboxyl terminus is phosphorylated. However, ROCK is not activated in this manner. It is activated when RhoA binds to the Rho binding domain [15]. Crystallography experiments also showed why ROCK inhibitors have greater selectivity for ROCK over other AGC kinases. ROCK inhibitors more specifically inhibit ROCK through subtle residue differences. For example, the presence of Ala215 in ROCK, in contrast to Thr183 in PKA, leads to a higher affinity for 4 different ROCK inhibitors (Y-27632, Fasudil, hydroxyfasudil and H-1152P). Additionally, while most of the biochemistry studies focus on monomers of ROCK, Chen et. al. found ROCK exists in the multi-mer form in the cytoplasm[16]. These studies illustrate that structure is of critical importance to ROCK function.

ROCK1 and ROCK2 Isoforms

There are two isoforms of ROCK, ROCK 1 and ROCK2. ROCK1 is also known as ROKβ and p160ROCK, while ROCK2 is also known as ROCKα. There are strong similarities between the isoforms. First, ROCK1 and ROCK2 are structurally alike. The two isoforms have 60% sequence homology and 90% kinase domain homology[6, 17]. Second, both isoforms are expressed ubiquitously in all tissues. Third, ROCK1 and ROCK2 are both activated by RhoA, RhoB and RhoC[18]. There are also redundancies in the substrates that they phosphorylate. Both ROCK1 and ROCK2 can phosphorylate the same downstream targets like MYPT1[19, 20].

Despite these similarities, there are important differences between ROCK1 and ROCK2. First, the isoforms are located on different chromosomes. ROCK1 is located on chromosome 18 (18q11.1) while ROCK2 is located on chromosome 2 (2p24)[2, 21]. Second, even though both isoforms are expressed, one isoform is often expressed at a higher level than the other isoform. For example, ROCK1 is more highly expressed in the lung, liver and kidney[4]. In contrast,

ROCK2 is the dominant isoform expressed in the brain and skeletal muscle[17, 22, 23]. Due to its higher expression in skeletal muscle, ROCK2 is the isoform that is investigated in this study. ROCK1 and ROCK2 also differ in their subcellular location. ROCK1 is expressed in the cytoplasm, where it colocalizes with E-cadherin complex and microtubule centers, and the plasma membrane[24]. ROCK2 is expressed in the cytoplasm, where it colocalizes with vimentin and F-actin, and also the nucleus[1, 16, 25]. ROCK1 and ROCK2 can be activated in isoform-specific ways. ROCK1 is activated when caspase 3 cleaves ROCK1's carboxyl terminus[26, 27]. In contrast, ROCK2 is cleaved by granzyme B[28]. Additionally, the isoforms can be inhibited in through different mechanisms. ROCK1 is inhibited when RhoE binds to the amino terminus and when Gem binds to its coiled coil region. ROCK 2 can be inhibited by Rad[29]. These biochemistry and cellular differences suggest that there are important functional differences between these isoforms.

Previous studies have shown that ROCK1 and ROCK2 have different functions. Homozygous genetic deletion of the either isoforms leads to premature death in 90% of offspring[30, 31]. However, the cause of death differs between the isoforms. ROCK1 deficient mice exhibit two phenotypes. They develop open eyes and omphalocele[30], a birth defect where internal organs remain outside the abdomen in a sack. ROCK2 deficient mice's death are attributed to placenta disruption and thrombus formation[31]. It is important to note that the original genetic deletion studies were conducted on a C57Bl/6 background. However, when ROCK deletion occus on different mouse backgrounds different phenotypes resulted. For example, ROCK1 deletion on a FVB background had a higher 40% survival rate with a normal phenotypes[32]. A ROCK2 deletion CD1 background had 70% of mice survival rate with normal phenotype[33]. This suggest that future *in vivo* studies should take into account the genetic mouse models used, especially with

regards to the choice of background strain[34]. Likewise, *in vitro* studies have shown that ROCK1 and ROCK2 have different functions in the same cell systems. For example, ROCK2 deletion in mouse embryonic fibroblast (MEF) exhibit enhanced adipogenesis, but there was no change in adipogenesis with MEF ROCK1 deletion[35]. Shi et. al also demonstrated that ROCK1 in MEF cells were for phosphorylating MLC phosphorylation while MEF ROCK2 was responsible for phosphorylating cofilin[36]. These functional differences highlight a need to have isoform-specific tools that can investigate ROCK on both *in vivo* and *in vitro* levels.

ROCK's Role in Cytoskeleton Regulation

Historically, ROCK was first investigated for its ability regulate cytoskeleton. Increased ROCK activity leads to an increase in F-actin formation (also known as stress fibers). ROCK can regulates actin cytoskeleton through its ability to phosphorylate Myosin Light Chain (MLC). ROCK modulates phosphorylated MLC in two separate manners. First, ROCK directly phosphorylates MLC at Ser19[11]. Second, ROCK decreases myosin phosphatase activity by phosphorylating Thr855 and Thr697 of myosin phosphatase-targeting subunit 1 (MYPT1). A decrease in MLC phosphatase activity results in increased phosphorylated MLC levels[37]. Increased phosphorylated MLC levels increases contractility through its ability to interact with actin.

ROCK also regulate actin cytoskeleton through LIMK. ROCK directly phosphorylate LIMK1 and LIMK2 at Thr508 and Thr505 respectively. This in turn increases LIMK phosphorylation of Cofilin at Ser3 which inhibits cofilin. Cofilin depolymerize actin. Inhibition of cofilin increase F-actin stabilization and formation. Thus ROCK activity increase F-actin formation[14, 38].

Chapter 2: Clinical Implications of ROCK

There has been a high level of interest in ROCK therapies since its discovery in the 1990s. ROCK activity has been correlated with a diverse range of diseases including Alzheimer's Disease[7, 39, 40], multiple forms of cancer[41-43], hypertension[44-46], glaucoma[47-49], metabolic disorder[50-52] and neuromuscular diseases[53-55]. In this chapter, we will discuss how ROCK inhibitors are used therapeutically. We will also discuss the current view on ROCK's role metabolic and motor diseases. We are focusing on these two disease areas as they are most relevant to the dissertation's study.

Current ROCK therapies

Over a dozen small molecule ROCK inhibitors have been tested in clinical trials. However, to date only two inhibitors are market approved, Fasudil and K-115. Fasudil is used to treat cerebral vasospasm in Japan. Fasudil is also known as AT-877 and HA-1077. The small molecule is composed of a isoquinoline ring and seven-membered homopiperazine[56, 57]. Fasudil, like most other chemical inhibitors in clinical trial, does not differentiate between ROCK1 and ROCK2 and therefore inhibits both isoforms of ROCK equipotently. It also moderately binds to AMPK and PKA at high concentration[57]. Fasudil was initially characterized as an intracellular calcium antagonist back in the 1980s[58, 59]. Fasudil-bound ROCK crystal structures have been studied. These studies show that Fasudil inhibit ROCK by binding to 5-specific residues within its ATP-binding site[56]. The active form of Fasudil is hydroxyfasudil (HF). HF has been has greater affinity for ROCK than Fasudil itself[60]. In this dissertation project, we modeled ROCK inhibition *in vitro* by treating C2C12 myotubes with 10 μM HF.

Japan approved Fasudil for the clinical treatment of cerebral vasospasm in 1995 and China followed suit in 2013. Cerebral vasospasm is a condition where arteries in the subarachnoid space are constricted for a prolonged period of time. It is the leading cause of death after subarachnoid hemorrhage[61]. Pre and post-market data have shown that Fasudil has an excellent safety profile and is well-tolerated in humans[62-64]. One of the first clinical trials with Fasudil occurred in 1992 across 60 neurosurgical centers in Japan. Patients who suffered a subarachnoid hemorrhage were treated with either a placebo or Fasudil. Fasudil-treated patients had 38% decrease in vasospasm and 54% decrease in poor clinical outcomes[65]. Later in 2006 Zhao et. al. conducted a head to head study that compared Fasudil with another cerebral vasospasm drug named Nimodipine. They found that Fasudil worked as well or better than Nimodipine on multiple measures of outcome[66]. More recently, a meta-analysis of 8 clinical trials found that Fasudil treatment decrease incidences of angiographic and clinical vasospasm across the board[65, 67, 68]. Thus, fasudil has been shown to be an effective treatment for cerebral vasospasm.

ROCK inhibitor K-115, also known as ripasudil, was recently approved for the treatment of glaucoma and ocular hypertension[69]. Glaucoma is an eye condition where intraocular pressure (IOP) continuously increases, eventually leading to blindness. Glaucoma is a worldwide leading cause of vision lost[70] ROCK inhibitors decreases IOP by lowering aqueous humor outflow via its effect on the trabecular meshwork[71]. In 2014, ROCK inhibitor ripasudil was approved for glaucoma treatment in Japan. Ripasudil is isoquinolinesulfonamid derivative with high selectivity for ROCK. Ripasudil is has higher affinity for ROCK2 with IC50 of 0.019 μM compared to ROCK1 with an a IC50 of 0.051 μM[72]. A Phase II clinical trial, featuring 210 glaucoma patients, showed significant reduction in IOP in ripasudil treated patients[48]. Multiples

studies have verified that ripasudil treatment reduces IOP in both humans and animal models[47-49, 69, 70, 72-76]. Altogether, ROCK inhibition has been shown to ameliorate vision loss.

ROCK & Metabolic Diseases

ROCK's role in metabolic disease has been extensively studied but conflicting results remain. Most metabolic studies focused on ROCK's ability to regulate glucose metabolism and insulin sensitivity. An excellent review ROCK's effect on metabolism has been written[77]. Several previous studies have indicated that ROCK activity is increased in metabolic disorders and ROCK inhibition is beneficial to metabolic health. Liu et. al. showed that leukocytes isolated from metabolic syndrome patients had increased ROCK activity[52]. Animal studies are in agreement that ROCK inhibition is beneficial. When obese Zucker rats were acutely treated with Fasudil, their glucose sensitivity improved[51]. Similarly, when diabetic rats were chronically treated with Fasudil, diabetic neuropathy outcomes improved[78]. Noda et. al. showed that ROCK inhibition improved metabolic outcomes through AMP-activated protein kinase (AMPK) activation[79, 80]. Tang et. al. further supported this notion be demonstrated ROCK1 regulates insulin insensitivity through AMPK signaling[81]. However, other studies found ROCK inhibition does not impact on systematic blood glucose. Specifically, Kolvaennu found that DB/DB mouse treated with Fasudil did not have significant changes blood glucose[82]. In contrast, other studies have suggested that ROCK inhibition is actually detrimental to glucose metabolism. An *in vitro* study demonstrate that when ROCK was inhibited in adipocyte and myotubes, glucose uptake was inhibited[50]. In a follow-up study, ROCK1 was knocked out globally in mice and insulin resistance resulted[83]. In a human study, Type 2 diabetic patients had defective ROCK1 activity[84]. Thus, despite over 13 years of experiments, there remain conflicting data and no clear consensus as to the role of ROCKs in metabolism.

It is possible that the conflicts in ROCK's metabolic studies are the result of limitations of the tools used in previous experiments. Most animal studies use metabolic disease models that have been treated with ROCK inhibitors. This approach is severely lacking as inhibitors do not differentiate between ROCK1 and ROCK2-specific effects. These studies also inhibit ROCK globally and thus one cannot determine tissue-specific effects. Elucidating tissue-specific effects is especially important in metabolic studies due to the high degree of intra-tissue signaling. Additionally, ROCK inhibitors can also inhibit other AGC kinases. Thus, chronic treatment with ROCK inhibitors may result in physiological responses that are downstream of non-ROCK kinases, such as PKA and PKC. This dissertation project overcomes these complications by have tissue-specific and isoform-specific genetic deletion.

ROCK & Neuromuscular Diseases

ROCK's effect on motor performance have not been thoroughly studied. To date only one study has been published on ROCK's association with exercise. Recently, Muñoz et. al showed that rats that were subjected to swimming exhibited improved insulin sensitivity[85]. Exercise increased both ROCK1 and ROCK2 expression as well as ROCK activity. In contrast, rats that were globally inhibited with Y-27632 demonstrated decreased insulin sensitivity. Our study is similarly focused on motor performance but there are critical differences. We used C56BL/6J mice while Muñoz used Fisher rats. ROCK inhibition methods were also different. Muñoz used an imprecise method where both isoforms were inhibited globally, while we had an isoform-specific, tissue-specific genetic (and thus chronic) deletion. There was also a difference in the type and length of exercise. Muñoz exercised the rats one week by swimming while our strongest phenotypes were observed after six weeks of treadmill running. These differences in experimental design could explain the differences between our experimental outcomes.

We could also determine ROCK's role in motor performance by examining how ROCK treatments affect neuromuscular diseases models. ROCK inhibition has been implicated in a wide range of neuromuscular diseases including spinal cord injury[86-88], spinal muscular atrophy (SMA)[53, 54], Parkinson's Disease[89, 90], Huntington's Disease[91], and amyotrophic lateral sclerosis (ALS)[92-94]. In general, ROCK inhibition improves motor function and increase survival in these disease models. However, the experimental methodology used can impact results. For example, Takata et. al. found that when a ALS mouse model (SOD1) were treated with Fasudil there was increased survival and improved motor performance as measured by rotarod test[92]. But when Gunther et. al. treated the same ALS model with Fasudil , there was no change in survival and motor performance was only improved in male mice treated with the higher dose[55]. Thus, while there have been some initial studies on ROCK's role in motor performance an isoform and tissue-specific tool is needed to elucidate its precise role.

Chapter 3: Skeletal Muscle Metabolism

In this chapter, we will introduce skeletal muscle physiology background needed to contextualize the dissertation project. We will discuss skeletal muscle fiber types, skeletal muscle substrate utilization and the physiological adaptation to exercise. A deeper understanding of skeletal muscle physiology is needed to comprehend how the dissertation project expand on current knowledge in skeletal muscle metabolism field.

Muscle Fiber Types

Skeletal muscle is comprised two general fiber types, Type 1 (known as Type 1A) and Type 2. Type 2 fibers can be further divided into three more classes, Type 2A, Type 2X and Type 2B. Multiple types of fibers are necessary for the diverse range of tasks muscles are used for. Most characteristics of fibers have a range with Type 1A on one extreme and Type 2B on the other end. There are multiple ways to classify fiber type, but we will focus on three categories: myosin heavy chain isoforms, contraction speed, and energy source.

Currently the most common way of identifying muscle fiber is through identification of myosin heavy chain (MHC) isoform. Each type of muscle fiber is correlated to a specific MHC isoform. Fibers usually consist of only one MHC isoform but some fibers express 2 different MHC isoforms. These hybrid fibers have physiological and biochemistry properties that are intermediate between the isoforms[95]. Fiber types are identified by immunofluorescence staining muscle slices with MHC isoform antibodies.

Historically fibers were classified through their contraction speed. Type 1A fibers contact slowly. Type 2A contracts at a moderate pace and Type 2B contracts quickly. Skeletal muscle contraction is initiated when muscle fiber is stimulated by a neuron and calcium is released from

the sarcoplasmic reticulum. Collectively, neuron and skeletal muscle form a motor unit. A single neuron can innervate multiple fibers. Neuronal stimulation is very important to muscle function. Slow-frequency neuronal stimulation can promote a fast-to-slow fiber type transformation[96]. Conversely, fast-frequency neuronal stimulation leads to a slow-to-fast fiber type transformation. Additional, denervation of motor neuron is known to cause muscular atrophy[97]. Thus, when we are studying skeletal muscle physiology it is important to consider non-muscular tissue's (such as vascular and nerves) effect on skeletal muscle.

It is also possible to fiber type according to the skeletal muscle fibers' metabolism. Type 1A and Type 2A have higher rates of fatty acid oxidation while Type 2X and 2B uses predominately glycolysis. Relatedly, Type 1A has the highest density of mitochondria followed by Type 2A and then Type 2X and then lastly Type 2B. One common form of fiber typing is through measuring ATPase activity. Type 1A fiber have the slowest ATPase activity and is the most fatigue resistance. Type 2B have the fastest ATPase activity and fatigues quickly. ATPase activity is also correlated to contractile speed. The higher the ATPase activity, the faster the muscle fiber contracts. Thus, Type 2B has the highest about of ATPase activity and fastest contractile speed[98]. Additionally, fiber typing can occur by measuring succinate dehydrogenase (SDH) activity. SDH, also known as Complex II, is an enzyme involved in oxidative phosphorylation. Increased amount of SDH activity is indicative of increased oxidative phosphorylation. Thus, Type 1A has the greatest amount of SDH activity while Type 2B has the least. While there is a high degree of correlation between fiber types identified through different methods, these methods do not necessarily match up. For example, exercise can increase SDH activity without changing the myosin heavy chain isoform expressed in the fiber[99].

The current dissertation study uses mice as the i*n vivo* model. Thus, it is important to point out the differences between human and mouse skeletal muscle fibers. First, humans have much higher proportion of oxidative fibers compared to mice. Additionally, humans are more likely to have heterozygous MHC isoforms expressed within a single muscle fiber. By comparison, rodents muscles predominately express a single MHC isoform[100]. Confusion may also exist in comparing human and mice studies due to changes in fiber type classification. Rodents have four types of fibers: Type 1A, Type 2A, Type 2X and Type 2B. This classification is based on expressions of specific MHC isoforms. Type 2X is the most recently discovered fiber types and has characteristics that are intermediate between Type 2A and Type 2B[101]. Genetic studies have found that human fibers that were traditionally categorized as Type 2B actually express Type 2X MHC isoform[101-103]. In fact, humans do not express Type 2B MHC isoform at all[101]. However, if human muscle fibers are classified using non-MHC isoform methods, it likely that human fibers will be misclassified as Type 2B instead of Type 2X. Thus when one is reading skeletal muscle literature, it is important to understand the method of classification[104].

Skeletal Muscle Substrate Utilization

Skeletal muscle can draw energy from multiple substrate pools. There are four kinds of stored energy: ATP, creatine phosphate, glycogen and fatty acids. Skeletal muscle's inherent pool of ATP and creatine phosphate are depleted within 5-6 second of work, so we will focus on the two larger fuel sources glycogen and fatty acids[105]. Glycogen is stored form of carbohydrates. Glycogen is stored in two depots in humans. It is stored in the liver (~100g) and skeletal muscles (~500g). The amount of glycogen in skeletal muscle is controlled multiple variables including

whether the body is in a fed/fast state, amount of carbohydrate intake and exercise[106]. Glycogen is broken down in a process called glycolysis which occurs under anaerobic conditions.

In contrast, triglycerides are the main form of stored FFA. It is mainly deposited in adipocytes. When fuel is needed, triglycerides is broken down into free fatty acids (FFA) and combined with other proteins to form chylomicrons. Chylomicron transport FFA to its target tissues through the circulatory system. Once FFA arrives at a skeletal muscle it is transported inside and esterified with acyl-CoA. Long-chain acyl-CoA is then transported into the mitochondria and fatty acid oxidation occurs. Fatty acid oxidation requires oxygen to occur[107].

Interestingly, in addition to accumulating in adipocytes, lipids can accumulate within a skeletal muscle. The purpose of intramuscular lipid accumulation (IMLA) in skeletal muscle is controversial. Some experts believe IMLA is detrimental to the body. Obese patients have increase IMLA[108]. IMLA is also inversely correlated with insulin sensitivity in both adults and children[109-112]. However, IMLA is currently believed to be beneficial in athletes. In comparison to untrained individuals, endurance athletes have increased lipid accumulation[113]. It has been postulated that IMLA form an easy-to-access reservoir of energy to use for the next time the athletes exercise[113]. Thus, more studies are needed to understand the molecular mechanism behind IMLA.

Skeletal muscle substrate utilization depends on the length and intensity of work. Glycolysis is the preferred catabolic method when the work is short term or of high intensity, which is defined as exercise that requires greater than VO_2 at 70% of maximum VO_2. Glycolysis draws first from skeletal muscle glycogen pool then from plasma glucose derived from liver glycolysis[106]. High-intensity exercise draws its energy from glycolysis part due to the faster speed of glycolysis. In contrast, longer-term exercise depends fatty acid oxidation. Fatty acid

oxidation draws first from intramuscular triglyceride stores and then pulls from plasma FFA. Fatty acid oxidation is also preferred in low to moderate intensity exercise (<70% VO2 max)[114]. The switch from sugar to fat fuel sources demonstrate that both pools of energy are important to skeletal muscle performance.

Fuel utilization constantly changes as the body responds to nutritional and physiological cues. This ability to toggle between different fuel sources is called metabolic flexibility. A healthy skeletal muscle has the ability to toggle between glycolysis and fatty acid oxidation effectively[115]. In contrast, the inability to toggle between different fuel sources is named metabolic inflexibility. Metabolic inflexibility has been linked to obesity and type II diabetes in multiple human studies[116-118]. The dissertation project will elucidate ROCK's ability to modulate skeletal muscle metabolism.

Physiological Adaptation to Exercise

Generally, exercise can be divided into two categories: endurance exercise and resistance exercise. Endurance exercise is characterized as series of long-term submaximal muscular contractions that improves aerobic-based work. Common endurance exercises include jogging, swimming and biking. In contrast, resistance exercise, also known as strength exercise, is characterized as short-term maximal muscular contraction that leads to skeletal muscle hypertrophy. Traditional strength exercises include training with free weights such as dumbbells and kettle balls and repetitions on weight machines[119-121]. We will focus on adaptation to endurance exercise as that is the phenotype observed in MYO R2KO mice.

Chronic endurance exercise leads to physiological adaptations in skeletal muscle. Exercise improves energy efficiency of skeletal muscle, making them more fatigue-resistant. During

exercise, skeletal muscle fatty acid oxidation is increased by 5-10 folds[122-124]. Endurance exercise also increases glycolysis. Exercise raises GLUT4 expression which in turn increases glucose uptake in skeletal muscle[125]. On a molecular level, exercise adaptions are regulated transcriptionally. A single bout of exercise can induce multiple exercise-related transcription factors, such as mitochondria biogenesis genes NRF-1 and PGC-1α and master metabolic regulators PPAR-α and AMPK. These transcription factors go on to signal dozens of downstream exercise-induced targets[126]. Multiple rounds exercise lead to an accumulation of exercise-induced protein expression/activity, which is the foundation to physiological adaptation to exercise.

In addition to skeletal muscle changes, endurance exercise stimulates cardiovascular adaptations. Both endurance and resistance exercise increase cardiac mass[127]. Endurance exercise also increase capillary density of within skeletal muscle[128] which reduces both systolic and diastolic blood pressure[129]. These cardiovascular benefits increase the efficiency of oxygen delivery to the skeletal muscle.

Endurance exercise is known to be beneficial in a disease context. Exercise reduces the risk of cardiovascular disease. Mason et. al. in a meta-analysis of over 70,000 patients, exercise were associated with reduction in coronary events[130]. A separate meta-analysis, featuring data from over 1.3 million patients, showed cardiovascular disease risk decreased with increasing percentiles of exercise[131]. Exercise is also beneficial to metabolic diseases. A study featuring over 110, 000 patients demonstrated exercise decreased diabetes incidence over a 6-year period[132]. A separate meta-analysis saw that 8 weeks of exercise decrease HbA1C levels significantly[133]. Exercise is also improved sarcopenia, which is age-related muscular decline. 12 weeks of physical training improved cardiovascular outcomes in older males[134]. A similar

study found that when sarcopenia patients underwent regular exercise 3 times per week, patients increased muscle mass, strength and function[135]. Therefore, it is important consider exercise as viable therapy for multiple diseases in both preventative and curative background.

Chapter 4 All Things Mitochondria

In the process of working on this dissertation project, we found that ROCK2 had a strong effect on mitochondria function and dynamics. In this chapter, we will discuss why mitochondria is critical for skeletal muscle physiology and summarize previous literature that studied ROCKS' effect on the mitochondria. This content presenting in this chapter underlies our mechanistic experiments.

Skeletal Muscle Mitochondria Function

Mitochondria plays a critical role in skeletal muscle physiology. Skeletal muscle mitochondrial are located in two subcellular pools: one in the sarcoplasmic reticulum and the other in an intramyofibril pool. Fundamentally, mitochondria are responsible for ATP production. This is very important in skeletal muscle because ATP demand raises 100-folds during maximum contraction[136]. Additionally, mitochondrial reactive oxygen species (ROS) plays a signaling role. ROS production increases 2-4 folds after muscle contraction[137]. Originally, this increase in ROS was assumed to drives diseases such as Duchenne Muscular Dystrophy and muscular atrophy[137, 138]. However, recently the concept of mitohormesis has emerged. Mitohormesis occurs when mild or acute levels of ROS instigate positive cytosolic and nuclear responses[139, 140]. Skeletal muscle ROS is also needed post-translational modifications regulation[141]. Furthermore, mitochondrial biogenesis is needed for fiber type transformation. Exercise induces PGC-1α which in turn increase the proportion of oxidative fibers[142, 143]. Thus, mitochondrial plays multiple roles in skeletal muscle physiology.

In addition to playing an important role physiologically, skeletal muscle mitochondria are important in pathophysiology. Dysfunctional mitochondrial is observed in muscular dystrophy patients. Specifically, increased apoptosis is observed in Ullrich congenital muscular dystrophy[144]. Mitochondrial structural abnormality were also observed in patients with congenital muscular dystrophy[145]. Additionally, skeletal muscle mitochondrial dysfunction is associated with metabolic diseases. A seminal paper by Kelley et. al demonstrated that Type 2 diabetic patients had impaired oxidative phosphorylation compared to control[146]. Mitochondrial dysfunction has been tied to multiple aspects of diabetes including low fatty acid oxidation, insulin resistance, oxidative stress and inflammation[147, 148]. Skeletal muscle mitochondrial dysfunction is also associated with sarcopenia. Declining motor performance have been associated with a decline in oxidative phosphorylation, DNA content, and mitochondria biogenesis[149]. Thus, previous studies therapies that can promote mitochondria function has great clinical potential.

ROCKS' Effect on the Mitochondria

Past studies have investigated ROCKS' effect on the mitochondria. Mitochondrial morphology is constantly changing and its dynamic shape is critical to its function. Fusion elongates mitochondria, while fission fragments the mitochondria. ROCK has been previously shown to promote mitochondria fission. While the mechanism behind fission still is not fully elucidated, it is known that DRP1 is a critical molecular regulator. DRP1 is a cytoplasmic protein that translocate to the mitochondrial when activated. DRP1 oligomerize to form a ring-like structure around the mitochondria and then constricts prompting the mitochondria to divide[150]. Wang et. al found that ROCK1 activity promotes mitochondrial fission in podocytes.

Mechanistically, Wang found that ROCK1 directly phosphorylate DRP1 at serine600 and ROCK activity leads to increased mitochondrial fission[151]. This study had a direct impact on the dissertation project as it suggests the ROCK2 may play a similar role in altering mitochondrial fission.

ROCK may be regulating fission through its control of the actin cytoskeleton. The actin cytoskeleton regulate fission through two distinct manners. First, actin cytoskeleton drives pre-constriction. Studies with ER-localized inverted formin 2 show that F-actin need to accumulate at ER/mitochondrial site for mitochondrial fission to be initiated[152, 153]. Second, actin-cytoskeleton mediate DRP1 mitochondrial recruitment. Actin-cytoskeleton disruption has led to decrease mitochondrial DRP1 recruitment[154, 155]. As previously discussed, ROCK is a critical regulator of actin cytoskeleton. Thus, ROCK has the ability mediate mitochondrial fission through its regulation of F-actin formation. Martorell-Riera et. al. found that ROCK activity in cortical neurons is necessary for mitochondria fission[156]. A separate study showed that Fasudil treatment of endotoxemia mice model prevented excessive DRP-1 phosphorylation and mitochondrial fragmentation[157]. This dissertation project builds on the previous experiments by identifying an isoform specific effect of ROCK on mitochondrial fission and how molecular change leads to *in vitro* and *in vivo* phenotypes.

In addition to its role on mitochondrial fission, ROCK play a role in apoptosis. However, whether ROCK promotes or inhibits apoptosis is still controversial. In the first paper published on this topic, Colemann et. al demonstrate that ROCK1 is both necessary and sufficient in promoting apoptosis in mouse fibroblast line. ROCK1 induced multiple aspects of apoptosis including contractile force generation, membrane blebbing and apoptotic-body formation[26]. In support of this original paper, inhibition of ROCK decreased apoptosis in aortic samples,

hepatocytes, cardiomyocytes, human embryonic stem cells[158-160]. However, contradictorily, inhibition of ROCK has also been found to increase apoptosis. Small molecule ROCK inhibition increased apoptosis in hepatic satellite cells, smooth muscle cells, corneal epithelium and urothelial cancer line[161-165]. Thus, ROCK's role on regulating apoptosis may be context dependent.

Section B

Experimental Results

Chapter 5 *In vivo* Phenotype of MYO R2KO

Section 2 of this dissertation will feature experiments conducted in the dissertation project. Every experiment will contain an introduction, method and results section. The project was approached in a top- down manner. First, I determined the whole-body phenotype, then I delineated tissue-specific changes in energy utilization and lastly, I classified molecular and cellular effects of ROCK2 in *in vitro* skeletal muscle models. In this chapter, I will describe the *in vivo* phenotype of a skeletal-muscle ROCK2 deficient mice. Specifically, I will 1) verify the creation of the novel transgenic mice 2) identify ROCK2's effects on motor performance 3) classify how ROCK2 deficiency change whole metabolism and 4) investigate if ROCK2 mediate glucose homeostasis. To my knowledge, these are the first studies studying how ROCK2 deficient in skeletal muscle affect *in vivo* motor and metabolic phenotypes.

Creation of Novel Skeletal Muscle-Specific ROCK2 Deficient Mice

The Liao lab created a novel skeletal muscle-specific ROCK2 deficient mice named MYO R2KO. MYO R2KO is created by crossing myogenin-Cre mice with $ROCK2^{flox/flox}$ mice. MYO R2KO has a C57BL/6 background. Myogenin-Cre is a transgenic mouse strain where Cre recombinase is driven by the myogenin promoter/enhancer[166]. Myogenin-Cre mice was generously provided to Dr. Liao from Dr. Eric N. Olson (UT Southwestern, Dallas, TX). $ROCK2^{flox/flox}$ mice were created on C57Bl/6 background as previously described[167]. Exon 3 of the ROCK2 gene was flanked by two loxP elements. MYO R2KO were bred to be heterozygous for the Cre and thus ROCK2 is only partially deleted. Myogenin Cre is used as control mice for every *in vivo* experiment in this project.

MYO R2KO was created as a tool to study ROCK2's role in a tissue- and isoform-specific manner. ROCK2 is the isoform of interest as it is the predominant ROCK isoform expressed in the skeletal muscle [17, 23]. The Liao lab is interested in studying the skeletal muscle ROCK2 because previous literature has shown that ROCK plays an important role in *in vitro* skeletal muscle differentiation [168-175]. Previous studies have also demonstrated that ROCK inhibitors improved outcomes in neuromuscular diseases [53-55, 85-94, 176]. Thus, our goal for the first part of the experiment is to determine how skeletal muscle ROCK2 affect whole body physiology.

Experiment 1.1 Western blot of skeletal muscle ROCK2 expression

Introduction: MYO R2KO features a skeletal muscle-specific deletion of ROCK2. To validate ROCK2 deficiency, we measured ROCK2 protein expression by Western blot in two different skeletal muscles, the tibialis anterior (TA) and soleus muscle. Previous work by Pelosi et. al. demonstrated that ROCK2 expression varies between different skeletal muscle[23]. Thus, we wanted to measure ROCK2 expression in different muscles. We chose the TA and soleus muscle as they represent the fast and slow-twitch muscle respectively. We normalized ROCK2 expression to housekeeping gene Alpha Tubulin. We hypothesized that ROCK2 expression would be decreased in muscles isolated from MYO R2KO animals.

Method: Mice were fed a normal chow (NCD) diet and studied at week 14. Mice were sacrificed via CO_2 chamber and TA and soleus muscles were harvested from MYO R2KO and Control mice. Muscles were immediately snap frozen in liquid nitrogen.

Sample Prep: To extract skeletal muscle protein, we homogenized samples of skeletal muscle in muscle lysis buffer (20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 % Triton X-100, 10 % (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol). Protease inhibitor (Research Product International) and phosphorylase inhibitor (Thermo Scientific) was added to muscle lysis buffer. Next, we centrifuged the samples at 14K rpm for 10 min in 4^{o}C and then transferred the supernatant to a new tube. Next, we conducted Bradford assays to determine the protein concentration. We mixed equal volumes of whole muscle lysate samples with 2x Laemlli Buffer (Bio-Rad) and boiled Laemlli samples in 95^{o}C heat blocks for 5 minutes.

Western Blot: We made 8% SDS-PAGE gels and loaded 80 µg of each sample into the wells. We also injected protein ladder (Thermo Fisher) into the gel. We then ran the gel at 120V for 1.5 hr at 22^{o}C. The gel was wet-transferred to a PVDF membrane (Millipore) at 70V for 1.5 hr in 4^{o}C. Afterwards, we stained with Ponceau S (Sigma Aldrich) to ensure even lane loading. Following, the membranes were washed in PBST and then blocked with 3% Bovine Serum Albumin/BSA (Research Product International) for an hour. We incubated the membranes in primary antibody overnight in 4^{o}C. We used a 1:1000 dilution of ROCK2 antibody (BD) and 1:2000 dilution of Alpha Tubulin (Abcam) in 3% BSA. The next day membranes were washed 3x with PBST and incubated the in secondary antibody for 1 hour at room temperature. Secondary antibodies used were either 1:10,000 dilution of Anti-Mouse (BioRad) or 1: 10,000 dilution of Anti-Rabbit (BioRad) depending on the primary antibody source. Membranes were then washed 3x with PBST. Next, membranes were incubated in mixed chemiluminescent solution (BioRad) for 1 min and then imaged with ChemiDoc (BioRad). Blots were analyzed by ImageLab (BioRad) and analysis was conducted in Prism (GraphPad).

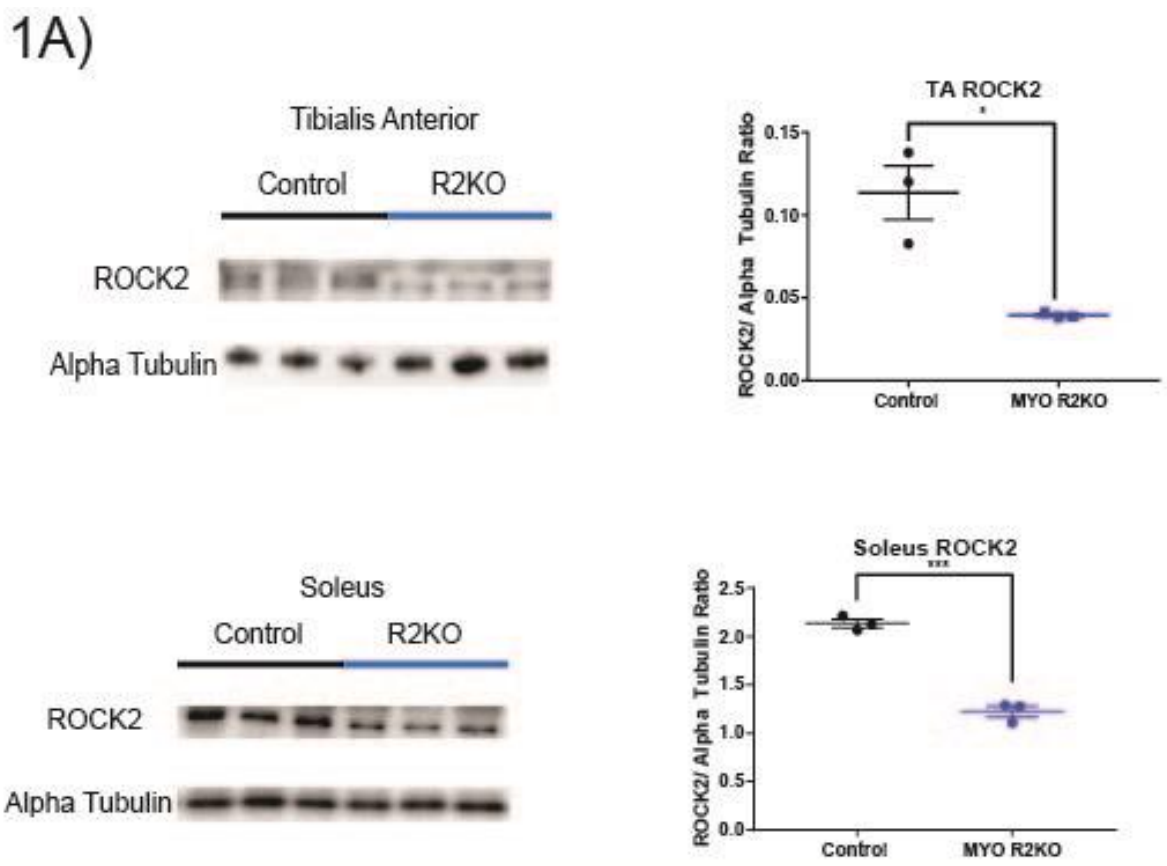


Figure 1A Skeletal muscle is deficient in ROCK2

Western blot of ROCK2 from whole muscle lysate extracted from tibialis anterior (TA) and soleus muscle. Muscles were extracted from male Week 14 mice. N=3 per genotype. ROCK2 expression was normalized to expression of housekeeping gene Alpha Tubulin. Significance is calculated through unpaired Student T-test.

Results: We observe a significant reduction in ROCK2 expression in both TA and soleus muscle both qualitatively and quantitatively. This experiment indicates that ROCK2 is deficient in skeletal muscles isolated from MYO R2KO.

Experiment 1.2 Western blot of skeletal muscle ROCK1 expression

Introduction: Next, we determined whether ROCK1 was upregulated in MYO R2KO skeletal muscles to compensate for ROCK2 deficiency. ROCK1 and ROCK2 typically regulate distinct downstream target but there are common targets like MYPT1[19, 20]. ROCK2 is not known to regulate ROCK1 expression. Thus, we hypothesized there is no difference between MYO R2KO and Control's ROCK1 expression.

Method: We blotted for ROCK1 using the same TA whole muscle lysate and soleus whole muscle lysate samples used in experiment 1.1. We also used the same western blot protocol as described in experiment 1.1, but with different primary antibodies. However, for primary antibodies we used 1:1000 dilution of ROCK1 (BD).

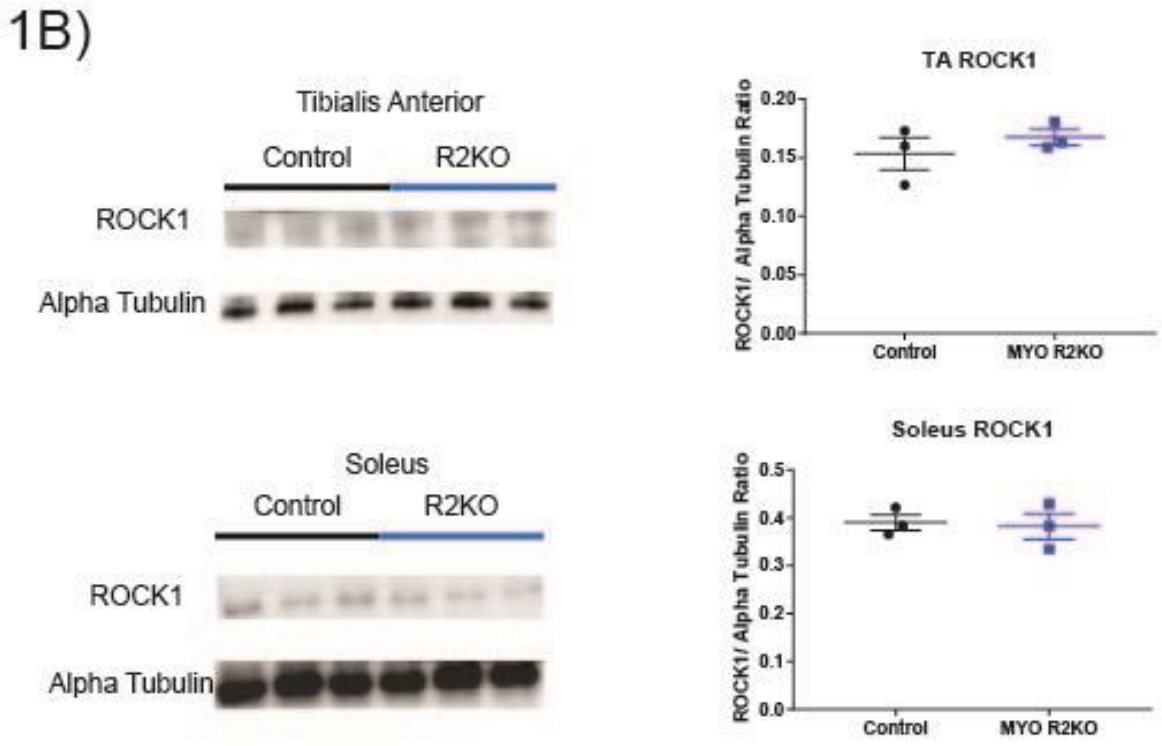


Figure 1B ROCK1 does not compensate for ROCK2 deficiency

Western blot of ROCK1 from whole muscle lysate extracted from tibialis anterior (TA) and soleus muscle. Muscles were extracted from male Week 14 mice. N=3 per genotype. ROCK1 expression was normalized to expression of housekeeping gene Alpha Tubulin. Significance is calculated through unpaired Student T-test.

Results: There was no difference in ROCK1 expression in muscles isolated from MYO R2KO and Control mice. This indicate that there was ROCK1 did not compensate for MYO R2KO skeletal muscle's ROCK2 deficiency.

Experiment 1.3 ROCK2 deficiency is skeletal muscle specific

Introduction: We needed to validate that ROCK2 deficiency was skeletal muscle specific. Thus, we blotted for ROCK2 expression in a variety of tissues: brain, heart, adipose, kidney and liver. Heart ROCK2 expression was particularly interesting because some muscle Cre mouse strains are leaky and impact both skeletal muscle and cardiac muscle. Thus, we wanted to ensure that ROCK2 deficiency was isolated to the skeletal muscle and did not affect any other types of tissues. The myogenin Cre is driven by a skeletal muscle differentiation gene (myogenin) and is expressed from embryonic day 8.5 [166] Therefore, we hypothesized there will be no changes in ROCK2 expression in non-skeletal muscle tissues.

Method: Mice were fed NCD and studied at week. Mice were sacrificed and heart, adipose, kidney and liver were harvest. Tissues were snap frozen in liquid nitrogen. Tissues were homogenized in RIPA buffer supplemented with protease and phosphorylase inhibitor. Samples were centrifuged at 14k RPM for 10 min at 4^{o}C. Supernatants were then transferred a new tube and protein concentration was determined via Bradford assay. We used the same western blot protocol as described in experiment 1.1. For primary antibodies we used 1:1000 dilution of ROCK2 (BD).

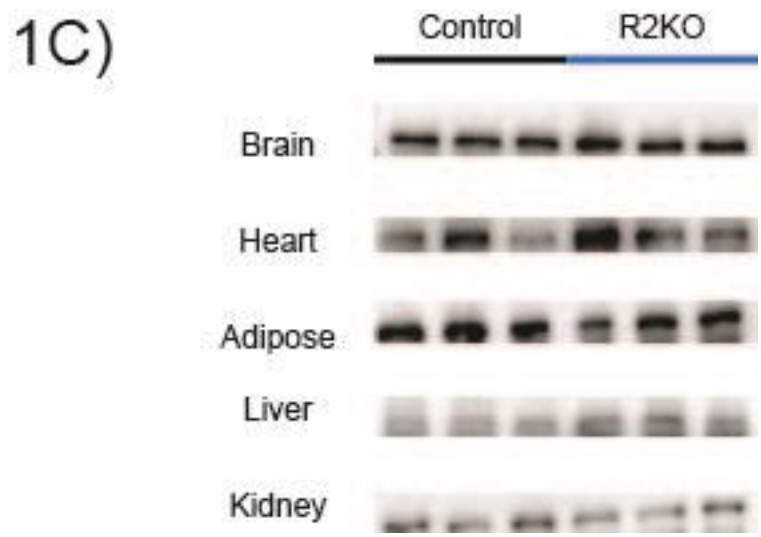


Figure 1C ROCK2 deficiency is specific to skeletal muscle

Western blot of ROCK2 in protein lysates isolated from brain, heart, adipose (subcutaneous), liver and kidney tissue. Tissues were extracted from male Week 14 mice. N=3 per genotype. ROCK2 expression was normalized to expression of housekeeping gene Alpha Tubulin. Significance is calculated through unpaired Student T-test.

Results: There was no change in ROCK2 expression between MYO R2KO and Control non-skeletal muscle tissues. This demonstrate that ROCK2 deficiency is specific to the skeletal muscle in our novel MYO R2KO mice strain.

Experiment 2.1 Forced Treadmill Endurance Test

Introduction: We wanted to determine ROCK2's impact on motor performance. There are 2 general classes of exercise: endurance and strength. To measure ROCK2's impact on endurance performance we performed a Forced Treadmill Endurance Test on our MYO R2KO mice. For behavioral tests it is critical that mice are comfortable in the instrument. Thus, we trained the mice 3 times before the test. The treadmill instrument (Columbus Instruments) only has 6 lanes so multiple cohorts of animals were trained and tested to achieve N>13 per genotype. ROCK inhibition has been shown to be beneficial to neuromuscular diseases previously. We hypothesized that ROCK inhibition would increase endurance performance.

Method: Mice were fed NCD and studied at week 12. Training schedule is defined below. In each training session, animals were allowed to acclimate in his individual lanes for 5 min followed by 50 min of running at 7 m/min. Mice were encouraged to run with puffs of air from an air canister. On test day, mice acclimated to their lanes for 5 min. After they were forced to run at an initial speed of 7 m/min for 5 minutes. Following, the speed was increased by 2 m/min every 2 minutes until exhaustion. Exhaustion occurs when a mouse is unable to run despite puffs of air from air canister. Running time was measured and total distance was calculated from running time. Analysis was conducted in Prism (GraphPad).

Day of Test	Type of Exercise	Protocol
Day 1 (Mice are Week 12)	Training	5 Min Acclimation in cage (no running) 50 Min at 7m/min
Day 2	Rest Day	None
Day 3	Training	5 Min Acclimation in cage (no running) 50 Min at 7m/min
Day 4	Rest Day	None
Day 5	Training	5 Min Acclimation in cage (no running) 50 Min at 7m/min
Day 6	Rest Day	None
Day 7	Test Day	5 Min Acclimation in cage 5 min at 7m/min Increase speed 2m/min every min until exhaustion

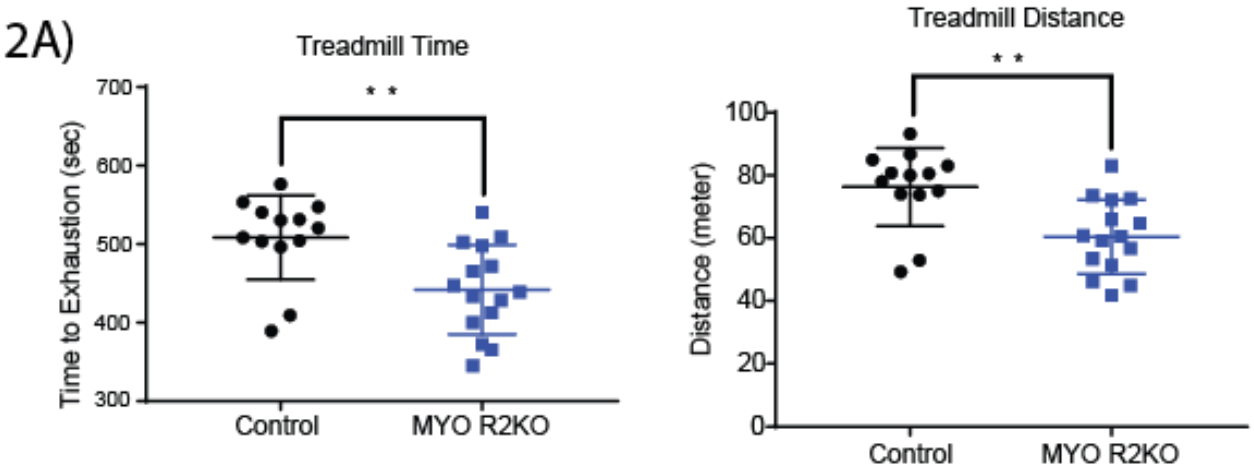


Figure 2A ROCK2 mediates endurance performance

Forced Treadmill Test was executed on MYO R2KO and Control mice. Mice were trained 3 times before test was executed. Male Week 12 mice were used. N=13 for Control and N=15 for MYO R2KO. Treadmill time is the total amount of time mice ran to exhaustion. Distance was calculated off of speed. Significance was calculated by unpaired Student T-Test.

Results: Surprisingly, we observed a highly significant ($P<0.001$) decrease in running time and running distance in MYO R2KO mice compared to Control. This suggests that ROCK2 mediates endurance performance.

Experiment 2.2 Voluntarily Ambulatory Motion via CLAMS

Introduction: Forced Treadmill Endurance Test is an example of a forced test as mice were encouraged to run through puffs of air from the air canister. We wanted to determine if volunteer movement was also altered in MYO R2KO animals. We measured MYO R2KO ambulatory motion in the CLAMS instrument (Columbus Instruments). We hypothesized that ambulatory movement would be lower in MYO R2KO mice compared to Control.

Method: Mice were fed NCD and studied at week 14. Mice were individually housed inside CLAMS instrument (Columbus Instrument) at room temperature ($22^{o}C$). Mice acclimated to individual chamber for ~6 hours before first dark cycle started at 18:00. We measured ambulatory motion for 48 hours or 2 dark and light cycles. Ambulatory movement was measured as the number of beam breaks. A beam break occurs when a mouse crossed a series of infrared beams on the chamber x-axis. Data exported from CLAX software (Columbus Instruments) and analyzed in Prism (GraphPad).

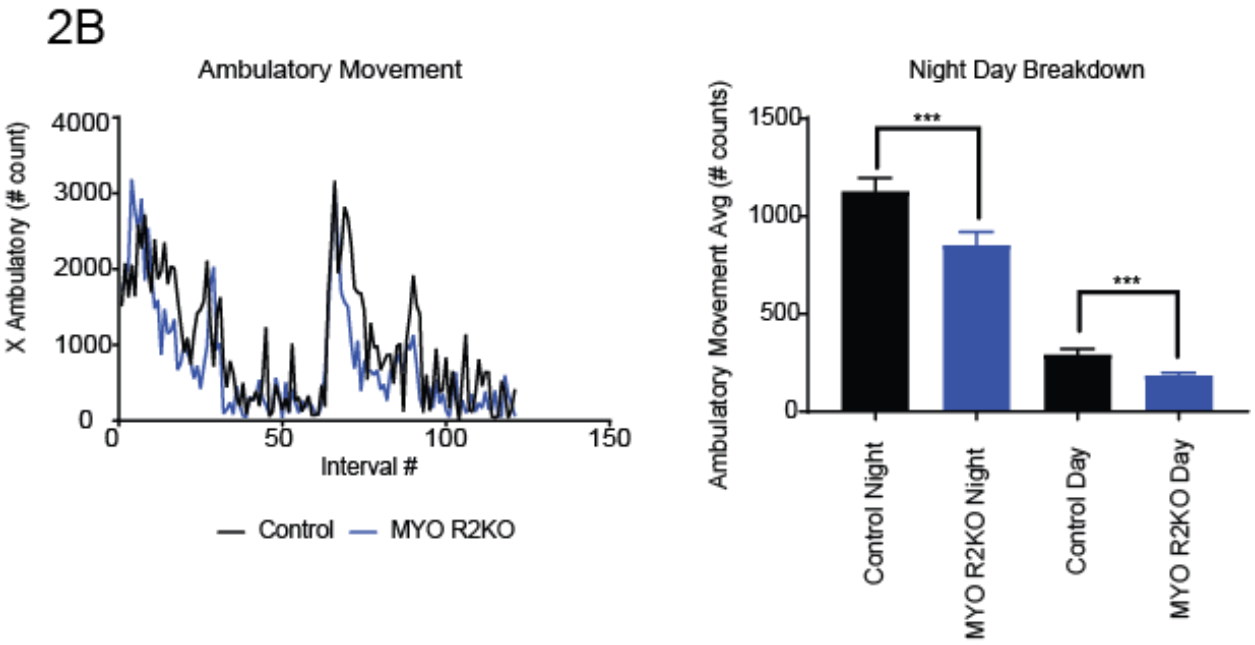


Figure 2B MYO R2KO have decreased ambulatory movement

Ambulatory movement was measured in CLAMS instrument (Columbus Instruments). Mice were individually housed in chambers and ambulatory movement was counted when a mouse crossed 3 or more infrared beams. Male Week 8 mice were used. N=4 per genotype. Left panel significance was calculated through paired Student T-Test. Right panel show mean value for each time period ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: There was a decrease in ambulatory motion in MYO R2KO mice. This decrease in motion was significant in both active night time period and day time period. This validates our previous observation that skeletal muscle ROCK2 modulates motor performance.

Experiment 2.3 4 Limb Hanging Test

Introduction: In addition to endurance, one can measure motor performance by measuring strength. To measure isometric strength, we performed 4 Limb Hanging Test on MYO R2KO and Control mice. Strength was measured as hanging time and impulse. Impulse accounts for differences in force required to hang that result from mice having different body weights. We hypothesize that MYO R2KO would exhibit a decrease in muscular strength.

Method: Mice were fed NCD and studied at week 14. 4 Limb Hanging Test was administered as previously described[177]. Mice were weighed before the test. Mice were then set in a cup with a grid on the bottom. The cup is raised to 35 mm above the ground and flipped so that mice are forced to hang upside down. Time was measured from the moment mice were inverted to the moment mice fell. Impulse was calculated as the product of hanging time multiplied by bodyweight. Analysis was conducted in Prism (GraphPad).

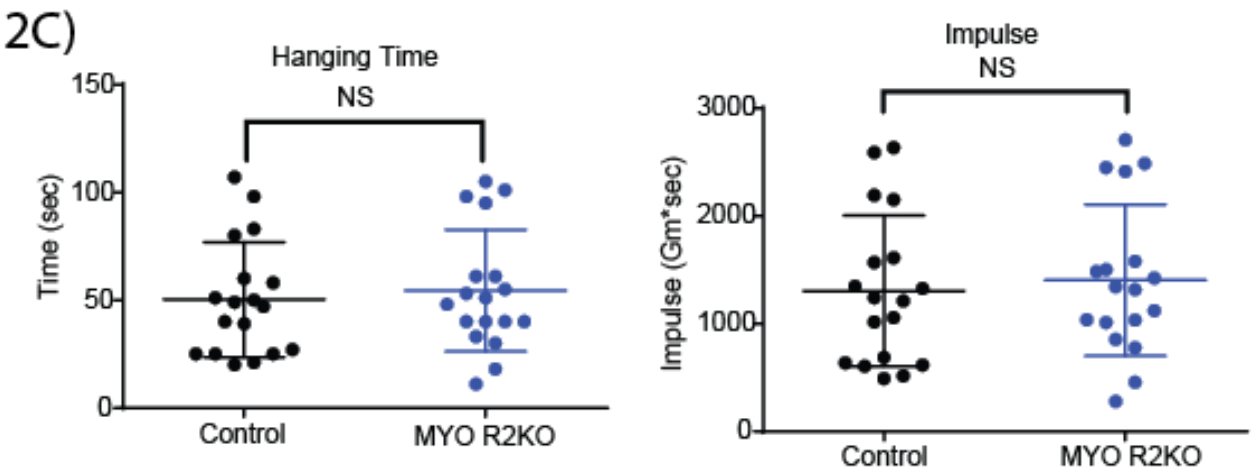


Figure 2C ROCK2 does not modulate muscular strength

4 Limb Hanging Test was executed. Male Week 14 mice were used. N=6 per genotype. Mice were hanged three times with 20 minutes of rest in between repetitions. Impulse was calculated by multiplying hanging time with body weight of mice. Error bars represent standard error mean Significance was calculated through unpaired Student T-Test.

Results: There was no difference in hanging time or impulse between MYO R2KO and control. This suggestion that skeletal muscle R2KO does not mediate muscular strength.

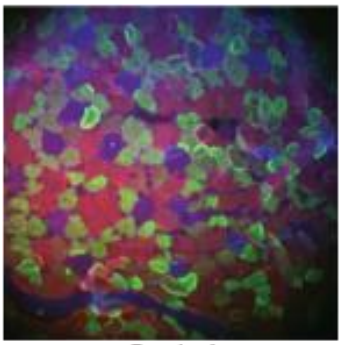
Experiment 2.4 MYO R2KO Fiber Type Composition

Introduction: We wanted to find the source of decrease in endurance. Skeletal muscles are composed of 3 classes of muscle fibers. Oxidative fibers (Type 1A and Type 2A) are more conducive for endurance exercise then glycolytic fibers (Type 2B). Similarly, endurance exercise increases oxidative fiber proportion [99, 102, 179]. Thus, we investigated if MYO R2KO mice had a different fiber composition. We subjected MYO R2KO and Control mice to 6-weeks of chronic exercise and fiber typed the gastrocnemius (GC) muscle. Due to the decreased endurance phenotype, we hypothesized there would be lower proportion of oxidative fibers in MYO R2KO GC muscle compared to Control.

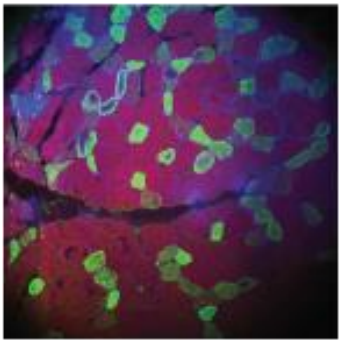
Method: Mice were fed a NCD. From Week 8 to Week 14, mice were forced to exercise 5 times a week. Each exercise session consists of 5 minutes of running at warm up speed of 5 meters/minute followed by 45 minutes of running at 8 meters/minute. After 6 weeks of exercise, mice were sacrificed by CO_2 inhalation. GC muscles were harvested and snap froze in liquid nitrogen. A cryostat (Leica) was used to cut the muscles at 8 uM thickness. Samples were transferred onto Super Frost Slides (Thermo Fisher). Slides were fixed with acetone (Fisher Scientific), blocked in 10% FBS (Gibco) for 1 hour at room temperature and washed with PBS (Gibco). Samples were then stained overnight in cocktail of myosin heavy chain antibodies (BA-F8/SC-71/BF-F3) manufactured by University of Iowa Developmental Studies Hybridoma Bank.

The following day, slides were washed and then stained for 1 hour in secondary antibody cocktail of Alexa Fluor-350/Alexa Fluor-488/Alexa Fluor-593 (Life Technologies). Images were taken on an DSU spinning disk confocal microscope (Olympus Corporation) with an Evolve EM-CCD camera (Photometrics, Tuscan, AZ). Images were analyzed via SlideBook (Intelligent Imaging Innovation) and ImageJ software (NIH). Data was analyzed in Prism (GraphPad).

2D)



Control



MYO R2KO

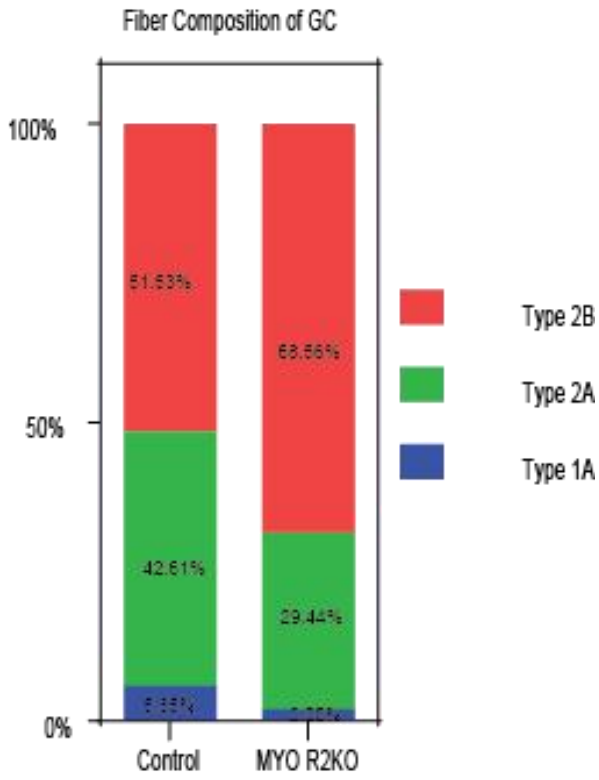


Figure 2D MYO R2KO have lower proportion of oxidative fibers

Male mice were chronically exercised for 6 weeks, from Week 8 to Week 14. N=6 per genotype. Gastrocnemius (GC) muscles were harvested after last bout of exercise. Images were captured on DSU spinning disk confocal microscope (Olympus) at 20x magnification. Representative images are shown in left panel. Significance was calculated by unpaired Student T-Test.

Results: There was a decrease in proportion of oxidative fibers (Type 1A and Type 2A) in MYO R2KO after 6 weeks of exercise. Thus, the decrease in endurance can be attributed to changes in muscle fiber composition. These results suggest that skeletal muscle ROCK2 may play a role in energy utilization. We will further explore this in Chapter 6.

Experiment 3.1 Body Weight on Normal Chow and 43% High Fat Diet (HFD)

Introduction: The skeletal muscle is a critical metabolic tissue. Thus, I hypothesized that skeletal muscle ROCK2 deficiency may changes to whole body metabolic measurement. To test this hypothesis, I measured the body weight of my Control and MYO R2KO animals on both normal chow and 43% HFD.

Method: Normal chow-fed mice were fed NCD for their entire life. 43% HFD fed mice were fed NCD from birth to week 8 and then HFD from week 8 to the time of sacrifice (~Week 24). The HFD used is Teklad Diet 97268. Body weight was measured on a weekly basis from age week 8 to week 20. Only male mice were used in this experiment. One cohort of normal chow fed mice was measured. Multiple cohorts of HFD-fed mice were weighed so that N>10 in the final figure. Data was analyzed by Prism (GraphPad).

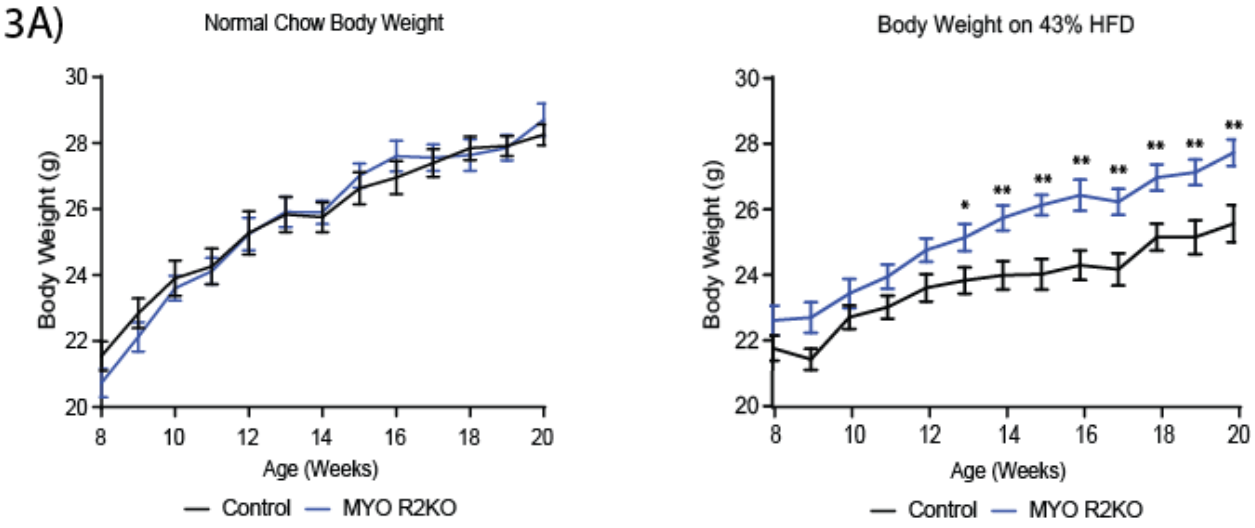


Figure 3A MYO R2KO mice have higher body weight on HFD

Only male mice were used for this experiment. Normal Chow fed mice were fed for normal chow their entire life. N=6 per genotype. Mice were weighed weekly. Significance was calculated through unpaired Student T-test based on each genotype mean body weight at each individual week. HFD fed mice were fed normal chow from birth to week 8. At week 8 mice were switched onto a 43% HFD and fed this HFD until sacrifice. N=10 for MYO R2KO and N=13 for Control mice. Values are means ± standard error mean. Significance was calculated through unpaired Student T-test. * indicates $P<0.05$. ** indicates $P<0.01$.

Results: There was no difference in body weight between MYO R2KO and Control mice normal chow. However, MYO R2KO mice had higher body weights on a 43% HFD. This suggests that skeletal muscle ROCK2 is mediating whole body metabolism.

Experiment 3.2 Body Composition by Duel Energy X-ray Absorptiometry (DEXA)

Introduction: We wanted to determine the source of the body weight increase seen in Experiment 3.1. We raised another cohort of mice on and fed them 43% HFD for 14 weeks, starting in adulthood (defined as Week 8). We submitted MYO R2KO and Control HFD-fed mice to University of Chicago's Diabetes Research and Training Core (DRTC) to undergo duel energy x-ray absorptiometry (DEXA) analysis. DEXA can determine % lean mass, % fat mass and bone mass density thus attribute the weight gain to a source. We hypothesized that there is secondary increase adiposity in MYO R2KO mice due to the fiber composition phenotype and differences in voluntary ambulatory movement and exercise endurance observed earlier.

Method: Mice were fed a normal chow diet from birth to week 8. Afterwards, mice were fed a 43% HFD until sacrifice. At week 22, body composition was measured at the DRTC at the University of Chicago. Data was analyzed by Prism (GraphPad).

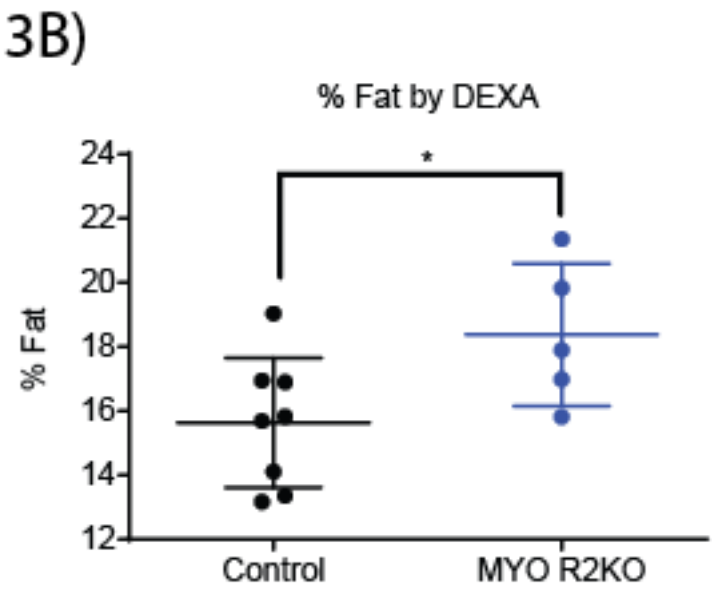


Figure 3B MYO R2KO mice have greater percentage fat

DEXA was measured by Dr. Hong at the Diabetes Research Training Center (DRTC) at the University of Chicago. HFD-fed mice were measured at Week 22 of age. Mice were fed normal chow from birth to week 8 and then HFD from Week 8 to time of sacrifice. Only male mice were used. N=5 for MYO R2KO and N=8 for Control mice. Values are means ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: DEXA results indicated that HFD-fed MYO R2KO mice had an increase in proportion body fat compared to control. This demonstrate increased body weight (observed in Experiment 3.1) was caused by an increase in body fat.

Experiment 3.3 Body Composition by NMR

Introduction: Body composition was verified by Nuclear Magnetic Resonance (NMR) study performed by University of Pennsylvania's Metabolism Core. NMR measures total fat weight, as opposed to DEXA's quantification of percentage body fat. This experiment provides an additional method to quantify body composition. We hypothesize that there is an increase in total fat weight in MYO R2KO compared to Control.

Method: Mice were fed a normal chow from birth to Week 8. Mice were then fed a 43% HFD until sacrifice. Body composition by NMR was measured at the University of Pennsylvania Metabolism Core when mice were 24-weeks old. Data was analyzed by Prism (GraphPad).

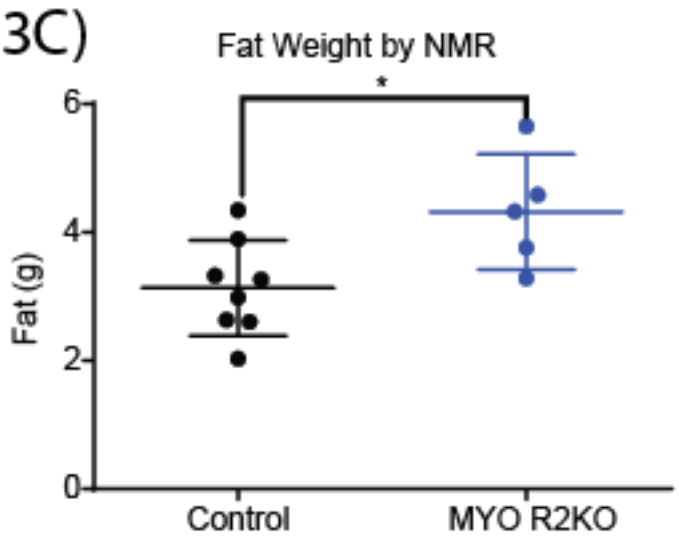


Figure 3C MYO R2KO mice have greater total fat weight

NMR was measured at the University of Pennsylvania Metabolic Center. HFD-fed mice were measured at Week 22 of age. Mice were fed normal chow from birth to week 8 and then HFD from Week 8 to time of sacrifice. Only male mice were used. N=5 for MYO R2KO and N=8 for Control mice. Values are means ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: MYO R2KO had increased total fat weight compared to Control. This validates DEXA body composition results and further support the notion that ablation of skeletal muscle ROCK2 expression results in increased adiposity, likely through a reduction in energy expenditure.

Experiment 3.4 Subcutaneous adipose cell size

Introduction: Decreased fatty acid oxidation has been linked to increase adipocyte size [178, 179]. The past two experiments demonstrate that MYO R2KO have increased adiposity. Therefore, we hypothesized that adipocyte cells are larger in MYO R2KO compared to Control.

Method: Adipocyte size was determined as previously described [180]. Briefly, subcutaneous adipose depots were harvested from HFD-fed mice at Week 24. HFD-fed mice were fed NCD from birth to Week 8 and then HFD from Week 8 until sacrifice at Week 26. Subcutaneous adipose depots were snap frozen in liquid nitrogen. Later, tissues were fixed in 10% formalin (Sigma Aldrich) for 36 hours at 4^{o}C. Tissue were then embedded in paraffin and sliced at 10 μM thickness. Tissues were processed by the University of Chicago Human Tissue Resource Center. Slides were stained with H&E and images were captured on BX41 microscope (Olympus). Adipocyte cell areas were measured by ImageJ (NIH) and data was processed with Prism (GraphPad).

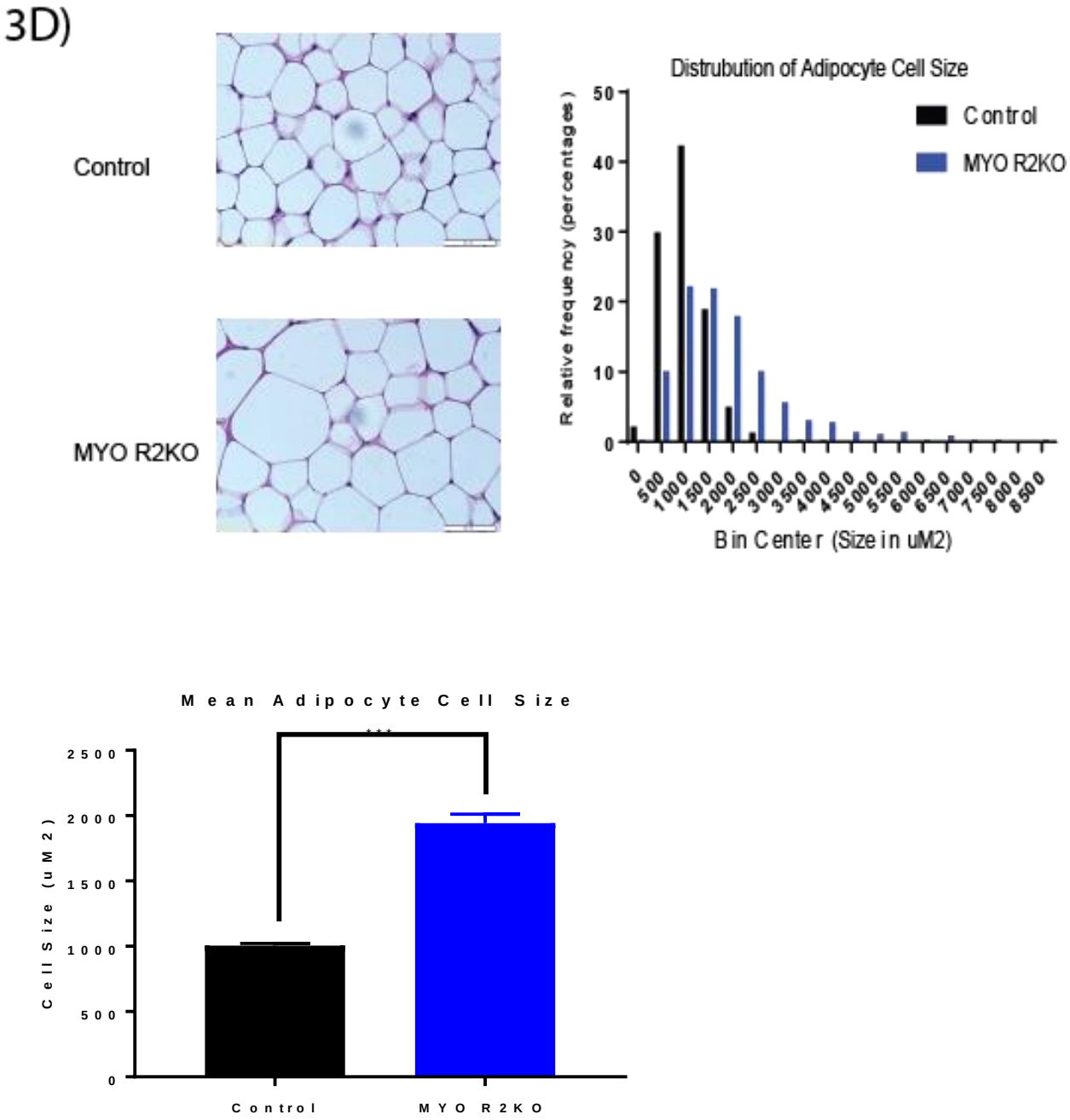


Figure 3D MYO R2KO mice have greater adipocyte cell size

Subcutaneous adipose depot harvested at Week 26. N=5 per genotype. Representative images were selected. Images were captured on BX41 microscope (Olympus) at 60x magnification. Significance was calculated by unpaired Student T-Test. *** indicates P<0.001.

Results: We observed an increase in adipocyte size in MYO R2KO compared to Control. This further validates the MYO R2KO obesity phenotype and suggest that skeletal muscle ROCK2 plays a critical role in fatty acid metabolism.

Experiment 4.1 Fasting Glucose Levels

Introduction: We initially believed that adiposity was caused by changes to whole body glucose metabolism. Circulating plasma glucose concentration is tightly regulated [181]. Therefore, we measured fasting plasma glucose to quantify glucose metabolism. We fasted both normal chow and HFD-fed mice for 6 hours to reduce post-prandial effects. We hypothesized that skeletal muscle ROCK2 deficient mice may have defects in glucose uptake and thus MYO R2KO would have a higher fasting glucose level than Control.

Method: Normal chow-fed mice were tested at Week 12. They were fed normal chow their entire lives. HFD-fed mice were tested at Week 24. They were fed normal chow from birth to Week 8 and 43% HFD from Week 8 to time of sacrifice. Mice were fasted for 6 hours with ad lib access to water. Plasma glucose was measured with Freestyle Lite Glucose Monitor (Abbott). Data was analyzed by Prism (GraphPad).

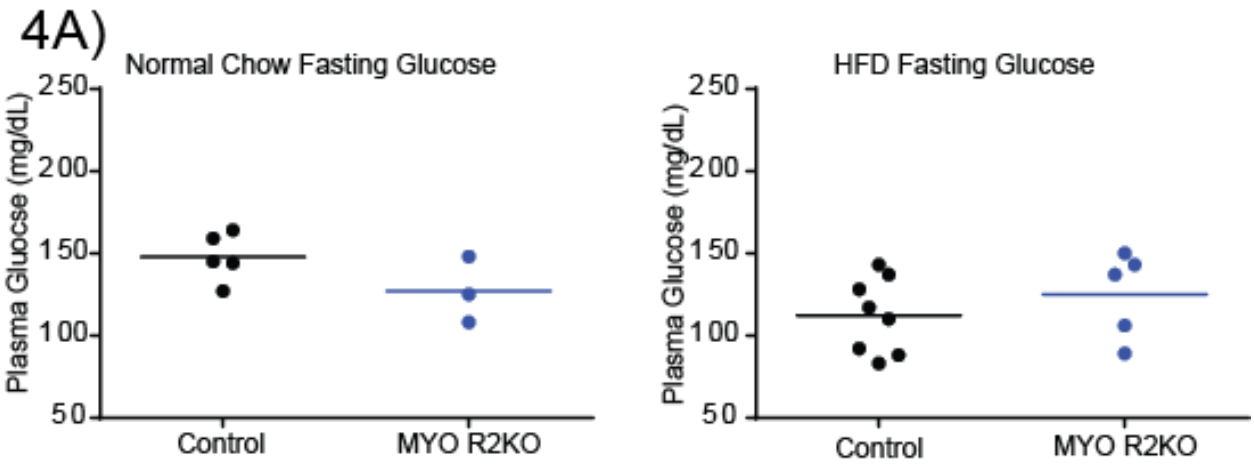


Figure 4A No difference in fasting plasma glucose

Male mice were fasted for 6 hours before plasma glucose was measured. Normal chow fed mice were measured at Week 12. N=3 for MYO R2KO and N=5 for Control. HFD fed mice were fed normal chow from birth to week 8 and then HFD from Week 8 to sacrifice. Fasting glucose was measured when mice were Week 24. N=5 for MYO R2KO and N=8 for Control. Values are means ± standard error mean. Significance was calculated by unpaired Student T-Test.

Results: There was no difference in fasting glucose levels between MYO R2KO and Control in both normal chow and HFD-fed cohorts. These surprising results suggest that skeletal muscle ROCK2 does not impact *in vivo* glucose homeostasis.

Experiment 4.2 Glucose Tolerance Test (GTT)

Introduction: GTT is golden standard method to measure glucose sensitivity in both mice and humans. The skeletal muscle is responsible for 70-90% of post-prandial glucose uptake and is critical regulator of glucose homeostasis [114, 182]. Previous studies are in conflict over whether ROCK increases or decreases glucose sensitivity[50-52, 77-84, 183]. Thus, we hypothesized that skeletal muscle ROCK2 deficient mice would have decreased glucose tolerance compared to control.

Method: GTT were administered on Week 12 mice for normal chow-fed animals and Week 26 mice for HFD-fed animals. Normal chow GTT were conducted at the University of Chicago, while HFD GTT were conducted by the University of Pennsylvania Metabolism Core. Normal chow-fed animals were fed normal chow their entire life. HFD-fed animals were fed normal chow from birth to Week 8 and then 43% HFD from Week 8 until time of sacrifice. Mice were fasted 6 hours prior to the test. Next, they were weighed and injected intraperitoneally with 1g/ 1U dose of 20% Dextrose (Sigma Aldrich). Blood glucose was measured pre-injection (0 min), 15, 30, 60 and 120 minutes post-injection with Freestyle Lite Glucose Monitor (Abbott). Data was analyzed by Prism (GraphPad).

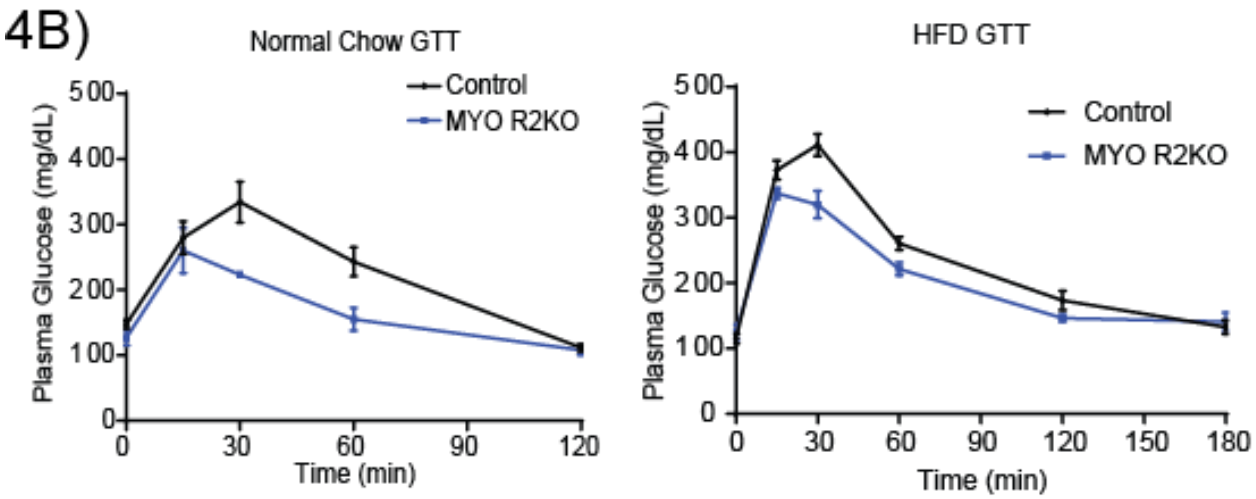


Figure 4B No difference in glucose sensitivity

Glucose Tolerance Test (GTT) was administered at Week 12 for Normal Chow-fed male mice. N=3 for MYO R2KO and N=5 for control. GTT was administered at Week 24 for HFD-fed male mice. N=5 for MYO R2KO and N=8 for Control mice. Values are means ± standard error mean. Significance was calculated for area under the curve through unpaired Student T-Test.

Results: There was no difference in glucose sensitivity between MYO R2KO and Control mice for both normal chow and HFD-fed mice cohort. While the MYO R2KO trended towards improved glucose sensitivity on both normal chow and HFD, the sensitivity was not significant measured paired student T-test. This validate the previous experiment conclusion that skeletal muscle ROCK2 does not regulate *in vivo* glucose homeostasis.

Experiment 4.3 Insulin Tolerance Test (ITT) on HFD Mice

Introduction: Insulin signaling is vital to glucose homeostasis. Tissues must be insulin sensitive to respond to changes in glucose demand. Insulin resistance is a key mechanism behind Type II diabetes [184-186]. ROCK has been previously connected insulin signaling but the specific role skeletal muscle ROCK2 plays is unknown [50, 84]. Thus, we wanted to investigate if skeletal muscle ROCK2 impacts insulin sensitivity via an ITT. We hypothesized that there was no difference in insulin sensitivity when ROCK2 is deficient in the skeletal muscle due to the lack of changes observed in Experiment 4.2.

Method: Insulin Tolerance Test were administered on Week 26 HFD male mice at the University of Pennsylvania Metabolism Core. Mice were fed normal chow from birth to Week 8 and then fed 43% HFD from Week 8 to time of sacrifice. One the day of the test mice were fasted for 6 hours. They were then weighed and injected interperitoneally with insulin at a 1U/ 1 kg dose. Glucose was measured at pre-injection (0 min), 15, 30, 60 and 120 post injection. Data was analyzed with Prism (GraphPad).

4C)

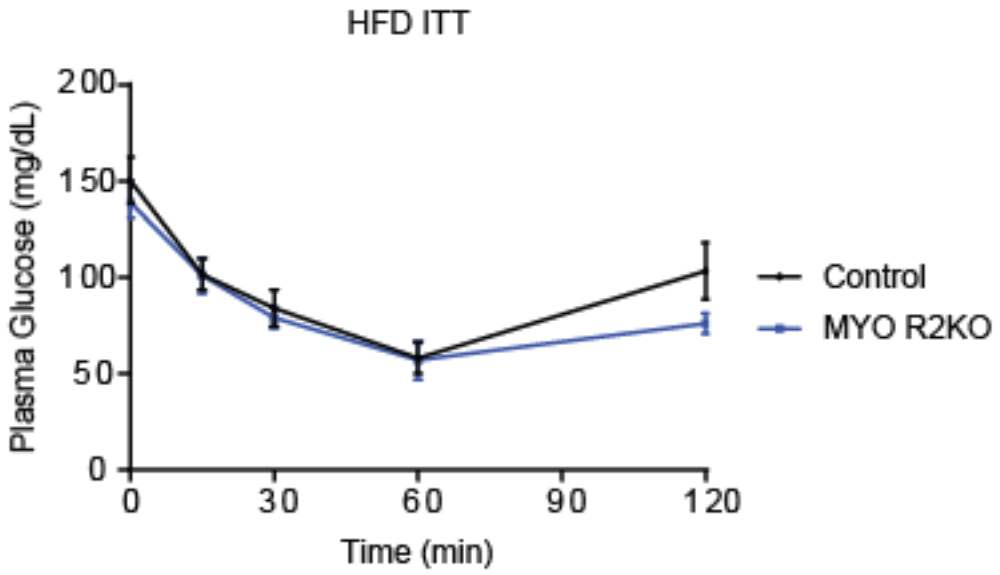


Figure 4C No difference in glucose sensitivity

Insulin Tolerance Test (ITT) was conducted on HFD-fed male mice. Animals were fed normal chow from birth to week 8 and then 43% HFD from week 8 until sacrifice. ITT was conducted on Week 24 mice. N=5 for MYO R2KO and N=8 for Control. Values are means ± standard error mean. Significance was calculated through paired Student T-Test.

Results: There was no difference between insulin sensitivity between MYO R2KO and Control mice. This suggest that skeletal muscle ROCK2 does not regulate insulin sensitivity *in vivo*. This result also suggests that insulin sensitivity could be isoform specific. It is foreseeable that changes in insulin signaling is downstream of ROCK1 and that is why in vivo glucose and insulin sensitivity are changed by pan-ROCK inhibitors.

Summary: This chapter catalogues the first set of experiments we conducted on the dissertation project. We started with *in vivo* studies because we wanted to test if skeletal muscle ROCK2 deficiency had a whole-body physiological effect before diving deeper into cellular and molecular experiments. First, we validated MYO R2KO had a tissue and isoform-specific deletion. Next, we discovered that skeletal muscle ROCK2 regulates endurance performance but does not affect muscular strength. We also found that ROCK2 deficiency leads to obesity but does not impact glucose homeostasis. In the next chapter, we will zoom in and determine how ROCK2 deficiency affects skeletal muscle energy utilization.

Chapter 6: ROCK2's Role in Skeletal Muscle Energy Utilization

This chapter explores skeletal muscle ROCK2's role in skeletal muscle energy utilization. We wanted to investigate skeletal muscle energy utilization because MYO R2KO endurance and obesity phenotype suggests ROCK2 is altering skeletal muscle metabolism. First, we will describe the two *in vitro* systems that I will use in for the rest of the dissertation project. Next, we will delineate the experiments we performed to investigate ROCK2's effect on glycolysis and fatty acid oxidation. These experiments connect the *in vivo* phenotype observed in Chapter 5 with the molecular mechanism observed in Chapter 7.

Two distinct *in vitro* skeletal muscle models

We use two different *in vitro* models in my cellular and molecular biology experiments. One model used features differentiated C2C12 myotubes. C2C12 is an immortalized mononucleate mouse myoblast cell line[187]. When C2C12 cells are switched from high serum media to low serum media (specifically 2% horse serum), the myoblasts start differentiating. C2C12 form multinucleated myotubes that express contractile proteins such as MHC, alpha actin and troponin [188]. This mimic muscle fibers in an *in vitro* setting. Typically, we would seed C2C12 myoblasts in a cell culture plate and allow them to grow in growth media (DMEM, 10% FBS, Pen/Strep) until they reach 100% confluent. Next, we would switch the cells to differentiation media (DMEM, 2% Horse Serum, Pen/Strep) and differentiate them for 6 days. We would change the media every 24 hours to fresh differentiation media. In the last 24 hours of differentiation we would treat the myotubes with a pan ROCK inhibitor, 10 µM HF. The advantage of this system is that it is easy to set up. However, the disadvantage of this system a

long length of time (~1 week) is needed to prepare the cells and ROCK inhibition through HF treatment is not isoform specific.

A second way to model skeletal muscle *in vitro* is to isolate primary myofibers from my mice. We use the protocol previously published [189]. Briefly, we sacrifice a mouse and extract the flexor digitorum brevis (FDB) muscle from both feet. We digested FDB in a collagenase solution for 1.5-2 hours in 37^{o}C. We then titrate the muscle bundle with a p1000 pipette until single myofibers are dissociated. The primary myofibers are transferred onto a Matrigel coated cell culture plate and allowed to settle overnight. The advantage of using primary myofibers is that this model has a ROCK2-specific deficiency, and thus is a better *in vitro* model of MYO R2KO mouse. The disadvantage of this system is the mouse is sacrificed during the isolation and thus continual mouse breeding is required. Thus, initial *in vitro* experiments were all conducted using C2C12 myotubes and later experiments were repeated on primary myofibers.

Experiment 5.1 Glycolysis Stress Test in C2C12 Myotubes

Introduction: Seahorse XF24 (Agilent Technologies) instrument was used to measure metabolic flux. Specifically, a glycolysis stress test was performed to measure glycolysis. Glycolysis is quantified by measuring how the extracellular acidification rate (ECAR) changes in response to a series of chemical compounds. Glycolytic muscle fibers are used in strength exercise. There was no difference in muscular strength between MYO R2KO and Control (observed in Experiment 2.3). Therefore, we hypothesized there would be no change in glycolysis between HF-treated and untreated C2C12 myotubes.

Method: Glycolysis stress test was conducted in XF24 Seahorse instrument (Agilent Technologies) per manufacturer's instructions. C2C12 were seeded in XF24 culture plate and differentiated for 6 days. Some wells treated with 10 μM HF for the last 24 hours of differentiation. On the day of the test, C2C12 myotubes was switched from differentiation media to glycolysis stress test media and placed in 0% CO_2 incubator for 30 minutes before the test. Next, a series of drugs were injected into the wells and ECAR was measured. Glucose (Sigma Aldrich) was the first injection. Glucose turns on glycolysis in because cells were starved for 30 minutes prior to the test. Oligomycin was the second injection. Oligomycin inhibits ATP Synthase. When oxidative phosphorylation is inhibited, glycolysis is promoted. The 3rd injection was 2-deoxyglucose (2DG). 2DG is a glycolysis inhibitor. Raw data was exported from Seahorse software and analyzed in Prism (GraphPad).

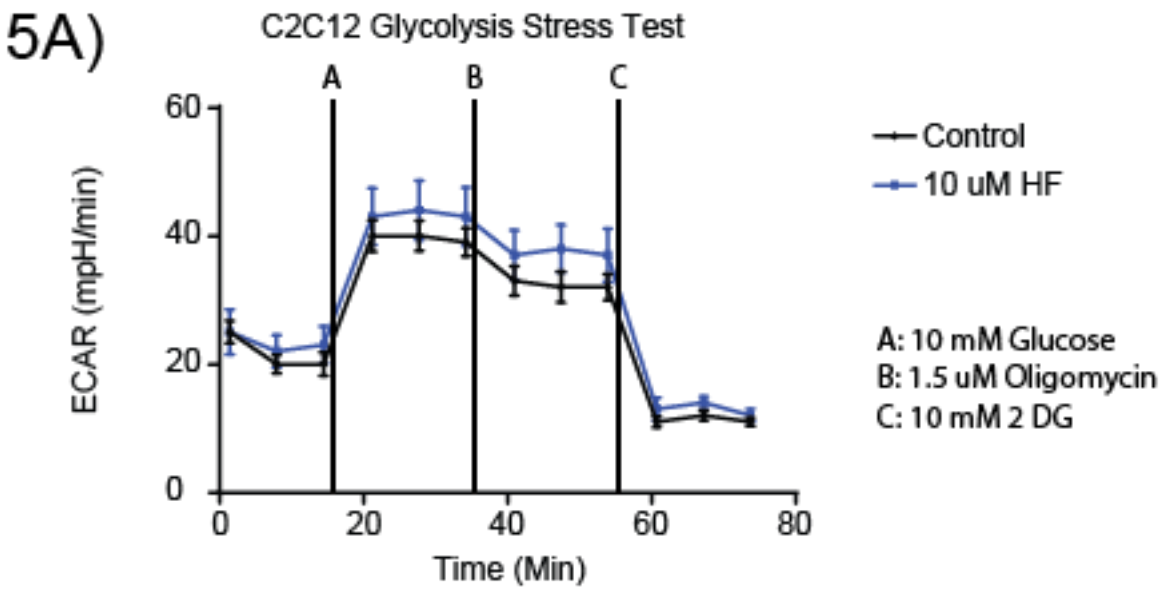


Figure 5A ROCK inhibition does not affect glycolysis

Seahorse Glycolysis Stress Test was conducted on C2C12 myotubes. ECAR was measured using XF24 Seahorse Instrument (Agilent). Glucose, Oligomycin and 2 deoxyglucose were injected at indicated time. Values are mean ECAR ± standard error mean. Significance was calculated from paired Student T-Test.

Results: There was no difference in glycolysis between ROCK inhibited C2C12 myotubes and untreated C2C12 myotubes. This suggest that skeletal muscle ROCK2 does not mediate skeletal muscle glycolysis.

Experiment 5.2 Glycolysis Stress Test in Primary Myofibers

Introduction: Although, there was no difference in glycolysis in ROCK inhibited myotubes, we were uncertain if ROCK2 had any isoform specific effect on skeletal muscle glycolysis. Thus, we performed a Glycolysis Stress Test on primary myofibers isolated from MYO R2KO and Control mice. We hypothesize that there is no change in glycolysis in ROCK2 deficient primary myofibers.

Method: Primary myofibers were isolated as previously described [189]. Briefly, animals were sacrificed by CO_2 and the flexor digitorum brevis (FDB) muscle was extracted. FDB was then digested in 40 mg/ mL of Collagenase Type II (Worthington) in 1% BSA/DMEM media for 1.5 hours in 95 ^{o}C incubator. Primary myofibers were titrated in primary myofiber media (DMEM, 20% FBS, 1 % Chicken Embryo, 1 % Pen Strep) [190]. Primary myofibers were then transferred to Matrigel coated cell culture plate and settled overnight in 37^{o}C. The following day we conducted a glycolysis stress test in the same manner as described in Experiment 5.1.

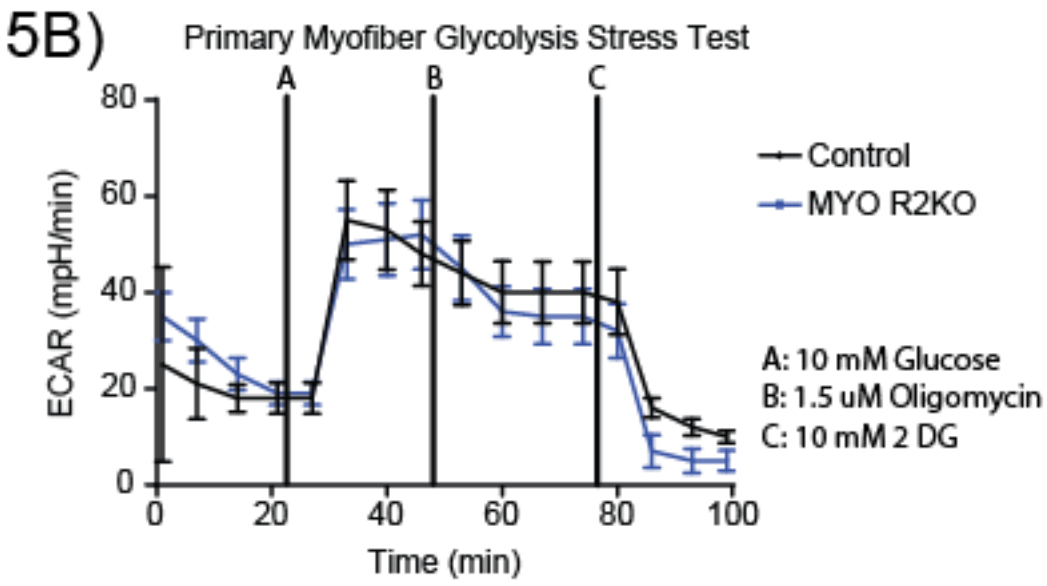


Figure 5B ROCK2 deficiency does not change glycolysis

Seahorse Glycolysis Stress Test was conducted on primary myofiber. N=10 per genotype. ECAR was measured using XF24 Seahorse Instrument (Agilent). Glucose, Oligomycin and 2 deoxyglucose were injected at indicated time. Values are mean ECAR ± standard error mean. Significance was calculated from paired Student T-Test.

Results: There was no difference in glycolysis stress test in between MYO R2KO and Control primary myofibers. This suggest that skeletal muscle ROCK2 specifically does not regulate skeletal muscle glycolysis.

Experiment 5.3 Intracellular lactate level

Introduction: Glycolysis is a ten-step process that converts glucose into pyruvate and produce 2 ATP. Pyruvate transform into lactate through the actions of lactate dehydrogenase. This action regenerates NAD+ which allows glycolysis to continue to cycle [191, 192]. Thus, intracellular lactate is a readout of glycolysis. Due to the lack of change in Seahorse experiments, I hypothesize that there will be no difference in intracellular lactate levels of ROCK inhibited and untreated myotubes.

Method: Intracellular lactate was measured following BioVision Lactate Kit instructions. C2C12 were seeded in 6-well plates and differentiated for 6 days with and without 10 uM HF treatment. Some wells of C2C12 myotubes were treated with 10 mM 2DG (Sigma Aldrich). 2DG is a glycolysis inhibitor and serve as a negative control. Cells were harvested with RIPA buffer and vortexed for 1 hours at 4^{o}C. Samples were spun at 14k RPM for 10 min at 4^{o}C and supernatants were transferred to a new tube. 10 uL of each sample were transferred to 96-well plate in duplicates. Lactate Assay Buffer and enzyme mix were added to plate. Reactions were incubated for 30 min. Next, fluorescence was quantified at Ex/Em=535/590 in microplate Reader (Teclan). Data was analyzed in Prism (GraphPad).

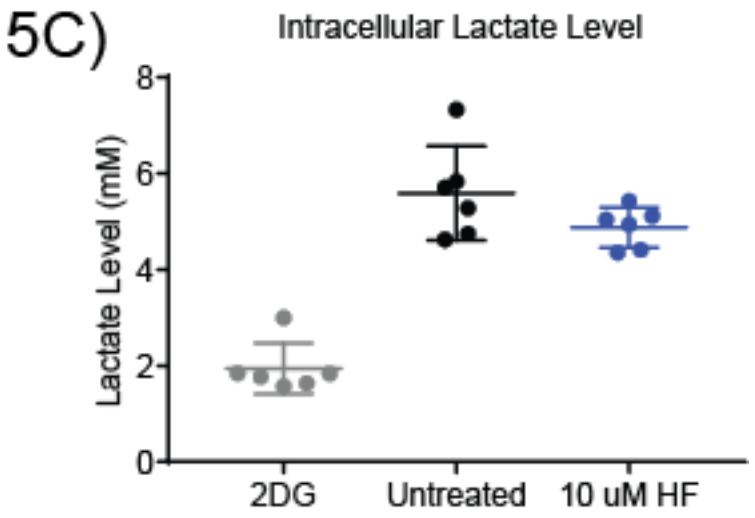


Figure 5C ROCK inhibition does not alter intracellular lactate level

Intracellular lactate level was measured per BioVision Lactate Kit instructions. N=6 per treatment. Values are mean ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: There was no difference in intracellular lactate levels between ROCK inhibited and untreated C2C12 myotubes. This experiment provides additional evidence that ROCK inhibition does not impact glycolysis.

Experiment 5.4 Glucose Uptake Assay

Introduction: Glucose uptake is an important indicator of glycolysis [191, 193]. There are multiple ways to measure cellular glucose uptake but we will approach it using a radioactive protocol. We are grateful to Dr. Brady's laboratory for allowing us to use their glucose uptake radioactive protocol. We hypothesize that there is no difference in glucose uptake in ROCK inhibited and untreated C2C12 myotubes.

Method: C2C12 were seeded and differentiated in 6-well plates. Cells were washed with PBS (GIBCO) twice and incubated in glucose-free media for 30 minutes (glucose free DMEM, 25 mM HEPES, 0.5% FBS). Myotubes were then treated with 2-deoxy-D [3H] glucose for 5 minutes at room temperature at a concentration of 2 μCi/mL. Radioactive glucose uptake was stopped by placing cell culture plate on ice and adding non-radioactive 200 mM 2-deoxy-glucose to all wells. Wells were washed 3x with cold PBS. Next. cells were scraped and mixed with 5 mL of Ecolume (MP Biomedical). Radioactivity was quantified with scintillation counter (Becker). Data was analyzed in Prism (GraphPad).

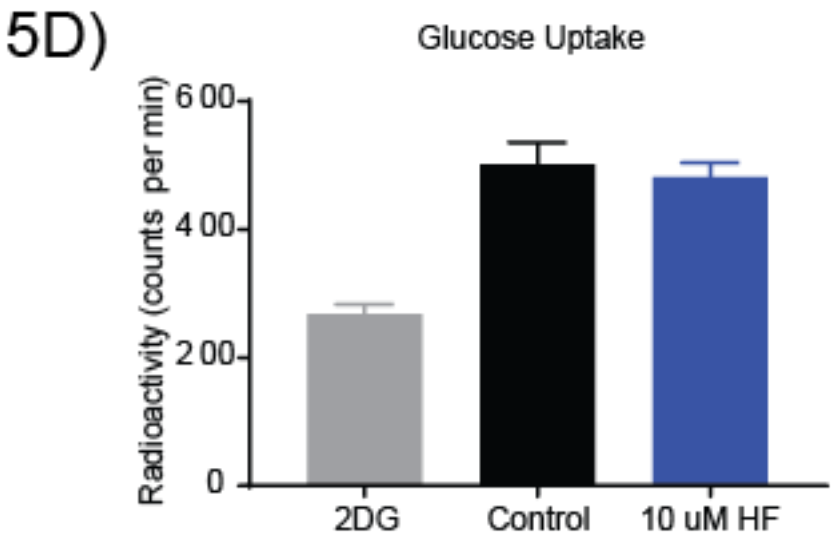


Figure 5D ROCK inhibition does not impact glucose uptake

Glucose uptake was measured through radioactive assay described in Method. N=3 per treatment. Values are mean radioactivity ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: There was no difference in glucose uptake between ROCK inhibited and untreated C2C12 myotubes. This experiment further indicates that ROCK2 does not mediate skeletal muscle glycolysis.

Experiment 6.1 Mito Stress Test in C2C12 Myotubes

Introduction: After determining skeletal muscle ROCK2 does not impact glycolysis, we investigated if skeletal muscle ROCK2 mediate fatty acid oxidation. Seahorse XF24 instrument is able to quantify oxidative phosphorylation through administration of mitochondrial (mito) stress test. Experiment 2.4 demonstrated that MYO R2KO mice had lower proportion of oxidative fibers. Thus, we hypothesized ROCK inhibited C2C12 will have decreased oxidative phosphorylation compared to untreated C2C12 myotubes.

Method: C2C12 were seeded and differentiated in XF24 cell culture plate. Some wells were treated with 10 μM HF during the last 24 hours of differentiation. C2C12 myotubes were switched out of differentiation media and into mitochondrial stress test media 1 hour prior to mito stress test. A series of drugs were injected into the well and oxygen consumption rate (OCR) was measured. Oligomycin (Sigma Aldrich) was injected to inhibit ATP synthase. Next FCCP (Sigma Aldrich) was injected. FCCP is an oxidative phosphorylation uncoupler. Lastly, rotenone (Sigma Aldrich) is added. Rotenone inhibits complex one in electron transport chain (ETC)and turns off oxidative phosphorylation completely. Raw data was exported from XF24 software and computed in Prism (GraphPad).

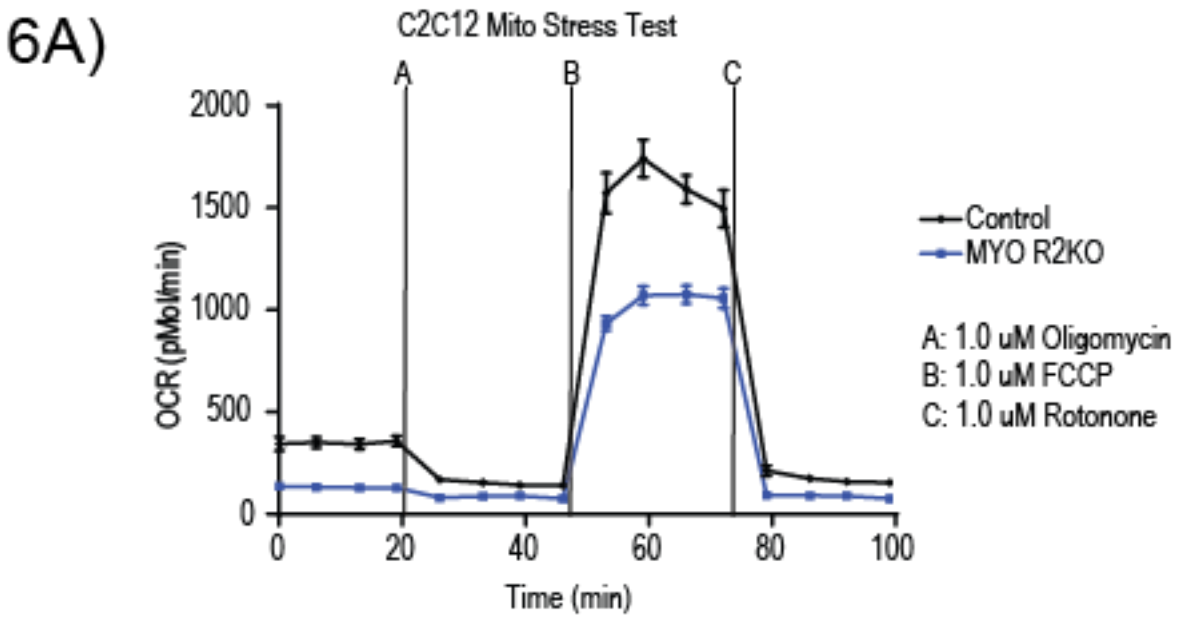


Figure 6A ROCK inhibition decrease oxidative phosphorylation

Mitochondrial Stress Test was conducted on C2C12 myotubes. N=4 per treatment group. OCR was measured using XF24 Seahorse Instrument (Agilent). Oligomycin, FCCP and Rotonone were injected at indicated time. Values are mean OCR ± standard error mean. Significance was calculated from paired Student T-Test.

Results: ROCK inhibited C2C12 myotube had lower OCR rate compared to untreated myotubes. This signifies that ROCK inhibition decreases oxidative phosphorylation. Thus, ROCK2 mediates skeletal muscle fatty acid oxidation.

Experiment 6.2 Mito Stress Test in Primary Myofibers

Introduction: Experiment 6.1 demonstrated ROCK mediates oxidative phosphorylation. However, it is unknown if ROCK2 alone can regulate skeletal muscle oxidative phosphorylation. To investigate this, we isolated primary myofibers from MYO R2KO and Control mice and subjected primary myofibers to a mito stress test. We hypothesized that MYO R2KO primary myofiber have lower OCR compared to Control primary myofiber.

Method: Primary myofibers are isolated as described in Experiment 5.2. Primary myofibers were plated on Matrigel coated XF24 cell culture plate and allowed to settle overnight. The following day I conducted the mito stress test as described in Experiment 6.1.

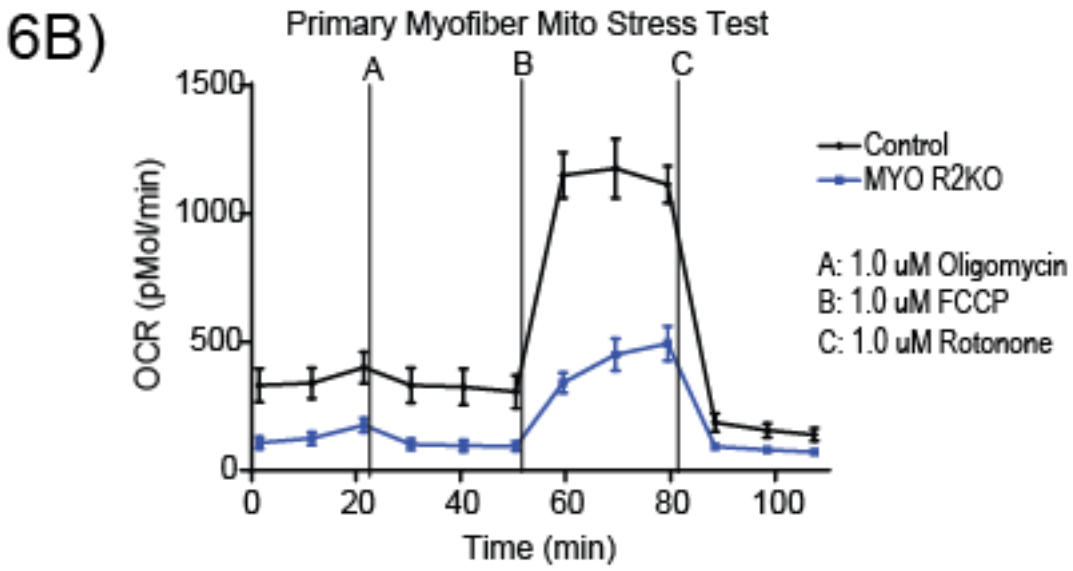


Figure 6B ROCK2 deficiency reduce oxidative phosphorylation

Mitochondrial Stress Test was conducted on primary myofibers. N=4 per treatment group. OCR was measured using XF24 Seahorse Instrument (Agilent). Oligomycin, FCCP and Rotonone were injected at indicated time. Values are mean OCR ± standard error mean. Significance was calculated from paired Student T-Test.

Results: We observe that MYO R2KO primary myofiber exhibited lower OCR compared to Control. This suggest that ROCK2 alone is sufficient to mediate skeletal muscle fatty acid oxidation.

Experiment 6.3 Oil Red O Stain of HFD-fed Adipocyte

Introduction: We wanted to use another method to validate fatty acid oxidation is decreased in ROCK2 deficient skeletal muscle. When skeletal muscle fatty acid oxidation is defective, lipid accumulates intramuscularly[112, 194]. Therefore, I measured intramuscular lipid accumulation through Oil Red O staining. We observed that MYO R2KO had an obese phenotype on a HFD and we tested the hypothesis that GC slices from MYO R2KO mice would have increased Oil Red O staining compared to GC slices from control mice.

Method: Oil Red O staining were conducted as previously describe [195]. Briefly GC muscle was extracted from HFD fed mice at Week 26, after 18 weeks of HFD. GC muscle was harvested and snap frozen in liquid nitrogen. Later muscles were sliced at 8 uM thickness in cryostat (Leica). Slides were then stained with Oil Red O. Images were captured on BX41 (Olympus).

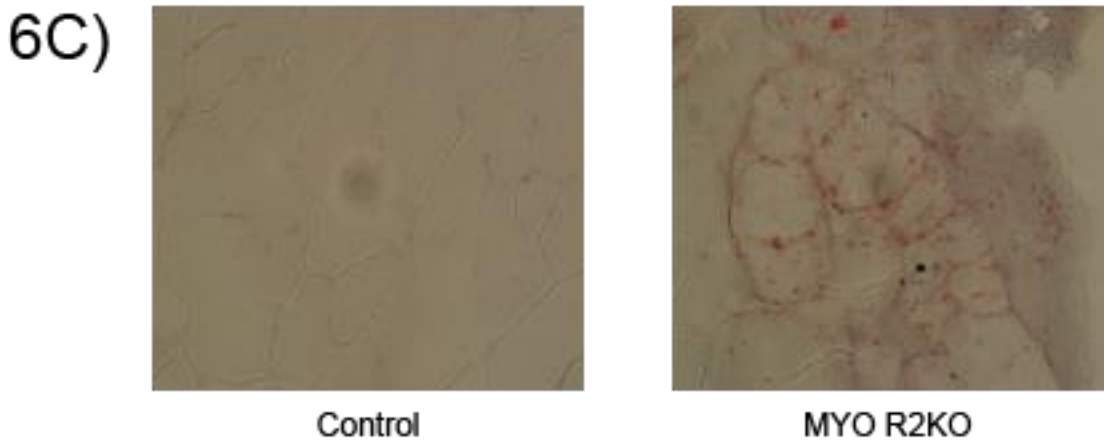


Figure 6.3 MYO R2KO mice have higher intramuscular lipid accumulation

HFD-fed male mice were fed normal chow from birth to Week 8, after they were fed high-fat diet from Week 8 until sacrifice. At Week 26 mice were sacrifice and GC was harvested. GC slices were stained with Oil Red O. N=3 per genotype. Images were captured on BX41 (Olympus) at 60x magnification. Representative images are shown.

Results: There was increase Oil Red O stain in GC isolated from HFD-fed MYO R2KO mice compared to control. This suggest that fatty acid accumulated as a result of decreased fatty acid oxidation. This further validates my conclusion that ROCK2 regulates skeletal muscle fatty acid oxidation.

Summary: In this chapter, I investigated how ROCK2 impacts skeletal muscle energy utilization. As we discussed in Chapter 3, skeletal muscle relays on sugar and fat as its predominant energy sources. By using two different *in vitro* systems, I have demonstrated that skeletal muscle ROCK2 does not impact glycolysis. However, skeletal muscle ROCK2 is necessary for skeletal muscle fatty acid oxidation. In the next chapter, I will investigate how ROCK2 impacts subcellular and molecular functions.

Chapter 7: ROCK2's regulation of mitochondrial function

In this chapter, we explored how ROCK2 regulates mitochondrial function. In Chapter 6 we found that MYO R2KO *in vivo* phenotypes are the results of decreased fatty acid oxidation. There are many potential reasons behind a decrease in fatty acid oxidation such as defects in fatty acid transporters, changes in transcriptional regulation and defects in the mitochondrial function[186, 196, 197]. We choose to focus on the mitochondrial dysfunction due to the insights drawn from Seahorse experiments. We hypothesized that decreased fatty acid oxidation was due to dysfunctional mitochondrial. In this chapter we investigated ROCK2's effect on 1) mitochondrial function, 2) mitochondrial mass and 3) molecular mechanism underlaying mitochondrial changes. This chapter zooms in further and focuses on ROCK2 subcellular and molecular role.

Experiment 7.1 Mitochondrial Copy Number

Introduction: Mitochondria have its own unique genome which replicates when the mitochondrial undergoes biogenesis. Decreased mitochondrial copy number has been linked to multiple diseases including cancer, diabetes and aging[198-200]. We hypothesize that ROCK2 mediates mitochondrial function and thus MYO R2KO mice have decreased skeletal muscle DNA copy number compared to control mice.

Method: Mice were grown to Week 26 on a normal chow diet. Mice were sacrificed and quadricep muscles were harvested and snap froze in liquid nitrogen. Total DNA (both nuclear and mitochondrial) was extracted from quadricep muscle with DNA Mini Kit (Qiagen) according to

manufacturer's instruction. DNA concentration was quantified by plate reader (Tecan). RT-PCR was performed on Applied Biosystem instrument using a one-Step RT-qPCR kit from BioRad. Mitochondria genes were normalized to expression of nuclear gene 18S. Analysis was conducted in Prism (GraphPad).

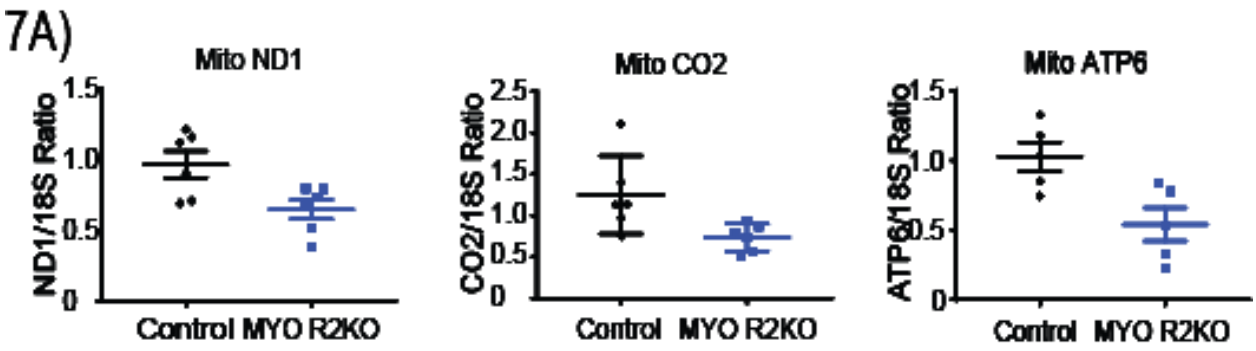


Figure 7A MYO R2KO mice have lower mitochondrial copy number

Mitochondrial DNA genome was extracted from quadricep of male Week 26 mice. N=6 per genotype. Expression of mitochondrial genes are normalized to nuclear gene 18S. Values represent mean normalized value ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: ND1 is a subunit of Complex 1. CO2 is a subunit of Complex 4. ATP6 is a subunit of ATP synthase. MYO R2KO had lower copy number in the three-mitochondrial gene. This indicates that the mitochondrial is dysfunctional in ROCK2 deficient mice and that ROCK2 mediates mitochondria function.

Experiment 7.2 Mitochondrial ROS Production

Introduction: In the previous experiment, we found MYO R2KO had lower mitochondrial copy number. Mitochondrial is one source of redox signaling. Increased ROS production has been associated with decreased mitochondrial copy number[201, 202]. Increased ROS production is also linked to multiple pathologies[202-204]. We hypothesized primary myofibers isolated from MYO R2KO have higher level of ROS production compared to control.

Method: Mice grew to Week 12 on normal chow diet. Mice were sacrificed and primary myofibers were isolated as described in Experiment 5.2. Primary myofibers were isolated from MYO R2KO and Control mice and plated in 96-well plate. Myofibers were allowed to settle in plate for 1 hr in 37^{o}C incubator. Myofibers were treated with 250 nM MitoSox for 15 minutes in 37 oC incubators. After they were washed with PBS (Gibco). Fluorescence was read with plate reader (Tecan) at an Ex/Em of 510/580 nM. Data was analyzed in Prism (GraphPad).

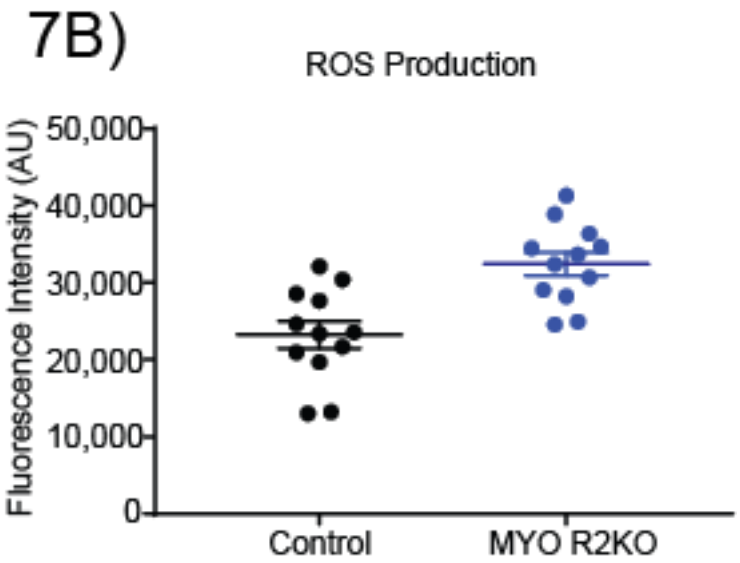


Figure 7B ROCK2 deficiency increases mitochondrial ROS production

Mitochondrial ROS production was measured through MitoSox fluorescence. Primary myofibers were isolated from week 14 normal chow fed male mice. N=12 per genotype. Values represent mean fluorescence ± standard error mean. Significance was calculated through unpaired Student T-Test.

Results: Primary myofibers isolated from MYO R2KO mice produced more ROS compared to Control myofibers. This indicates that there MYO R2KO have dysfunctional skeletal muscle mitochondrial and that ROCK2 mediates skeletal muscle mitochondrial function.

Experiment 7.3 Mitochondrial Membrane Potential

Introduction: Another method to measure mitochondrial function is through measuring membrane potential. Mitochondria have a gradient that is comprised of both electron gradient and pH gradient. Functional mitochondrial are negatively charged in the matrix. The presence of this gradient is necessary to drive ATP Synthase and produce ATP. Tetramethylrhodamine ethyl ester (TMRE) is a positive-charge stain that accumulates only in correctly potentiated mitochondrial. Therefore, a decrease in TMRE fluorescence indicates a decrease amount of active mitochondria[205]. We hypothesize that ROCK inhibited C2C12 myotubes will have decreased membrane potential compared to untreated myotubes.

Method: C2C12 were seeded and differentiated in 96 well plate for 6 days as described in experiment 5.1. During the last 24 hours of differentiation, cells were treated with 10 μM HF. Several wells were also treated with 20 μM FCCP for 15 minutes as negative control. Myotubes were then stained with 200 nM TMRE for 20 min at 37^{o}C. Myotubes were washed PBS (Gibco) and fluorescence read with plate reader (Tecan) at an Ex/Em of 549/575 nM. Data was analyzed by Prism (GraphPad).

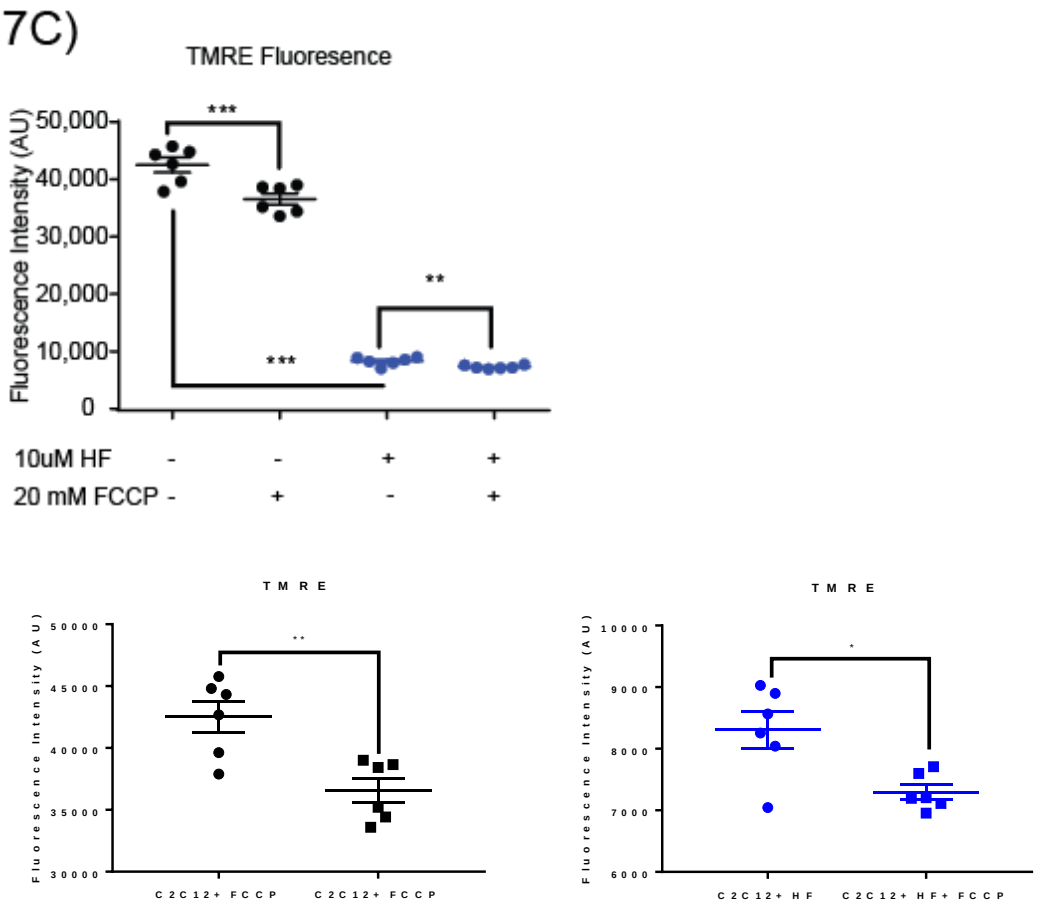


Figure 7C ROCK inhibition decreases membrane potential

Mitochondrial membrane potential was measured through TMRE staining on C2C12 myotubes. N=6 per treatment. Values represent mean fluorescence ± standard error mean. Significance was calculated through unpaired Student T-Test. * indicate $p<0.05$. ** indicate $P<0.01$. *** indicate $P<0.001$.

Results: ROCK inhibited myotubes exhibited lower membrane potential compared to untreated myotubes. This suggest that ROCK2 is an important mediator of mitochondrial membrane potential and ROCK2 deficiency cause mitochondrial dysfunction.

Experiment 7.4 ATP Production

Introduction: Mitochondria's classic role is to produce ATP. This is especially important in the skeletal muscle where ATP demand increases 100 folds in maximum contraction [136]. Due to previous experimental results, we hypothesize that ATP production will be lower in ROCK inhibited myotubes compared to untreated myotubes.

Method: C2C12 cells were seeded and differentiated in a 96 well plate for 6 days. During the last 24 hours of differentiation several wells were treated with 10 μM HF. 30 minutes prior to assay, some wells were treated with 2.5 μM FCCP in 37^{o}C incubator to serve as negative control. Assay then proceeded per manufacturer's instruction (Perkin Elmer). Media was partly removed so that 100 μL of media remained in the cell. Mammalian cell lysis buffer was added to the plate and the plate was shaken for 5 minutes. Substrate solution were added next and the plates were shaken for 5 minutes. Plate was then transferred to the plate reader and allowed to dark-adapt for 10 minutes. Luminosity was then read by the plate reader (Tecan). Data was analyzed by Prism (GraphPad).

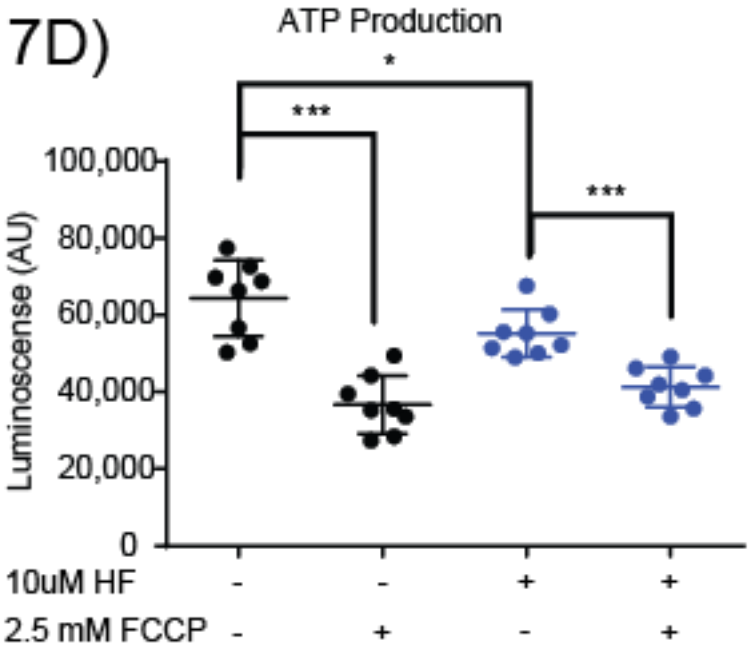


Figure 7D ROCK inhibition decrease ATP production

ATP production was quantified per Perkin Elmer's ATP Luminescence Kit instructions. Experiment was conducted with C2C12 myotubes. N=8 per treatment. Values represent mean luminosity ± standard error mean. Significance was calculated through unpaired Student T-Test. * indicate $p<0.05$. ** indicate $P<0.01$. *** indicate $P<0.001$.

Results: ATP luminance assay demonstrated ROCK inhibited myotubes produced less ATP compared to untreated myotubes. These results indicate that ROCK2 mediates mitochondrial function.

Experiment 8.1 Mitochondrial mass by mitotracker green

Introduction: The previous set of experiments demonstrates that ROCK2 deficiency leads to mitochondrial dysfunction. At this point we wanted to understand how ROCK2 was mediating mitochondrial dysfunction. We initially hypothesized that ROCK2 might mediate mitochondrial mass and MYO R2KO had lower mitochondrial mass compared to control. A decline in mitochondrial mass could explain the dysfunction. We seek
measured mitochondria mass by quantifying mitotracker green fluorescence. Mitotracker green is a molecular probe that labels mitochondria regardless of membrane potential. We quantified mitochondrial mass through flow cytometry. We hypothesized ROCK inhibited C2C12 myotubes would have lower amount of mitotracker green stain.

Method: C2C12 were seeded in 6 well plates and differentiated for 6 days. Some cells were treated with 10 μM HF for the last 24 hours. On the day of the assay, some wells were treated with 2.5 mM FCCP for 30 minutes. Myotubes were then stained with 100 nM of mitotracker green for 30 minutes. Cells were suspended in 1 % BSA in PBS and fluorescence was analyzed by LSR 3-8 (BD). Data was exported from FACSDIVA software and analyzed by FlowJo and Prism (GraphPad).

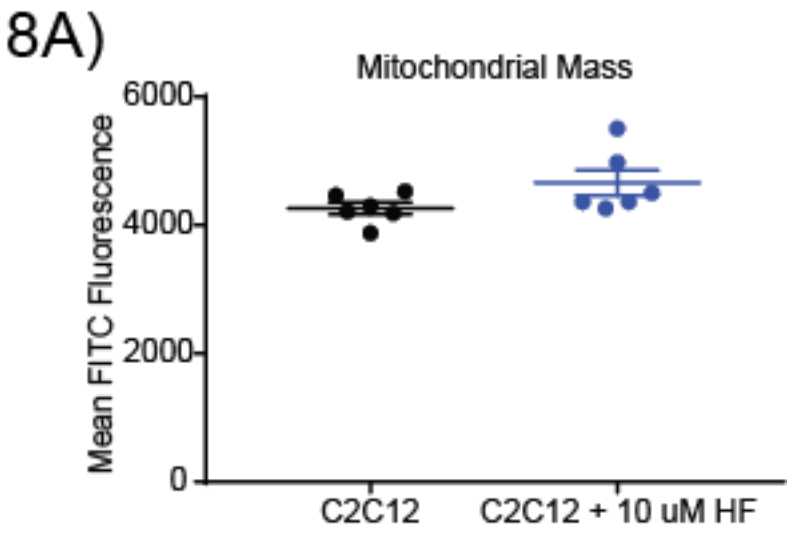


Figure 8A ROCK inhibition does not change mitochondrial mass

Mitotracker Green fluorescence was measured in C2C12 myotubes. N=6 per treatment. Fluorescence was quantified by flow cytometry. Values represent Significance was calculated through unpaired Student T-Test.

Results: Surprisingly, there was no difference in mitotracker green fluorescence between ROCK inhibited and untreated myotubes. This suggest that ROCK2 is not regulating mitochondrial mass in skeletal muscle. Therefore, differences in mitochondrial mass is not the cause of the mitochondrial dysfunction.

Experiment 8.2 ETC Complex Protein Expressions

Introduction: In light of Experiment 8.1 we wanted to confirm the lack of change in mitochondrial mass with an additional method. Therefore, we blotted for ETC protein expression in GC harvested from MYO R2KO and control mice. We hypothesized there was no difference in ETC protein expression levels between MYO R2KO and Control.

Method: Male mice grew to week 14 on normal chow diet. GC were then harvest and snap froze muscles in liquid nitrogen. Mitochondrial protein was extracted per BioVision Mitochondria/Cytosol Fractionation kit instruction. Next, I added cytosol exaction buffer and incubate on ice for 10 minutes. Protein concentration was determined via Bradford assay and western blot proceeded as described in Experiment 1.1. Abcam Total OXPHOS Rodent WB Antibody Cocktail at a 1:1000 dilution. Images were analyzed by Image Lab (BioRad) and Prism (GraphPad).

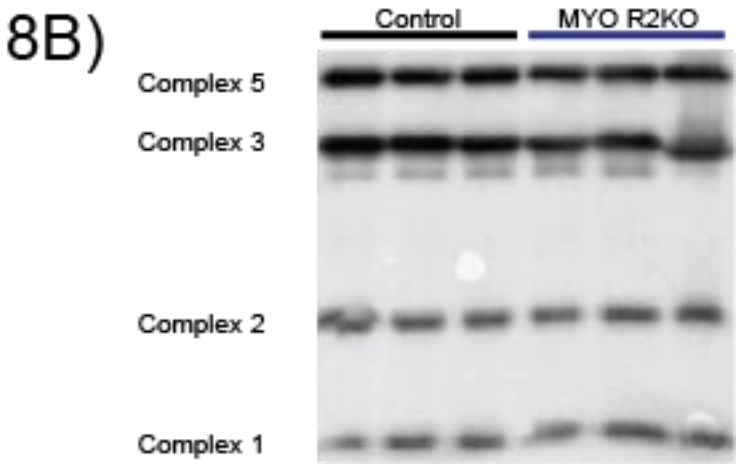


Figure 8B ROCK2 deficiency does not change ETC protein expression

Western blot of ETC complex protein using Abcam OXPHOS antibody cocktail. GC mitochondrial protein samples isolated from week 14 male mice. N=3 per genotype.

Results: There was no difference in ETC proteins expression between MYO R2KO and Control. This indicates there is no difference in mitochondrial mass between MYO R2KO and Control. This indicate that ROCK2 does not mediate mitochondrial mass.

Experiment 9.1 Mitochondrial Length

Introduction: Experiment 8.1 and experiment 8.2 demonstrated that ROCK2 does not mediate mitochondria mass. However, the cause of mitochondria dysfunction still needed to be elucidated. We dove back into the literature and found several studies that suggest ROCK mediates mitochondrial fission[151, 156, 157]. Fission occurs when the mitochondrial divides into 2 or more daughter mitochondrial and is necessary mitochondrial function. We hypothesized that MYO R2KO skeletal muscle mitochondria have less fission compared to Control and therefore MYO R2KO myofibers would have increased mitochondria length.

Method: Primary myofibers were isolated from NCD-fed male week 14 mice in the same manner described in Experiment 5.2. Primary myofibers were plated in Matrigel coated chamber slides (Lab Tek) and allowed to settle overnight in 37^{o}C incubator. The next day, myofibers were stained with 200 nM Mitotracker Deep Red for 30 min in 37 ^{o}C. Myofibers were washed with PBS (Gibco) and fixed with 4% paraformaldehyde (Sigma Aldrich) and covered with cover slip. Image were captured on an DSU spinning disk confocal microscope (Olympus Corporation). Data was analyzed using ImageJ (NIH) and Prism (GraphPad) software.

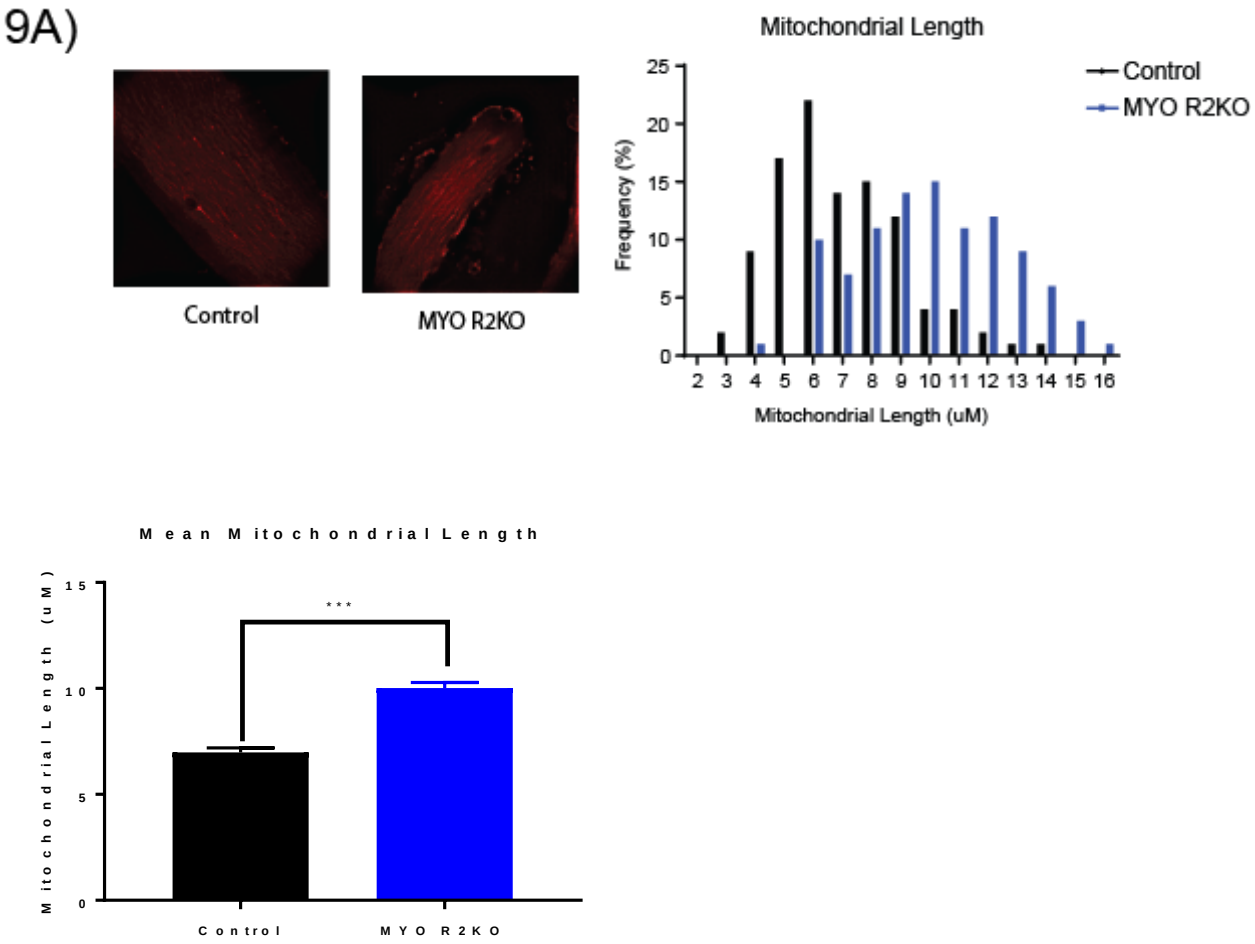


Figure 9A ROCK2 deficient myofiber have increased mitochondrial length

Primary myofibers isolated from Week 14 NCD-fed mice. N=5 per genotype. Over 100 mitochondria length measured per genotype. Images were captured on DSU spinning confocal microscope (Olympus) at 100x magnification. Representative images are shown. Significance was calculated through unpaired Student T-Test. *** indicates P<0.001.

Results: MYO R2KO mice had increase in mitochondrial length in primary myofibers compared to Control. This suggest that there is a defect in mitochondrial dynamics and ROCK2 could potentially mediate fission.

Experiment 9.2 Mitochondrial DRP-1 Expression

Introduction: Wang et. al. demonstrated ROCK1 increases mitochondrial fission in podocytes[151]. We wanted to test if ROCK2 has a similar effect on mitochondrial fission and if so if this effect was true in the skeletal muscle. The molecular basis of mitochondrial fission is still unclear but it is known that DRP-1 is an important regulatory. DRP-1 is a cytoplasmic protein that translocate to the mitochondria when fission is occurring. DRP-1 oligomerize and form a ring around mitochondria that constricts and drives the division of the mitochondria[150, 154]. To test if ROCK2 mediates mitochondrial fission, we isolated mitochondrial protein and blotted for DRP-1. We hypothesize that MYO R2KO have decreased mitochondrial fission and therefore MYO R2KO will have lower amount of mitochondrial DRP-1 compared to Control.

Method: GC mitochondrial protein samples extracted in Experiment 8.2 were used for this experiment. Western blot was completed using the protocol described in Experiment 1.1. DRP1 (Cell Signaling Technology) and TOM20 (Santa Cruz Biotechnology) were used for primary antibodies, both at 1:1000 dilution. Images were analyzed by Image Lab (BioRad) and Prism (GraphPad).

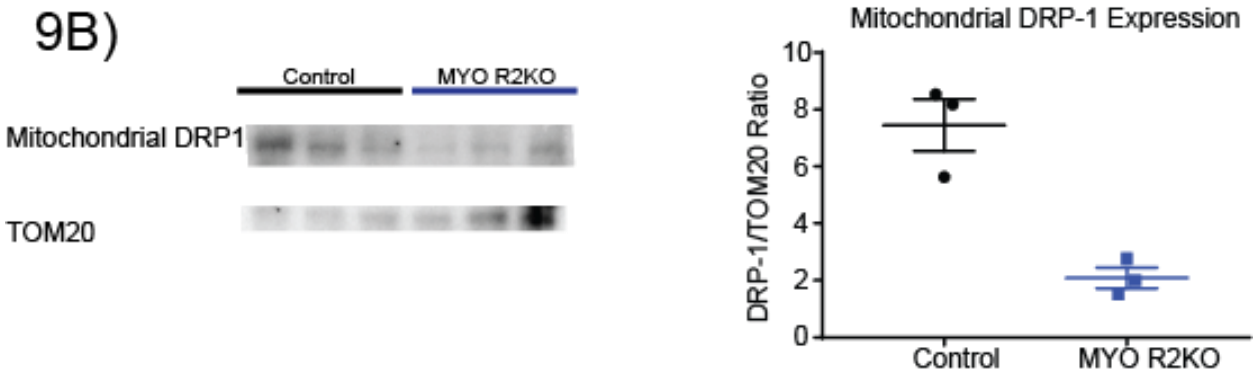


Figure 9B MYO R2KO mice have decreased mitochondrial DRP-1

Mitochondrial DRP1 was measured through western blot. Mitochondrial protein was isolated from GC harvested from Week 14 male mice. N=3 per genotype. DRP1 expression was normalized to mitochondrial housekeeping protein TOM20. Significance was calculated by unpaired Student-Test. Values represent mean ± standard error mean.

Results: MYO R2KO had lower expression of mitochondrial DRP-1 compared to Control. This suggest that ROCK2 is mediating mitochondrial fission and inhibition of fission is causing mitochondrial dysfunction in MYO R2KO.

Experiment 9.3 Phosphorylated DRP-1 Expression

Introduction: Wang et. al. showed that ROCK1 directly phosphorylate Serine 600[151]. Therefore, we wanted to test if skeletal muscle ROCK2 has the ability to phosphorylates Serine600. We hypothesized that ROCK2 was directly phosphorylating Serine 600 and that was the signaling mechanisms behind ROCK2 effect on mitochondrial fission.

Method: GC mitochondrial samples isolated in Experiment 8.2 was used for this experiment for the Phospho-DRP1 blot. Whole GC muscle lysate was used for the total DRP1 blot. Whole muscle lysate was extracted in the following manner. Male mice were grown to week 14 on normal chow diet. GC muscles were harvested and snap frozen in liquid nitrogen. 30 mg of GC was submerged in in muscle lysis buffer (20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 % Triton X-100, 10 % (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol) and homogenized for 1 minute. Samples were centrifuged at 14K rpm for 10 min in 4oC and supernatant were transferred to new tube. Western blot was completed using as described Experiment 1.1. For primary antibodies I used Phospho-DRP1 (Ser637) (Cell Signaling Technology) and total DRP1 (Cell Signaling Technology) at 1:1000 dilution. Images were analyzed by Image Lab (BioRad) and Prism (GraphPad).

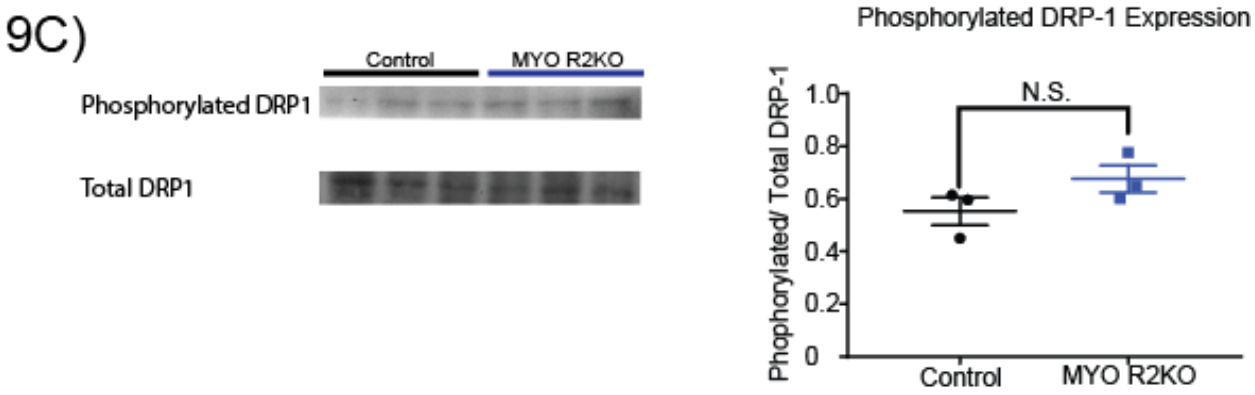


Figure 9C ROCK2 does not phosphorylate DRP-1 at Serine600

Phosphorylated DRP1 was measured by western blot. Mitochondrial protein was used to measure phosphorylated DRP1. Whole muscle lysate was used to measure Total DRP1. Proteins were extracted from week 14 male GC. N=3 per genotype. Phosphorylated DRP1 expression was normalized to total DRP1. Significance was calculated by unpaired Student- Test. Values represent mean ± standard error mean.

Results: Surprisingly, there was no changes in phosphorylated DRP-1 expression in between MYO R2KO and control. This suggest that ROCK2 is not phosphorylating DRP-1 at Serine 600. This result indicate ROCK2 was not regulating mitochondrial fission by phosphorylating Serine 600 but rather through a different molecular language.

Experiment 9.4 F-Actin Immunofluorescent Stain

Introduction: Previous studies suggest F-actin is necessary for fission to occur[152-154, 206]. This was highly relevant due as ROCK2 was historically studied for function in actin cytoskeleton. ROCK activity promotes F-Actin formation[11, 12, 14]. Therefore, we hypothesized that ROCK2 inhibition decreases F-Actin formation and this was the mechanisms that drives the decline in mitochondrial fission.

Method: Primary myofibers were isolated as described in Experiment 5.2 from week 12 normal chow fed mice. Myofibers were settled overnight onto Matrigel-coated chamber slides (Lab Tek) in 37^{o}C. The next day, myofibers were fixed with 4% formaldehyde (Sigma Aldrich) and permeabilized with 1% Triton X-100 (Sigma Aldrich) for 5 minutes. Samples were washed with PBS (Gibco) and stained with 1:1000 dilution of phalloidin (Thermo Fisher) for 30 minutes. Slides were covered with coverslip and images were captured on DSU spinning disk confocal microscope (Olympus Corporation). Images were analyzed by ImageJ (NIH) and Prism (GraphPad).

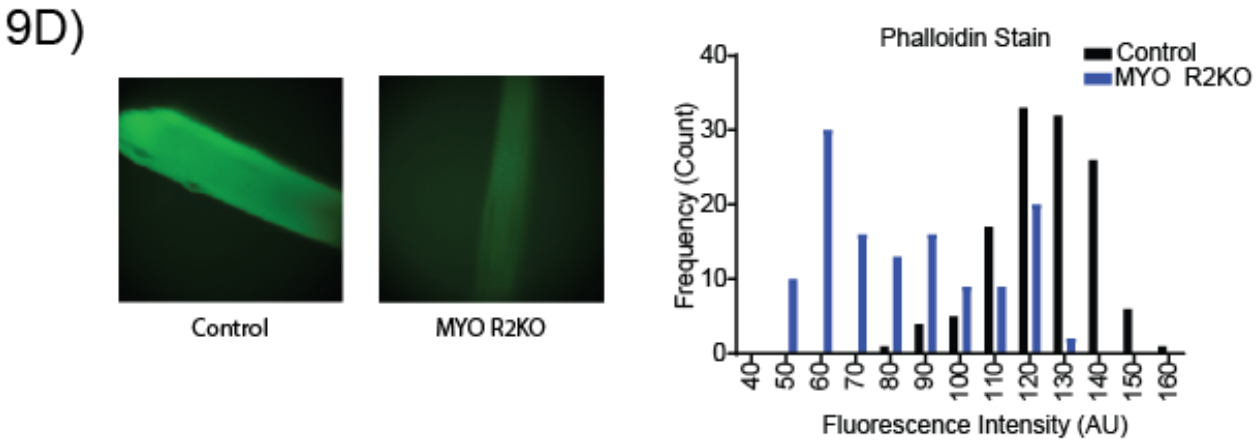


Figure 9D ROCK2 deficient myofibers have lower F-actin formation

F-Actin was measured by phalloidin staining primary myofibers. Primary myofibers were isolated from Week 14 male mice. Over 100 F-actin filaments were measured per genotype. Images were captured on DSU spinning disk confocal microscope (Olympus) at 100x magnification. Representative images are shown.

Results: There was less F-Actin fluorescence, as measured by phalloidin staining, in primary myofibers isolated from MYO R2KO compared to Control. This suggests that the decrease in mitochondrial fission could be caused by lower F-actin formation. Thus, ROCK2 deficient skeletal muscle have less F-actin which results in a reduction in mitochondrial fission which leads to mitochondrial dysfunction.

Summary: This chapter focused on how skeletal muscle ROCK2 deficiency affects the mitochondria. We wanted to study mitochondrial function as we believed that mitochondria dysfunction underlies decreased fatty acid oxidation observed in Chapter 6. We found that the mitochondrial is dysfunctional and ROCK2 deficient myofibers had lower mitochondrial copy number and ROS production. Additionally, ROCK inhibited C2C12 myotubes had decreased membrane potential and ATP production. Surprisingly, there was no difference in mitochondrial mass between MYO R2KO and Control animals. Thus, mitochondrial dysfunction could not be explained by changes in mitochondrial mass. We found that MYO R2KO had less mitochondrial fission and this reduction may be attributed by a decline in F-actin formation. In the next chapter, we will report on an earlier side project featuring a skeletal muscle specific constitutively active ROCK mice strain.

Chapter 8: CA-ROCK, the other mouse strain

This chapter presents our preliminary work on phenotyping MYO CAROCK. When we started this project, there were originally two novel genetic mice strains I was tasked with phenotyping, MYO CAROCK and MYO R2KO. As the project progressed, we focused on MYO R2KO based on thesis committee's advice. MYO R2KO was chosen as it was a more specific genetic model and an easier mouse strain to model *in vitro*. In this chapter, we will describe MYO CAROCK metabolic phenotype.

Creation of Novel Skeletal Muscle-Specific Constitutively Active Mice

The Liao lab created a novel skeletal muscle-specific constitutively active ROCK mice strain named MYO CAROCK. MYO CAROCK was created by crossing a myogenin-Cre mice with a constitutive-active human ROCK-floxed mice (CA-ROCK $^{flox/flox}$). As previously stated myogenin-Cre is a transgenic mouse strain where Cre recombinase is driven by the myogenin gene [166]. caROCK flox/flox was generously provided by Farhad R. Danesh (Baylor College of Medicine). caROCK construction features an amino-terminal kinase domain of human ROCK1 under the control of ubiquitin-C promotor which was deactivated by loxP-Stop-loxP cassette inserted into murine ubiquitous ROSA26 locus. Because the ROCK1 expressed was shorten, CA-ROCK is expected to phosphorylate both ROCK1 and ROCK2 downstream target. MYO CAROCK over activates ROCK in only the skeletal muscle.

Experiment 10.1 MYO CAROCK Body Weight

Introduction: We measured the body weight of MYO CAROCK mice on both normal chow and HFD. This was the first step to took to determine the metabolic phenotype of MYO CAROCK. Previously, I observed that MYO R2KO had an increase in body weight. Therefore, we hypothesize overexpression of ROCK in MYO CAROCK would have the opposite effect and MYO CAROCK would have lower body weight compared to control.

Method: 43% HFD fed mice were fed normal chow up until week 8 and then fed the HFD from week 8 to the time of sacrifice (~Week 24), while control mice were fed chow throughout. HFD used is Teklad Diet 97268. Body weight was measured on a weekly basis from age week 8 to week 20. Data was analyzed by Prism (GraphPad).

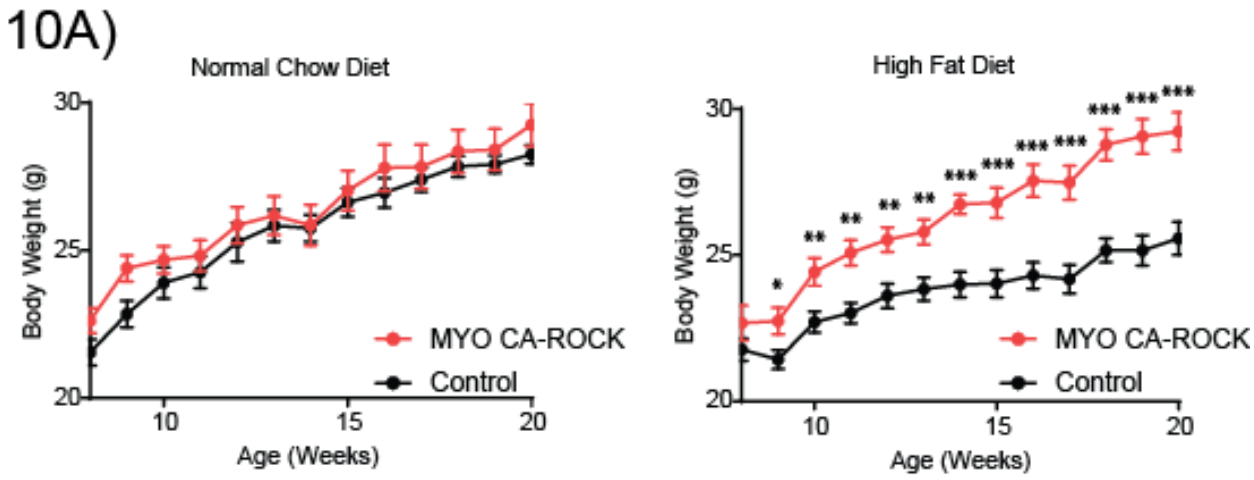


Figure 10A MYO CAROCK have increased body weight on HFD

Body weight were measured weekly for both normal chow fed and high fat diet fed mice. Normal chow mice were fed normal chow for their entire life. N=6 per genotype. High fat diet fed mice were fed normal chow from birth to week 8, after they were fed 43% HFD until sacrifice. N=9 for MYO CAROCK. N=13 for Control mice. Values represent mean weight ± standard error mean. Significance was calculated through unpaired Student T-Test. * indicates $p<0.05$. ** indicates $p<0.01$. *** indicates $p<0.001$.

Results: Surprisingly, I found that MYO CAROCK had an increased body weight on Normal chow compared to control. This difference in weight was statistically significant ($P < 0.001$) as measured by the Paired Student T-Test. MYO CAROCK had an even larger weight gain a 43% HFD diet. This study indicates that there are metabolic disturbances when ROCK is constitutively active in skeletal muscle.

Experiments 10.2 MYO CAROCK Body Composition

Introduction: Next, we determined the cause of the change in body weight. To that end we measured body composition via DEXA at DRTC in the University of Chicago. We hypothesized that the increase in body weight was due to an increase in adiposity for both groups of mice.

Method: Normal chow fed mice were fed normal chow their entire life. HFD fed mice were fed a normal chow diet from birth until week 8. Afterwards they were placed on a 43% HFD for starting from week 8 until sacrifice. At week 22, body composition was measured at the DRTC at the University of Chicago. Data was analyzed by Prism (GraphPad).

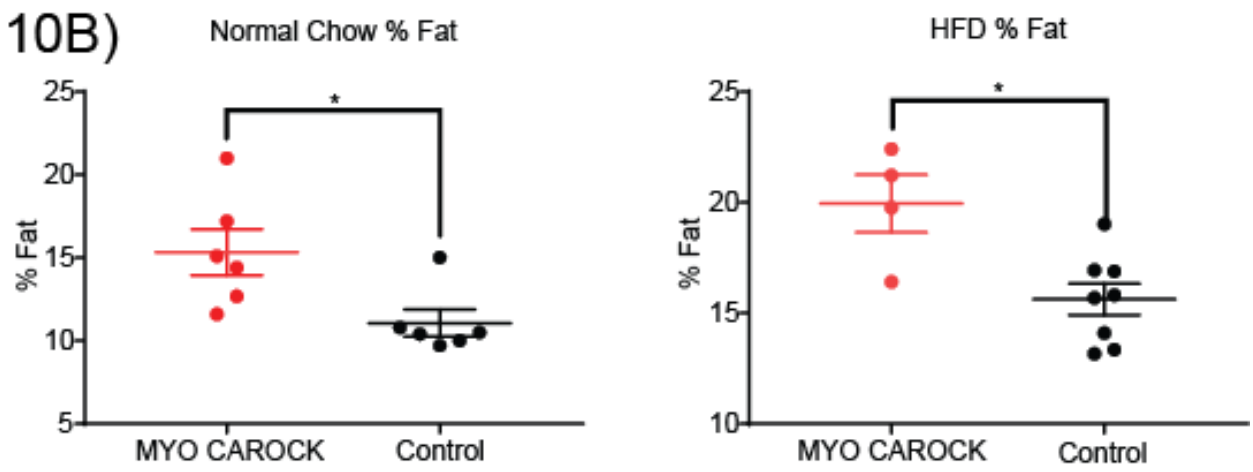


Figure 10B MYO CAROCK mice have increase adiposity

DEXA was measured by Dr. Hong at the Diabetes Research Training Center (DRTC) at the University of Chicago. Only male mice were used. Normal chow-fed mice were measured at Week 14. N=6 per genotype. HFD-fed mice were measured at Week 22 of age. N=5 for MYO R2KO and N=8 for Control mice. Values are means ± standard error mean. Significance was calculated through unpaired Student T-Test. * indicate p-value<0.05.

Results: MYO CAROCK had an increase in % Total Fat on both normal chow and HFD compared to control. These results also indicate that the increase in body weight comes from an increase in adiposity. ROCK overactivation leads to obesity

Experiment 10.3 MYO CAROCK GTT

Introduction: Previous studies demonstrate that patients with metabolic syndrome display increase ROCK activity in their leukocytes[52]. Similarly, obesity is a risk factor for diabetes[207-209]. Therefore, I hypothesize that MYO CAROCK have decreased glucose sensitivity compared to control.

Method: Normal Chow GTT was conducted on male mice that were 12 weeks old. These mice have been fed a normal chow diet for their entire life. Normal Chow GTT were conducted at the University of Chicago while the HFD GTT was conducted by the University of Pennsylvania Metabolic Core. HFD animals were fed normal chow for the first 8 weeks of their lives and then fed a 43% HFD from Week 8 until time of sacrifice. HFD GTT were administer on week 26 male mice. Mice were fasted 6 hours prior to the test. Mice were then injected intraperitoneally with 1g/ 1U dose of 20% Dextrose (Sigma Aldrich). Blood glucose was measured pre-injection (0 min), 15, 30, 60 and 120-min post-injection with Freestyle Lite Glucose Monitor (Abbott). Data was analyzed by Prism (GraphPad).

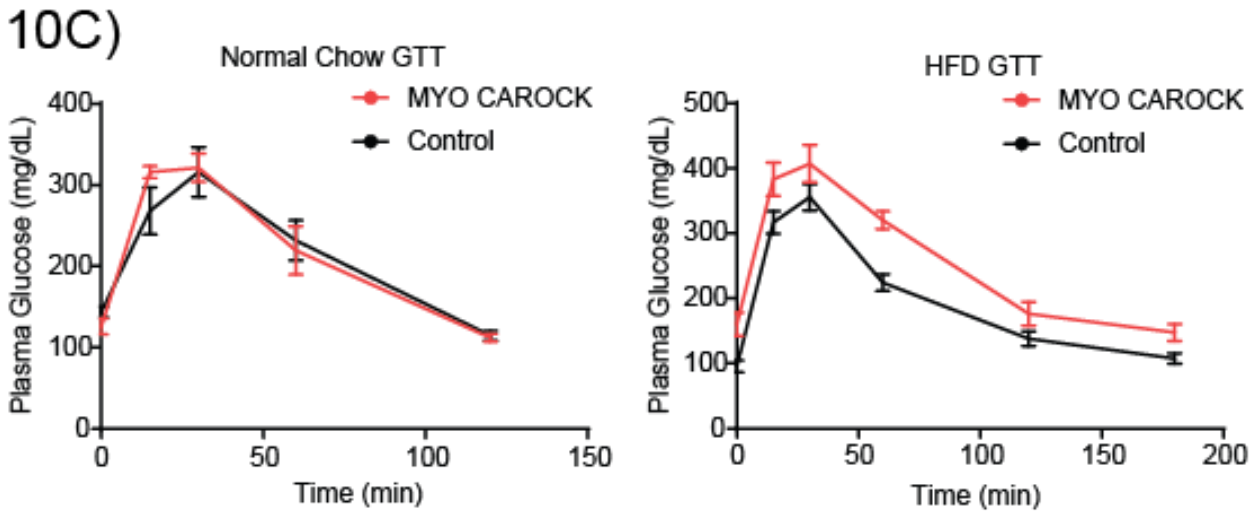


Figure 10C MYO CAROCK have decreased glucose sensitivity on HFD

Glucose Tolerance Test (GTT) was administered at Week 12 for Normal Chow-fed male mice. N=4 per genotype. GTT was administered at Week 24 for HFD-fed male mice. N=4 for MYO CAROCK and N=6 for Control mice. Values are means ± standard error mean. Significance was calculated for area under the curve through unpaired Student T-Test.

Results: There is no difference in GTT on for normal chow fed mice. However, HFD-fed MYO CAROCK demonstrated decreased glucose sensitivity. This suggests that skeletal muscle ROCK overactivation mediates glucose homeostasis. It is important to contrast these results with Experiment 4.2 results. The fact that there is decreased insulin sensitivity when ROCK is overactivated but there no improvement when ROCK2 is deficient suggests that there may be isoform-specific effects on glucose sensitivity. Potentially, ROCK1 is the isoform responsible for glucose signaling while ROCK2 affects mitochondrial function. Additional experiments are needed to validate this hypothesis.

Experiment 10.4 MYO CAROCK Insulin Tolerance Test (ITT)

Introduction: Glucose insensitivity can be caused by changes in insulin signaling. To test this ITT was performed on HFD-fed MYO CAROCK. Previous studies have indicated that ROCK mediate glucose signaling through insulin[50, 83, 84]. We hypothesize that MYO CAROCK animals would have decreased insulin sensitivity compared to control.

Method: ITT were administered on Week 26 HFD male mice at the University of Pennsylvania Metabolism Core. HFD-fed mice were initially fed normal chow from birth to week 8 but then were fed 43% HFD from Week 8 to time of sacrifice. On the day of the test, mice were fasted for 6 hours. Next, they were injected interperitoneally with insulin at a 1U/ 1 kg dose. Glucose was measured at pre-injection (0 min), 15, 30, 60 and 120 post injection. Data was analyzed with Prism (GraphPad).

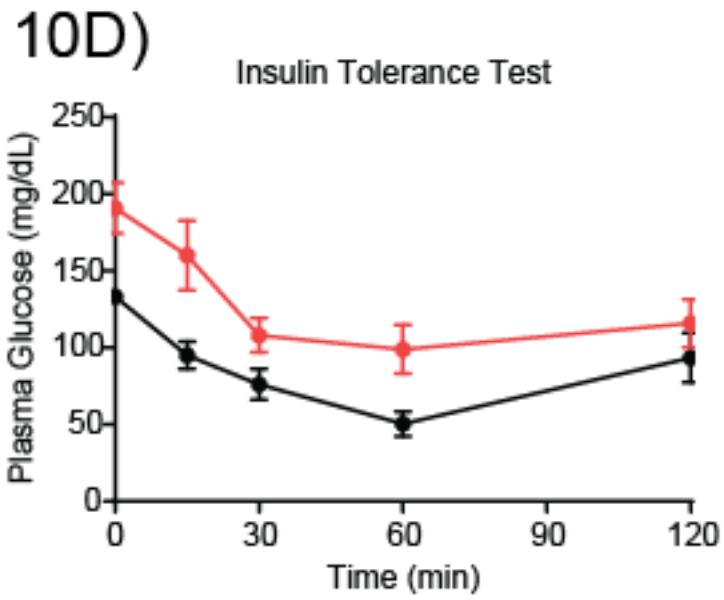


Figure 10D MYO CAROCK have poorer insulin sensitivity

Insulin Tolerance Test (ITT) was conducted on HFD-fed male mice. Animals were fed normal chow from birth to week 8 and then 43% HFD from week 8 until sacrifice. ITT was conducted on Week 24 mice. N=4 for MYO CAROCK and N=6 for Control. Values are means ± standard error mean. Significance was calculated through paired Student T-Test.

Results: MYO CAROCK animals are less insulin sensitive compared to Control. This suggests that ROCK plays a direct role in insulin signaling and this leads to changes in glucose homeostasis. Additionally, these results suggest that ROCK could be a therapeutic target to treating obesity and diabetes.

Summary: In this chapter, we summarized my studies on a novel *in vivo* model featuring constitutively active skeletal muscle ROCK. We found that MYO CAROCK mice had increased body weight which resulted from increased adiposity. MYO CAROCK also had poorer glucose sensitivity due to their decreased insulin sensitivity. These results serve as a stark contrast to the *in vivo* phenotype observed in MYO R2KO animals (as discussed in Chapter 5). We did not see a change in glucose homeostasis in MYO R2KO. Therefore, it is possible that ROCK1 and ROCK2 plays different role in skeletal muscle. ROCK1 can be more important for glucose signaling while ROCK2 is more vital for fatty acid oxidation. If this were true it could explain the conflicting results of observed in previous metabolic studies of ROCK and those studies used pan ROCK inhibitors[50-52, 77-84, 183].

Section C

Discussion

Chapter 9: Discussion

The goal of this dissertation project was to elucidate the physiological role of skeletal muscle ROCK2. We examined ROCK2's role *in vivo* and at the tissue, cellular and molecular level. We found that skeletal muscle ROCK2 deficiency leads to a decline in endurance performance and an increase in adiposity on a 43% HFD. Surprisingly, we did not observe any differences in strength performance. To account for the decline in endurance, we investigated whether skeletal muscle ROCK2 impacts skeletal muscle energy utilization. We found that ROCK2 does not regulate glycolysis but it can mediate fatty acid oxidation. Specifically, skeletal muscle ROCK2 deletion leads to lower fatty acid oxidation. To determine the cause of the decrease in fatty acid oxidation we investigated whether skeletal muscle ROCK2 deficient mice had more dysfunctional mitochondrial. Indeed, we found greater mitochondrial dysfunction and this dysfunction was associated with improper mitochondria dynamics. ROCK2 deficient skeletal muscle exhibited less mitochondrial fission potentially through ROCK2's role in regulating F-actin formation. In contrast to MYO R2KO, we also studied a skeletal muscle-specific constitutively active ROCK mice model. MYO CAROCK displayed an obesity phenotype on both NFD and HFD. However, we believe MYO CAROCK's obesity is caused by a different mechanism than what was observed with MYO R2KO's obesity. MYO CAROCK have significant decline in glucose and insulin sensitivity compared to Control, suggesting that obesity is caused by changes to glucose metabolism. In contrast, MYO R2KO obesity phenotype appear to be caused by dysfunctional fatty acid oxidation. These results suggest that skeletal muscle ROCK1 and ROCK2 may play distinct roles in skeletal muscle physiology. Thus, this dissertation project asserts that more precise, isoform-specific and tissue-specific tools should be used in future studies on ROCK.

Albert Einstein once said “the important thing is to never stop questioning.” While the dissertation has answered the original question of “What is ROCK2 physiological role in the skeletal muscle?” across multiple levels of analysis, our results gathered opens up even more questions and areas to explore. In this discussion, we will focus on three potential questions, 1) Does skeletal muscle ROCK2 directly signal adipocytes? 2) How does gender factor into skeletal muscle ROCK2 physiological effect? 3) How can we translate the dissertation project mouse results into human studies? We will discuss the rationale behind each question and potential experiments that could be executed to answer these questions.

1)Does skeletal muscle ROCK2 directly signal adipocytes?

One unexplored topic of research is to determine whether skeletal muscle ROCK2 directly signals adipocyte and thus participate in skeletal muscle-adipose cross talk. Experiment 3.4 demonstrated that MYO R2KO mice had adipocytes with larger cell size compared to adipocytes in Control mice. We attributed the increased cell size to decreased skeletal muscle fatty acid oxidation. However, an alternative explanation is possible. It is possible ROCK2-deficient skeletal muscle produces a hormone (also known as myokine) that could signal other non-skeletal muscle tissues. Thus, the increase in adipocyte could be caused by indirect hormonal signaling rather than a secondary effect of decreased skeletal muscle energy utilization. There is a need to study skeletal muscle-adipose cross talk due to the obesity epidemic in the United States[210]. Identifying potential molecules that could mimic exercise in a therapeutic form would have the potential to help millions of patients.

Examples of hormones that effect skeletal muscle-adipose cross talk have been established in the literature. Exercise is known to induce skeletal muscle to secrete IL-6. Increased circulatory IL-6 levels has been associated with increase fatty acid oxidation and glucose sensitivity[211-213]. Similarly, mRNA levels of IL-15 increase post-exercise. IL-15 treatment in rats have been shown to reduce white adipose tissue mass and decrease circulating triacylglycerol levels[214, 215]. Perhaps, the most well-known myokine is the hormone Irisin. Irisin is secreted from skeletal muscle post-exercise and promote white adipocyte beiging [216]. However, the wide application of Irisin is still under debate as Irisin has been found to be only upregulated active elderly subjects[217]. By determining whether skeletal muscle ROCK2 affect adipocytes via a myokine, we would be increasing the basic knowledge of myokines in general.

We propose three separate experiments that will determine if the adipocyte increased size is a result of decreased skeletal muscle fatty acid oxidation or effects of myokine. The first experiment would be to measure circulating fatty acid oxidation. We would collect serum from age-matched MYO R2KO and Control mice on both NCD and HFD. We could measure free fatty acid by using a commercial kit such as Abcam's Free Fatty Acid Assay Kit which detects long-chained free fatty acid. We would measure free fatty acid in both NCD and HFD mice because obesity was only observed in MYO R2KO mice when they were fed a 43% HFD. Therefore, it is possible that mice would need to be stressed on HFD before significant changes are observed in plasma free fatty acid concentration. We hypothesize that increased adiposity is due to a decrease in skeletal muscle fatty acid oxidation and not due to effects of myokines. Thus, we would expect that there is higher concentration of free fatty acid in HFD-fed MYO R2KO compared to HFD-fed Control. However, a lack of difference in free fatty acid levels between HFD-fed MYO R2KO

and HFD-fed Control mice would suggest that the increase in adipocyte cell size could be caused by myokine secretion.

To test if myokine secretion affected adipose cell size, we could conduct a condition media experiment. We could grow NCD and HFD-fed MYO R2KO and Control mice up to adulthood (~Week 12). We would then isolate primary myofibers from the mice and plate the myofibers in cell culture plates for 24 hours. We would collect the media after 24 hours of plating and apply the condition media wells of mature adipocytes. After 6 hours we could fix the adipocytes and image the cells with a microscope. Next, we would measure the adipocyte cell use using ImageJ as we did in Experiment 3.4. Potentially, there could be no difference in adipocyte size between adipocytes that have been treated with MYO R2KO condition media and adipocyte that have been treated with Control condition media. This would indicate that ROCK2 deficient myofibers are not secreting hormones that affects adipocyte cell size. However, if adipocyte treated with MYO R2KO condition media are larger in size that would suggestion MYO R2KO may be secreting myokines that can target adipocytes. We would then try to identify the myokine that is causing the size change.

One method we could use to identify the hormone would be to use commercial kit like the R&D System Proteome Profile Mouse Adipokine Kit. This kit can quantify the level of 38 proteins that are obesity related. We could collect the serum from NCD and HFD-fed MYO R2KO and control mice and apply the serum to the kit. If there is a change in expression levels of one of these 38 known proteins, we could proceed to molecular studies that will determine how ROCK2 regulates the protein. If there are no significant changes in these 38 proteins expression, but we observed a difference in condition media experiment we could try to identify the myokine through other methodologies such as proteomics, metabolomics and lipidomics.

Altogether these three experiments will be able to determine if the changes to adipocytes are due to skeletal muscle changes or myokine secretion. It is important to note that both options could be simultaneously true, that the adipocyte size increased is caused by both deficient skeletal muscle fatty acid oxidation and downstream effects of myokines. If both options were true, it would demonstrate skeletal muscle ROCK2 is a critical regulator of obesity as ROCK2 would be able to mediate adiposity through multiple mechanisms.

2) How does gender factor into skeletal muscle ROCK2 physiological effect?

A distinct approach is to investigate if and how gender can alter skeletal muscle ROCK2 physiological role. It is important to acknowledge that only male mice were used in *in vivo* experiments. Due to time and resource constraints, we limited experiments to male mice to reduce complexity and to obtain initial results. However, we planned on undertaking further studies to understand skeletal muscle ROCK2's effect on both gender. Therapeutically, the ROCK inhibitors that are already on the market are being used by both men and women. Clinical trials feature ROCK inhibitors are also being conducted on both genders. Additionally, NIH recently introduced a policy that requiring researchers to identify the effects of gender on experimental results. Accordingly, all future pre-clinical studies need to consider the impact of gender.

Previous rodent studies have already demonstrated that there are differences in ROCK inhibitor effect. As we mentioned in Chapter 2, there were gender differences in Fasudil treatment results. Takata et. al. treated an ALS mouse model with Fasudil and observed increased survival and improved motor performance. Because Takata did not specify gender, we assume these beneficial results are true in both males and females [92]. In contrast, when Gunther et. al. treated the same ALS mouse model with Fasudil improvement in motor performance only occurred in

the male mice [55]. This suggests that gender may bifurcate ROCK2 therapeutic effects. Thus, these studies demonstrate a need to stratify future ROCK clinical trial results by gender to determine if there are gender-specific phenotypes.

Similarly, gender is likely to modulate skeletal muscle ROCK's effect due to established differences in endurance performances between the two sexes. Endurance exercise relies heavily on fatty acid oxidation. Women athletes use more lipid and less carbohydrate compared to men during exercise[218, 219]. Similarly, endurance performance increases proportion of oxidative fibers. Women have greater proportion of oxidative fibers compared to men[220, 221]. Also, by definition endurance exercise refers to longer period of exercise. While men are faster at short distance runs, women are more fatigue-resistance and do better in longer distances races such as ultramarathon[222]. The predominant phenotype observed in our MYO R2KO mice was the decline in endurance performance. Therefore, we hypothesize that we would observe an even greater reduction in endurance performance in female MYO R2KO mice compared to male MYO R2KO.

Extending the results of this thesis, we propose two additional experiments to study how gender affects skeletal muscle ROCK2's physiological effect. First, we would determine whether gender changes endurance performance on a forced treadmill test. We would raise 4 groups of mice, male Control mice, female Control mice, male MYO R2KO and female MYO R2KO mice. We would follow these mice into adulthood (~Week 12) on NCD. Next, we would train these mice three separate times to acclimate them to the treadmill instrument. Afterwards, we would test the mice by forcing them to run to exhaustion with encouragement from compressed air cannister and measure total running time and total running distance. We hypothesize that both male and female MYO R2KO mice would run less compared to Control counterpart. Also, we

predict that female MYO R2KO mice would have lower running time and distance compared to male MYO R2KO. Alternatively, no change in in total running time and distance between male and female MYO R2KO would suggest that gender does not impact endurance performance.

Additionally, we could test whether gender affects *in vitro* skeletal muscle function. This is possible by executing a Mitochondrial Stress Test on primary myofibers isolated from male Control, female Control, male MYO R2KO and female MYO R2KO mice via the Seahorse instrument (Agilent). Specifically, we can test if gender impacts skeletal muscle substrate utilization. Similar to experiment 6.2 we would use OCR as an output fatty acid oxidation. We hypothesized that, similar to Experiment 6.2, both male and female MYO R2KO myofibers would have lower OCR compared to Control. Additionally, we hypothesize that female MYO R2KO myofibers will have lower OCR compared to male MYO R2KO myofibers. In contrast, no differences between male MYO R2KO and female MYO R2KO OCR would indicate that gender does not change skeletal muscle fatty acid oxidation.

It would be important to connect the *in vivo* and *in vitro* experiment. If female MYO R2KO animals displayed decreased endurance performance and lower fatty acid oxidation rate, we hypothesize that the decline in endurance performance is caused by defective substrate utilization. However, it is possible that the two experiments do not align. Female MYO R2KO may exhibit lower endurance performance compared to their male counterparts, at the same time there is no difference in oxidative phosphorylation between female and male myofibers. If this were to occur it would indicate that female MYO R2KO decline in endurance performance is mediated by changes that are independent of fatty acid oxidation. Potential alternative regulators of endurance performance include changes downstream of female hormones like estrogen and progesterone or lack of male hormone testosterone in female MYO R2KO mice. Additionally, there are inherent

differences in adipose biology between males and females that may play a role in endurance performance[223]. Determining if gender effects skeletal muscle ROCK2 physiological role will lead to exciting new directions of research.

3) How can we translate the dissertation project mouse results into human studies?

A purpose of biomedical research is to identify basic mechanisms that can ultimately be translated into the clinical setting in order to treat diseases and improve human health. As we mentioned in Chapter 2, there is already interest in using ROCK inhibitor clinically to treat a diverse range of diseases. While the dissertation project used mouse *in vivo* and i*n vitro* models, these rodent results have clinical implication.

The dissertation project highlights the need to develop more precise inhibitors. Current therapeutic ROCK small molecules can inhibit both isoforms of ROCK. The dissertation demonstrates ROCK has isoform-specific effect. Our research indicates ROCK1 and ROCK2 have separate metabolic roles. Overaction of ROCK1 changes glucose sensitivity while ROCK2 deficiency lowers fatty acid oxidation. Therefore, isoform-specific inhibitors to target signaling mechanisms more precisely. Additionally, isoform-specific inhibitors may ameliorate adverse side effects. While ROCK inhibitors are generally well tolerated, systematic ROCK inhibition have led to adverse effects such as abnormal hepatic function, intracranial hemorrhage and hypotension[224-226]. Also, current ROCK inhibitors have been shown inhibit other kinases. More precise inhibitors would limit non-ROCK effects.

The dissertation project also implies ROCK should only be inhibited for short durations of time. Currently, ROCK therapies are administered acutely. Fasudil is administered in an intravenously for only 14 days[68]. Ripasudil is administered locally via eye drop at low

dosages[69]. Our mouse studies further encourage short term administration of ROCK inhibitors. We showed that chronic inhibition, such as genetic ROCK2 deletion, will cause mitochondrial dysfunction. Mitochondrial dysfunction is extrapolated into whole body effect of lower motor performance and increase adiposity. Therefore, ROCK inhibition should be kept to acute duration to prevent mitochondrial dysfunction.

As we consider clinical implications of our studies, we should acknowledge that mouse models are limited tools to study skeletal muscles function. While biomedical researchers often use mouse models, most pre-clinical studies do not translate into clinical results as they are rarely reproducible. A recent meta-analysis found 0 out of 70 ALS mice studies were reproducible [227]. It is also difficult to translate therapies validated from mice models into humans due to the differences between mouse and human skeletal muscle. One such difference lies in human vs mouse satellite cells. Satellite cells are adult skeletal muscle stem cells. Bareja et. al. showed that mouse satellite cells have a different transcriptome compared to human satellite cells[228]. Satellite cells are responsible for skeletal muscle adaptation to exercise. Differences in human and mouse satellite cells thus could complicate translating mice exercise studies into humans. Importantly, human and mouse skeletal muscle have different fiber compositions. Humans have much higher proportion of oxidative fibers compared to mice[229]. Oxidative fibers are necessary for endurance exercise. Therefore, we hypothesize that if ROCK2 is critical for endurance exercise we would see larger effect sizes in humans compared to mice.

To start translating our mice results into humans, we propose three preliminary experiments that use human tissue. First, to determine if skeletal muscle ROCK2 is critical for human's endurance performance, we could measure ROCK2 expression levels in skeletal muscle biopsies of endurance trained (e.g. marathon runners) and untrained humans. We would collect muscle

biopsies of soleus muscles of male untrained, female untrained, male trained and female trained human subjects. By screening both men and women we are able to determine if there are any changes in ROCK2 expression between genders. We would try to limit complexity by eliminating individuals with pre-existing conditions and age-matching our samples. We will biopsy the soleus muscle as that is a predominant slow-twitch muscle. We could then assess ROCK2 expression though IHC staining of slices of biopsied tissue. We expect ROCK2 expression to be highest in the trained female, followed by the trained male followed by the untrained female and then the untrained male. If this were true it would indicate that ROCK2 is an important regulator of endurance performance in humans. Alternatively, it is possible that there will be no difference in ROCK2 expression level between the different groups of patients. This is possible because ROCK may be acting through its phosphorylation activity instead of its protein expression. If we do not see a change in ROCK2 protein expression level we could stain with phosphorylated target of ROCK2 such as vimentin. This experiment can be followed up by varying patient conditions such as age or training amount (5k vs marathon runners) and seeing if those conditions affect ROCK2 expression. Much remains to elucidated in molecular mechanism behind exercise physiology.

In addition to adaptation to exercise, it would be useful to determine if skeletal muscle ROCK2 expression is altered in pathological conditions. We found that skeletal muscle ROCK2 is necessary for mitochondria function in mouse. If this extrapolates into humans would expect to observe decreased ROCK2 expression in diseases that feature mitochondrial dysfunction such as diabetes and aging. To test this hypothesis, we can blot for ROCK2 expression in skeletal muscle biopsies taken from patients with Type II Diabetes Mellitus and older individuals. We would try to age-match individuals and eliminate those with co-morbidity. We would take muscle biopsies from the soleus muscle and IHC stain for ROCK2. At the same time, we would also stain for

succinate dehydrogenase (SDH) activity. SDH is complex II in the mitochondrial electron transport chain. SDH activity is used as a readout of mitochondrial activity. Based off the mouse data, we expect that levels of ROCK2 would correlate to SDH activity. Thus, we hypothesize that patients suffering from diabetes and old age would have love levels of skeletal muscle ROCK2 and low levels of SDH activity. However, it is possible that we can observe low levels of ROCK2 in the biopsies but no change in SDH activity. This would indicate that ROCK2 is impacted in skeletal muscle disorders but through mitochondrial independent activity.

Additionally, we could try to bridge the gap from mouse to human studies experimenting on primary human myofibers. It is technically feasible to turn skeletal muscle biopsies into plated cells[230]. The advantage of using cell lines instead of conducting histology studies, as described above, is we can measure energetic flux with *in vitro* systems. For example, we could isolate primary myofibers from healthy individuals. We could inhibit the cells in vitro with siRNA again ROCK2 and determine how OCR changes with Seahorse instrument. We would expect that ROCK2 inhibited myofibers would have lower OCR. siRNA treated myofibers may not change their OCR. If this were to happen his would suggest that ROCK2 may not be an important regulator of fatty acid oxidation in human samples. Human myofibers can also be inhibited by siRNA that target ROCK1. Thus, Seahorse experiments may have the potential to alleviate isoform-specific effects of skeletal muscle ROCK in clinically relevant samples.

In conclusion this dissertation found that the physiological role of skeletal muscle ROCK2 is to regulate endurance performance by modulating mitochondria function. Many new areas of research remained to be explored including skeletal muscle-adipocyte cross talk, gender impact on ROCK2 function and clinical implications of our mouse model. We hope that experiments conducted in this dissertation project will lay the groundwork for such future efforts.

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